Phytochemistry, Vol. 23, No. 3, pp. 519–524, 1984.
Printed in Great Britain.

0031–9422/84 $3.00 + 0.00
Pergamon Press Ltd.

FREE STEROLS AND GLYCOLIPIDS IN THE ALEURONE TISSUE OF GERMINATING WHEAT

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(*Revised received* 5 *August* 1983)

Key Word Index—*Triticum aestivum*; wheat; Gramineae; aleurone tissue; sterols, glycolipids; gibberellic acid.

Abstract—The action of gibberellic acid on wheat aleurone tissue led to reduced levels of free sterols and glycolipids compared with control tissue. Radio-labelled precursors were not incorporated into sterols or glycolipids, although mevalonate and glycerol were incorporated into polyisoprenoid hydrocarbon and phospholipid respectively. It is concluded that sterols and glycolipids are not synthesized in the tissue during germination; this is in contrast to earlier reports of the active, gibberellin-regulated metabolism of phospholipids.

INTRODUCTION

When quiescent wheat seeds imbibe water, their aleurone and embryonic tissues quickly begin to synthesize phospholipids and to form endoplasmic reticulum [1, 2]. In the aleurone tissue at least, these processes appear to require only the imbibition of water for their initiation, and they continue for about 24 hr into germination. Similar events occur in the barley aleurone tissue during germination, and in this species it has been proposed that the events are induced by gibberellin secreted from the embryo [3–6]. This claim has, however, been refuted in the cases of both barley and wheat [7, 8]. Instead, it has been proposed that gibberellin induces the increased turnover of phospholipids in pre-existing membranes during a period starting from about 18 hr of germination [7–9]. This hormone-induced turnover of phospholipids is believed to occur mainly in the endomembranes of the aleurone cell.

Sterols and glycolipids occur along with the phospholipids in plant cellular membranes [10]. The glycolipids, particularly the glycosyl diacylglycerols, are major constituents of plastid membranes [11], whereas sterols and steryl glycosides appear to be present in all subcellular membranes with the highest concentrations occurring in the microsomal fraction [12–14]. These lipids might therefore be involved in the formation and turnover of the endomembranes of the cereal aleurone tissue during germination. With this possibility in mind, we have determined the concentrations of free sterols and glycolipids in wheat aleurone tissue, as well as the incorporation of radioactive precursors into them. We have also investigated the possibility that gibberellic acid (GA) might control the metabolism of these lipids in the aleurone tissue.

RESULTS

Concentration of free sterols

Changes in total free sterol concentration in the aleurone tissue are shown in Fig. 1. The tissue from ungerminated grains contained about 110 μg sterol/30 grains. This value decreased to about 70 μg sterol/30 grains at 96 hr germination. In contrast, the concentration in embryoectomized grains did not change significantly during incubation for up to 96 hr. Incubation of the embryoectomized grains in the presence of GA caused a decrease in free sterols to about 80 μg/30 grains, similar to that seen in the whole germinating grains.

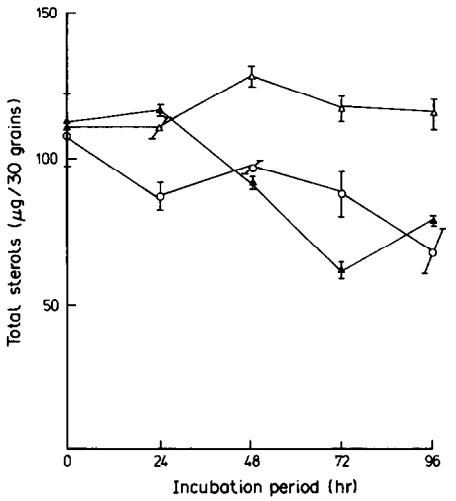

Fig. 1. Total free sterol concentrations in aleurone tissue. (○) Whole grain; (△) embryoectomized grain; (▲) embryoectomized grain + GA. Each value is the average from five experiments ± s.e.

