Phytochemistry, Vol. 20, No. 12, 2669–2674, 1981.
Printed in Great Britain.

0031-9422/81/122669-06 $02.00/0

PHOSPHOLIPID METABOLISM IN COTYLEDONS OF WILD AND CULTIVATED PEAS

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(*Received* 23 *February* 1981)

Key Word Index—*Pisum sativum*; *P. elatius*; Leguminosae; cotyledons; germination; phospholipids; endoplasmic reticulum; plasma membranes; choline; incorporation of choline and glycerol.

Abstract—The amount of phospholipids in the membrane fractions of the endoplasmic reticulum (ER) and plasma membrane of cotyledons of *Pisum sativum* and *P. elatius* was followed during germination. Incorporation of [^{14}C-methyl]choline into the ER and the plasma membrane was followed as well as [U-^{14}C]glycerol into the ER. The pool size of endogenous choline was determined and found to be much greater in the wild pea and to increase early in germination. In both species membranes are synthesized in large quantities early in germination and both turnover and synthesis of the backbone and the phosphatidyl tail occur throughout 48 hr of germination. In *P. sativum* incorporation peaks earlier than in *P. elatius* and degradation also begins earlier than in *P. elatius*. This is consistent with the general behaviour of the two species.

INTRODUCTION

The sites of metabolic control, which regulate germination, are still unclear [1]. One of the ways of studying this problem is to compare the metabolism of two related species of the same genus, one wild and the other cultivated, which differ in the rate of their germination. Such comparative studies on the two species of *Pisum*, *P. elatius* and *P. sativum*, have indicated appreciable differences in their germination behaviour [2–6]. The role of phospholipids in the metabolism of seeds is becoming increasingly clear and it is likely that membrane synthesis is a very early process in germination [7, 8]. Phospholipid metabolism in plant tissues including seeds has recently been reviewed [8]. Although the synthesis of organelles in the endosperm of germinating castor beans has been the subject of very detailed and extensive studies [9], the relation between germination as a process and phospholipid synthesis and turnover has been studied only in a few cases. Perhaps the most detailed analysis is that of Cuming and Osborne [10, 11] who attempted to detect differences in phospholipid metabolism in dormant and non-dormant seeds of *Avena fatua*. In these studies turnover of membrane compounds was maintained for long periods of time. Much attention has also been paid to the behaviour of membranes in the endoplasmic reticulum (ER), especially of the aleurone of cereals and the possible relation between phospholipid metabolism and hormone effects and especially gibberellic acid [12].

In view of the marked differences in the rate of metabolism of the two species of peas we decided to investigate the metabolism of phospholipids in the cotyledon of these two species. We felt that this might be one of the earliest measures of the rate of metabolic activity of the seeds and provide an indication of sites at which the rate of germination might be controlled. The following report presents the results of such a study.

RESULTS

The change in phospholipid content in the total membrane fraction as phospholipid phosphate and relative to the amount present in dry seeds (see Experimental) during germination is shown in Figs. 1 and 2, taking the phosphate content as a measure of the phospholipids. It is clear that the cotyledons of *P. elatius*, which had a lower initial content of phospholipid, synthesize them more rapidly especially during the first 5 hr. The phospholipid content continues to rise in both species up to *ca* 15–20 hr. Thereafter the phospholipid content remains steady in *P. elatius* but falls in *P. sativum*.

The free choline content of the cotyledons of the two species shows rapid and large changes, the wild pea *P. elatius* always having a markedly higher content of free choline. In both species during the rapid rise of membrane phospholipid, the free choline content also rises very rapidly (Table 1). Increases in the choline content of seeds have been reported in germinating wheat [13]. As germination proceeds, the free choline content drops, presumably due to incorporation into membranes.

The membrane fractions which were prepared were characterized enzymatically, and compared with a mitochondrial fraction prepared from a 13 000 *g* (15 min) precipitate. Zone 1, density 1.17–1.19, should represent primarily a plasma membrane fraction while zone 2, density 1.11–1.13, should contain mainly ER. The data in Table 2 clearly indicate that the enzymatic contents of the bands correspond to this presumed origin, with, however, a certain amount of cross-contamination. The index of purity of the fraction is the relative amounts of activity, rather than their absolute activity [14, 15]. Zone 1 is enriched in ATPase indicating its origin in the plasma membrane while zone 2 is enriched in NADPH cytochrome *c* reductase indicating the presence of ER. The membrane fractions of the two species are similar in their enzyme activities.