Sitosterol was the predominant sterol in the germinating grains and in the incubating embryoectomized grains; it accounted for about 60% of the total free sterols at all stages of germination or incubation. Campesterol accounted for about 19% of the total in the ungerminated grains, falling only slightly to about 17% after 96 hr. There were no significant differences between the germinating grains and the embryoectomized grains in this respect. Isofucosterol accounted for a further 11% in the ungerminated grains and incubating embryoectomized

grains (±GA). The remainder of the free sterols was accounted for by cholesterol and stigmasterol; they each accounted for about 5% of the total in the ungerminated grains. Within the limits of our experimental method, the proportion of cholesterol did not change significantly during germination. The proportion of stigmasterol, on the other hand, appeared to increase to about 9% at 96 hr in both germinating grains and incubating embryoectomized grains (±GA). The concentrations of the individual free sterols are presented in Table 1. It can be seen that the decrease in total free sterols in germinating grains can be mostly accounted for by decreases in the concentrations of the two major sterols, sitosterol and campesterol. Cholesterol and isofucosterol accounted for the remainder of the decrease. Stigmasterol was outstanding, because its concentration did not change significantly during germination. In the embryoectomized grains incubated without GA, the concentrations of cholesterol, campesterol, sitosterol and isofucosterol did not change significantly. The concentration of stigmasterol appeared to increase slightly from about 5.5 μg/30 grains to about 10 μg/30 grains. When embryoectomized grains were incubated with GA, a pattern of changes was obtained which was essentially the same as that seen in the germinating grain. In particular, the concentration of stigmasterol did not change significantly, while the concentrations of sitosterol, campesterol and isofucosterol decreased.

The abnormal behaviour of stigmasterol with respect to the other sterols is of particular interest in view of earlier reports that the sitosterol: stigmasterol ratio decreases during the growth and development of plant tissues (see Discussion). Sitosterol: stigmasterol ratios in the wheat aleurone tissue are therefore presented in Fig. 2. Two features are immediately obvious. Firstly, the ratio fell during incubation from a value of about 12 in ungerminated grains to about 6 in grains germinated for 96 hr. Secondly, the fall was not prevented either by removing the embryos from the grains or by applying GA to the embryoectomized grains.

Concentrations of glycolipids

In the aleurone tissue of the germinating grain, the glycolipid concentration remained constant up to at least 72 hr (Table 2). Thin-layer chromatography of the glycolipid fraction allowed the tentative identification of two classes of glycolipid, namely monoglycosyl diacylglycerol and steryl glycoside. These co-chromatographed with authentic lipids and gave the relevant colours with chromogenic reagents. A third spot migrated just behind diglycosyl diacylglycerol on the chromatogram. It reacted positively with chromogenic reagents for carbohydrates; the spot was therefore considered to contain a glycolipid, possibly a glycosyl monoacylglycerol. Both monoglycosyl monoacylglycerol and diglycosyl monoacylglycerol have been reported as present in the wheat aleurone tissue [15, 16] and they have similar chromatographic properties to our unknown. Diglycosyl diacylglycerol appeared to be absent from the aleurone tissue at all times during germination. The nature of the glycosyl moiety was not investigated, but the presence of galactose and glucose in the glycolipids of the wheat embryo [17] suggest that the same sugars would be found in the aleurone tissue glycolipids.

The concentrations of the individual glycolipids through germination are also presented in Table 2. Considerable difficulty was experienced in obtaining reproducible estimations of the glycolipids recovered from the developed TLC chromatograms, so that only major changes in the relative proportions of the glycolipids could be reliably identified. Within this limitation, however, the data suggest that the relative amounts of the individual glycolipids did not change significantly during the experimental period of germination.

The effects of removing the embryo and of applying GA were studied. Removal of the embryo, followed by incubation of the embryoectomized grains for 72 hr, did not affect the concentration of the total glycolipids in the aleurone tissue compared with that in the intact germinating grain. Incubation of the embryoectomized grains in

Table 1. Free sterol concentrations (μg sterol/30 grains) in the aleurone tissue of germinating grains and grains incubated either with or without 1 μM GA

		Incubation time (hr)				
	Sterol	0	24	48	72	96
Germinating grains	Cholesterol	6.9±1.8	2.0±0.8	6.5±0.3	6.6±2.1	3.2±0.3
	Campesterol	21.1±1.5	18.1±0.8	15.7±1.0	14.7±0.7	11.9±1.2
	Stigmasterol	5.5±0.8	4.4±0.2	7.1±0.6	5.5±0.7	6.9±0.8
	Sitosterol	64.7±4.2	55.8±2.3	56.6±2.9	52.8±5.6	41.5±4.9
	Isofucosterol	11.7±0.8	6.8±0.5	10.4±0.5	7.4±1.7	4.8±0.5
Embryoectomized grains	Cholesterol	6.9±1.8	7.2±1.0	5.0±0.4	5.3±0.8	6.8±1.2
	Campesterol	21.1±1.5	20.0±0.4	31.2±1.5	24.1±3.0	21.4±1.5
	Stigmasterol	5.5±0.8	7.1±0.6	7.4±1.4	8.8±1.6	9.9±0.5
	Sitosterol	64.7±4.2	66.0±1.5	84.3±0.4	82.7±4.5	76.9±3.9
	Isofucosterol	11.7±0.8	11.2±1.9	11.0±0.2	6.6±1.4	10.3±0.9
Embryoectomized grains + GA	Cholesterol	6.9±1.8	9.6±2.5	3.5±0.4	3.8±0.6	4.9±0.3
	Campesterol	21.1±1.5	20.0±0.4	23.8±1.1	10.8±1.1	13.5±0.2
	Stigmasterol	5.5±0.8	6.3±0.3	5.0±0.2	5.1±0.1	6.0±0.1
	Sitosterol	64.7±4.2	69.9±2.3	54.4±1.6	39.8±1.4	47.0±0.2
	Isofucosterol	11.7±0.8	12.1±0.3	6.6±0.7	2.9±0.8	6.4±0.3

Each value is the average from at least four separate experiments ± s.e.

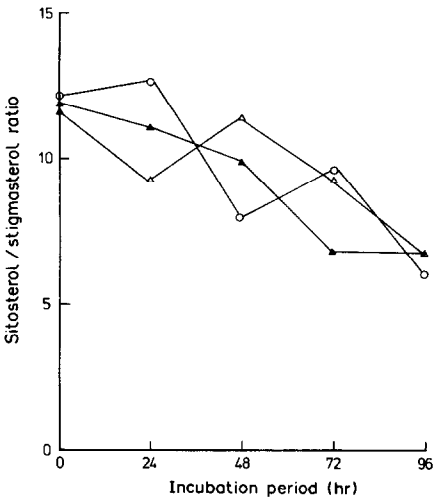

Fig. 2. Sitosterol/stigmasterol ratios in aleurone tissue. (○) Whole grain; (△) embryoectomized grain; (▲) embryoectomized grain + GA. Each value is the average from five experiments.

Table 2. Glycolipid concentrations (μg lipid galactose/30 grains) in the aleurone tissue of germinating grains

	Germination period (hr)			
	0	24	48	72
Monoglycosyl diacylglycerol	11	nd	12	16
Steryl glycoside	18	nd	13	18
Unknown	28	nd	27	22
Total glycolipid	57	62	59	58

nd, Not determined. Each value is the average from two experiments.

the presence of GA resulted, however, in a 33% decrease in the total glycolipid concentration (Table 3). Analysis of the individual glycolipids by TLC showed that their relative proportions were not affected by the GA action.

Incorporation of [^{14}C] *mevalonate into polyisoprenoid compounds*

Uptake of [2-^{14}C] mevalonate into isolated aleurone tissue followed a linear course for at least 60 min, and a standard 30 min incubation period was used for the present studies. Most of the radioactivity taken up (*ca* 60%) was incorporated into the neutral lipid fraction from silicic acid column chromatography. The aleurone tissue from quiescent grains incorporated 0.43 × 10^6 dpm/30 grains (Fig. 3A). This value increased to 1.57 × 10^6 dpm at 24 hr of germination and then declined slowly to 0.88 × 10^6 dpm at 96 hr. Aleurone tissue from embryoectomized grains incubated without GA gave a similar pattern of incorporation. In embryoectomized grains incubated with GA, a similar increase was observed between 0 and 24 hr. Beyond 24 hr, however, the decrease in incorporated radioactivity was significantly greater than that observed in the germinating grain; at 96 hr only

Table 3. Effect of GA on glycolipids (μg lipid galactose/30 grains) in aleurone tissue

	−GA	+GA
Monoglycosyl diacylglycerol	10 ± 1	8 ± 1
Steryl glycoside	19 ± 1	14 ± 1
Unknown	19 ± 5	11 ± 3
Total glycolipid	58	38

Each value for monoglycosyl diacylglycerol, steryl glycoside and the unknown glycoside is the average from three experiments ± s.e. Each value for total glycolipid is the average from two experiments.