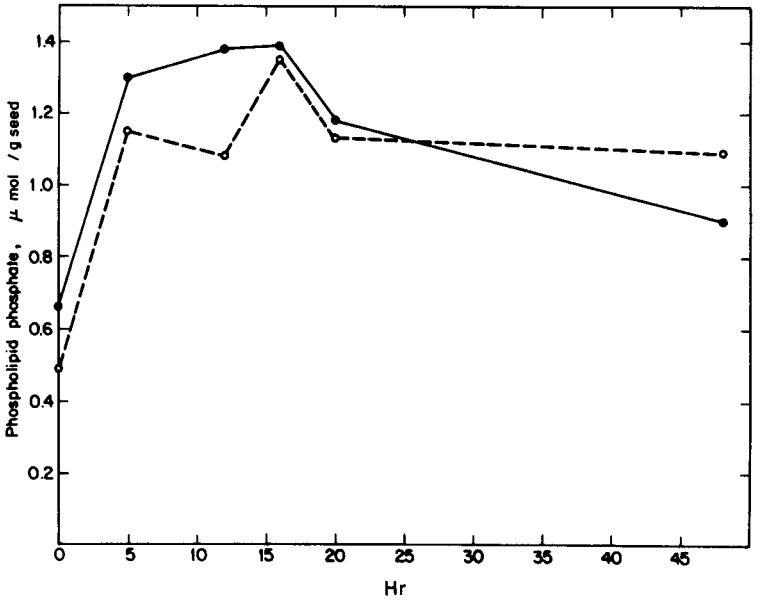

Fig. 1. Changes in phospholipid phosphate in cotyledons of two species of *Pisum*. ●——●, *P. sativum*; ○----○, *P. elatius*. Results as μmol Pi/g seed.

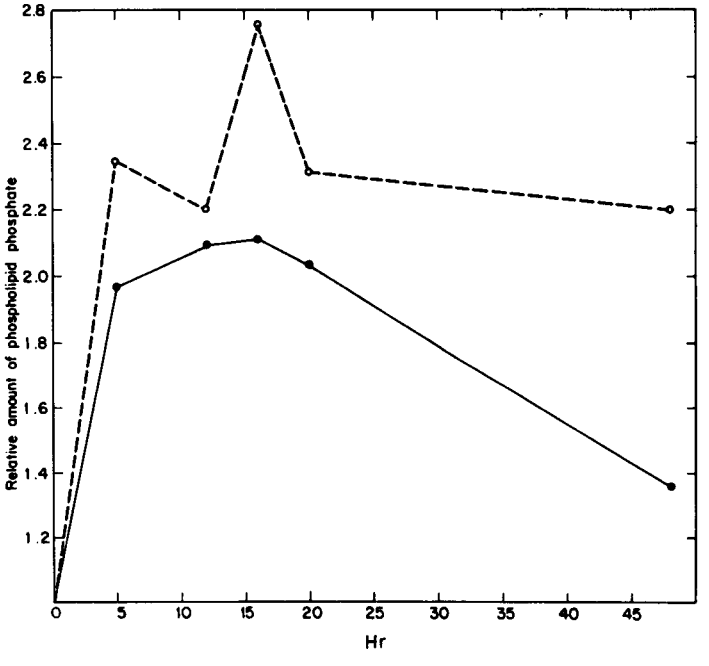

Fig. 2. Changes in phospholipid phosphate relative to initial content of cotyledons of dry seeds. Symbols as in Fig. 1.