0.41 × 10^6 dpm were incorporated.

Smaller quantities of radioactivity were found in the glycolipid fraction from silicic acid column chromatography. Careful examination of this fraction on two-dimensional TLC showed, however, that none of the radioactivity was located in sterol glycosides. Hydrolysis of the fraction in dilute acid (to hydrolyse mevalonate lactone to the free acid), followed by re-chromatography on TLC showed that the radioactivity was entirely associated with unmetabolized mevalonate.

Two-dimensional TLC of the radio-labelled neutral lipid fraction revealed radioactivity in four spots. Three of the spots stained green with *p*-anisaldehyde, and they were tentatively identified as short-chain polyprenols, long-chain polyprenols and ubiquinone + tocopherols by co-chromatography with authentic lipids. The fourth spot co-chromatographed with authentic squalene. This hydrocarbon spot contained 40–80% of the neutral lipid radioactivity, and the remaining activity was evenly divided among the other three polyprenoid spots. No significant radioactivity was detected in the region corresponding to free sterols. Levels of radioactivity in the hydrocarbon fraction from germinating grains and incubating embryoectomized grains are shown in Fig. 3B. They follow very similar patterns to those found for the neutral lipid fraction (Fig. 3A).

Incorporation of [^{14}C] *glucose,* [^{3}H] *galactose and* [^{14}C] *glycerol into glycerolipids*

Radio-labelled glucose, galactose and glycerol were investigated as possible precursors for glycolipid synthesis. The first two were chosen because they should be rapidly incorporated into the sugar moiety of the glycolipid and less rapidly into other parts of the molecule or into other lipids. Glycerol is a precursor of the glycerol moiety of all glycerolipids including the glycolipids. Preliminary experiments showed that it was not possible to saturate the uptake of the precursors into the aleurone tissue, even in the presence of 100 mM unlabelled compound. Therefore, the carrier conditions were chosen arbitrarily; glucose and galactose incorporations were determined with 20 mM unlabelled carrier, and glycerol incorporation was determined in the absence of unlabelled carrier.

The data for glucose and glycerol are presented in Table 4. Incorporation of radio-labelled glucose into lipids was small, and it even declined through the experimental germination period. There was no evidence

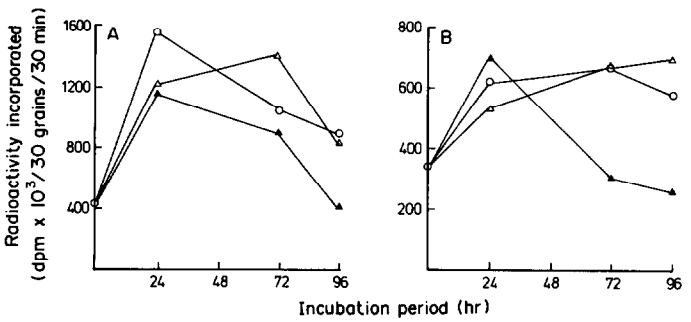

Fig. 3. Incorporation of radiolabelled mevalonate into total neutral lipids (A) and hydrocarbon (B). (○) Whole grain; (△) embryoectomized grain; (▲) embryoectomized grain + GA. Each value is the average from at least two experiments.

Table 4. Incorporation (dpm × 10^3/30 grains) of radiolabelled glucose and glycerol in aleurone tissue from germinated grains

Precursor	Product	Germination period (hr)					
		0	6	18	24	48	72
D-[U-^{14}C]Glucose	Neutral lipids	nd	0.43	nd	0.33	0.35	0.26
	Glycolipids	nd	0.36	nd	0.31	0.25	0.25
	Phospholipids	nd	0.39	nd	0.29	0.23	0.21
	Amino acids	nd	14.8	9.0	nd	16.4	8.0
	CO_2	nd	16.5	12.9	nd	16.4	18.4
D-[U-^{14}C]Glycerol	Neutral lipids	5.8	nd	nd	6.2	12.0	9.8
	Glycolipids	0.46	nd	nd	0.35	0.52	0.47
	Phospholipids	2.8	nd	nd	3.0	6.1	12.9

nd, Not determined. Incorporation of glucose was measured over 2 hr in the presence of 20 mM unlabelled substrate. Incorporation of glycerol was measured over 30 min in the absence of unlabelled substrate. Each value is the average from at least two experiments.

at any time during germination for specific incorporation of glucose into the glycolipid fraction. Similarly, incorporation of [^{3}H]galactose into the lipids was low (< 600 dpm/30 grains) and there was no evidence for the specific incorporation of this sugar into glycolipids. In contrast, radioactivity from [^{14}C]glucose was actively incorporated into carbon dioxide and into the amino acid fraction of the tissue. These results indicate that the sugars were satisfactorily taken up into the aleurone tissue, but that they were not incorporated into lipids. Radio-labelled glycerol was also taken up by the aleurone tissue, but it was not incorporated into glycolipids. Radioactivity from [^{14}C]glycerol was, however, incorporated into neutral lipids and into phospholipids. Further experiments (data not presented) showed that the [^{14}C]glycerol was incorporated specifically (97%) into the glycerol moiety of the phospholipids.

DISCUSSION

The metabolism of sterols and glycolipids is in marked contrast to that of the phospholipids in the aleurone tissue during germination. Thus, phospholipid concentration increases by about 30% during the first 24 hr of germination, this increase being independent of any hormones emanating from the embryo [1]. The glycolipid concentration remained constant during this time however, and the free sterols even decreased slightly. Embryoectomy of the grains only prevented this decrease, and it did not induce any obvious synthesis. Furthermore, there is an active incorporation of radio-labelled choline and phosphate into the phospholipids during this period [8], whereas incorporation of precursors into the sterols and glycolipids appears to be negligible. The contrast continues beyond 24 hr of germination, when GA-induced α-amylase synthesis takes place in the aleurone tissue. During this period of germination, GA action leads to increased phospholipid turnover [9]. This is associated with an overall decrease of phosphatidyl choline concentration, but the tissue concentrations of the other phosphatides are not affected. Although GA also causes a decrease in the concentrations of the free sterols and the glycolipids (Fig. 1 and Table 3), the extent of this decrease is not as great as that of phosphatidyl choline (cf. [9]). Moreover, we have been unable to identify any significant precursor incorporation into either the sterols or the glycolipids during this period. Thus, the present results indicate that both the free sterols and the glycolipids are metabolically inactive components of the aleurone tissue during germination. The decreases in their concentrations, which are brought about by GA, presumably reflect the general decrease in the quantity of endoplasmic

reticular membrane which occurs following the hormone action [2, 18].

Demonstration that the sterols and glycolipids are metabolically inactive does not mean, of course, that they have no function in the aleurone cell during germination. On the contrary, the sterols and sterol glycoside, at least, may have structural roles in the endomembranes as has been suggested in the case of other plant tissues [12, 13, 19–21]. If this is so, then they must be incorporated into these membranes as the endoplasmic reticulum is formed during early germination. Ultrastructural evidence indicates that this endoplasmic reticulum is formed from oleosomes [22]. Future chemical analysis of the oleosome and microsomal fractions from germinating seed tissues should provide interesting data for such a mechanism.

The failure of the aleurone tissue to incorporate radiolabelled mevalonate into sterols is similar to the situation described for several other seeds during early germination. Thus, peas incorporate mevalonate predominantly into squalene and β-amyrin, with little incorporation into sterols, during the early stages of germination [23, 24]. A similar situation has been found in hazel seeds [25]. Both the embryo and the endosperm of germinating pine seeds incorporate mevalonate into squalene but not into sterols during the first 24 hr of germination [26]. After 24 hr there is a rapid incorporation into sterols while the relative amount of radio-label in squalene falls rapidly. We have found an identical situation in the embryos of germinating wheat [27]. The wheat aleurone tissue differs from these other seed tissues in one respect, however, its sterol synthesis appears to be permanently blocked at the level of squalene (we have followed the incorporation up to 144 hr with consistently negative results). This could reflect the fact that the aleurone tissue does not undergo cell division during germination.

Our data for the glycolipids in the aleurone tissue differ somewhat from those reported earlier by Morrison and his colleagues [15, 16]. Their analysis of aleurone tissue from ungerminated wheat tentatively identified monoglycosyl diacylglycerol, monoglycosyl monoacylglycerol, diglycosyl diacylglycerol and diglycosyl monoacylglycerol. Despite using several solvent systems for TLC, we have found only three spots on our chromatograms. We tentatively identified two of these spots as monoglycosyl diacylglycerol and steryl glycoside. The third spot could not be identified, but it could have been diglycosyl monoacylglycerol. Diglycosyl diacylglycerol was conspicuously absent from our aleurone tissue. These differences between the two laboratories could simply reflect differences between the cultivars of wheat studied. Alternatively, they could arise from the use of different chromatographic procedures. Resolution of the differences awaits further studies.

EXPERIMENTAL

Chemicals and solvents. Our sources of authentic lipids, as well as solvents for lipid extraction and chromatography, have been described previously [17, 28]. Radio-labelled compounds were obtained from Amersham International (Amersham, Bucks, U.K.).

Plant material. High vigour wheat of the soft winter cv. Atou were used in batches of 30 grains (1.8 g). Grains or embryoectomized grains were sterilized and allowed to germinate or incubate in the dark at 25° by our routine procedure [29, 30]. Where the effect of GA was to be investigated, embryoectomized grains were incubated with 1 μM GA. Upon harvesting, the grains were dissected to obtain their aleurone layers for analysis.

Extraction and analysis of lipids. The methods which we use routinely for the extraction of lipids and for their fractionation into classes have been described in detail previously [17, 28]. Separation and analysis of free sterols and glycolipids by TLC have also been described previously [17]. Separation and analysis of the free sterols by GC was carried out on a liquid phase of 3% OV-17 supported on Chromosorb W-AW-DMCS (100–120 mesh) at 275° [31].

Incorporation of radiolabelled glucose into CO_2 and amino acids. Aleurone layers from 30 grains were incubated for 120 min in 5 ml 100 mM K phosphate buffer, pH 6.6, containing 2.5 μCi D-[U-^{14}C] glucose and 10 mM unlabelled glucose (sp. act. 50 μCi/mmol) in a reciprocating incubator at 25°. The incubation was carried out in an 18 ml glass vial containing an empty microcentrifuge tube and stoppered with a 'subaseal' cap. At the end of the incorporation incubation, 0.5 ml 1 M hyamine hydroxide in MeOH was injected into the microcentrifuge tube and 0.5 ml 3 M $HClO_4$ was injected into the medium. The vial was left in the incubator, with shaking, for a further 60 min. After this, the hyamine soln, containing absorbed CO_2, was transferred to a scintillation vial and its radioactivity determined by scintillation spectrometry.

The tissue was then removed from its medium and ground in 2 ml ice-cold 3 M $HClO_4$ with a pestle and mortar. It was then homogenized further for 1 min using a Polytron homogenizer (Kinematica AG, Luzern, Switzerland) operated at maximum speed. This homogenate was filtered through a sintered-glass filter, which was then rinsed through with a small vol. of H_2O. The filtrate was combined with the incubation medium and adjusted to pH 2.5 with 30% KOH. Cation exchange chromatography of the extract was carried out through a column of Amberlite IR 120 (14–20 mesh), 1 cm diameter × 9 cm high. The whole of the extract was transferred to the prepared column. Radioactive sugars and anions were eluted with 20 ml 10 mM glucose followed by 50 ml H_2O at a flow rate of 0.5 ml/min. Amino acids were then eluted with 50 ml 5% (w/v) NH_4OH and their radioactivity was determined by scintillation spectrometry.

Incorporation of radiolabelled precursors into lipids. Aleurone layers from 30 grains were incubated at 25° in 10 ml 100 mM K phosphate buffer, pH 6.6. For the measurement of mevalonate incorporation into terpenoid lipids the medium contained 5 μCi DL-[2-^{14}C]mevalonic acid (sp. act. 22 mCi/mmol) and no unlabelled mevalonic acid. The incorporation period was 30 min. For the incorporation of glucose and galactose the medium contained 5 μCi D-[U-^{14}C] glucose or 5 μCi D-[1-^{3}H] galactose and 20 mM of the appropriate unlabelled compound (sp. act. 25 μCi/mmol); the incubation period was 120 min. For the incorporation of glycerol the medium contained 2.5 μCi D-[U-^{14}C] glycerol (sp. act. 37 mCi/mmol), and no unlabelled glycerol; the incubation period was 30 min.

Acknowledgements—We are indebted to the Rank Prize Fund for the award of postgraduate studentships to F.G.B. and E.McD. and a research support grant to D.L.L. We are also indebted to the Science and Engineering Research Council for the award of a postgraduate studentship to M.C.W. We wish to thank Miss W. Jones for her technical assistance.

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