Table 1. Free choline content of cotyledons of *P. elatius* and *P. sativum*

Time of germination	μg free choline/g cotyledons	
	P. elatius	*P. sativum*
0	60	15
5	647	254
20	93	49

In order to study the metabolism of a definite membrane component, seeds were supplied with [^{14}C-methyl]choline chloride during 5 hr of imbibition, and incorporation into the phosphatidylcholine, present in the membrane fraction was then determined after various periods of germination. Choline uptake was measured, by determining differences between the amount taken up and the amount supplied. In both species 26 nmol choline was taken up during imbibition/g seed. However, despite the equal uptake, incorporation into phosphatidylcholine was quite different in the two species (Fig. 3). Because of the different pool sizes of choline in the two species it is

Table 2. Enzymatic characterization of membrane fractions

	Enzyme activity					
	ATPase		NADPH cyt *c* reductase		Cyt *c* oxidase	
	P. sativum	*P. elatius*	*P. sativum*	*P. elatius*	*P. sativum*	*P. elatius*
Zone 1	83.6	56.4	2.8	4.0	6.5	7.6
Zone 2	27.2	28.4	6.6	10.8	1.6	2.6
Mitochondrial fraction	—	—	—	—	11.1	15.6

All enzyme activity/mg protein. ATPase as μg Pi/hr; cyt *c* reductase as μM cyt *c*/min; cyt *c* oxidase as μM cyt *c*/min. Activity determined in fraction obtained from cotyledons of seeds germinated for 22 hr.

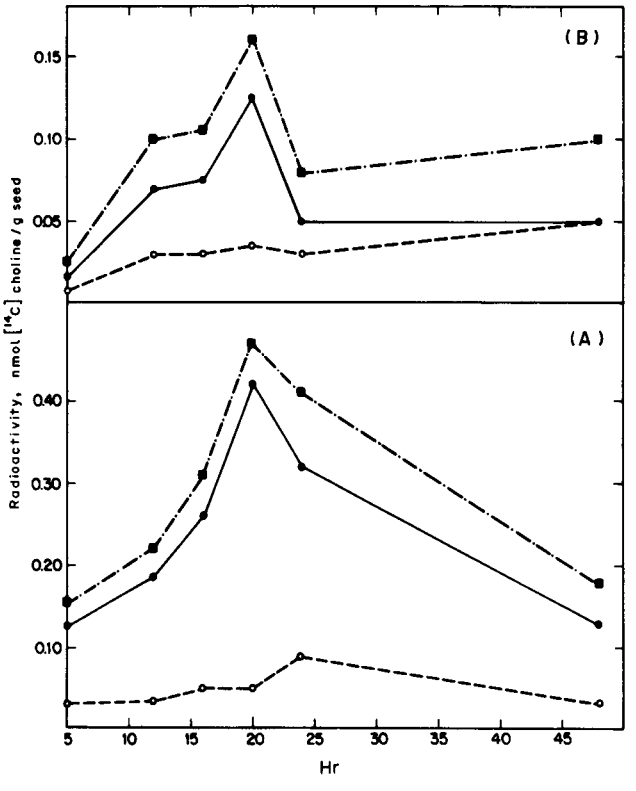

Fig. 3. Incorporation of [^{14}C]choline into membranes of cotyledons of species of *Pisum*. (A) *P. sativum*. (B) *P. elatius*. ■–·– ■, Total incorporated; ○ – – – ○, plasma membrane; ●——●, ER. Results as nmol/g seed.

difficult to compare precisely the behaviour of the two species. However, it is clear that the rate of incorporation after the first 5 hr in *P. sativum* is lower than that in *P. elatius* (Fig. 4) and the time course is also very different. The data in Fig. 4 analyse only the incorporation into the 'ER' fraction since incorporation into the plasma membrane was very low and did not show very dramatic changes during germination. It is perhaps misleading to consider incorporation on a per wt basis. However, expression on a protein basis is also unsatisfactory since the amount of protein differs in the two kinds of membrane and is also known to change during germination.

In order to obtain a better insight into membrane metabolism an attempt was also made to label the ER membrane with [U-^{14}C]glycerol. The results in Fig. 5 clearly show that much more label is incorporated into phosphatidylcholine than into phosphatidylethanolamine. It also shows that the peak amount of incorporation in the wild pea occurs later than in the cultivated pea, the difference being *ca* 4 hr. This is further borne out by expressing the labelling by glycerol into phosphatidylcholine as the amount relative to that obtained after 5 hr. A careful comparison of Fig. 6 with Fig. 4 indicates that the glycerol backbone of phosphatidylcholine is not metabolized at the same rate as the choline moiety linked to the phosphatidyl group, but in both cases metabolism is initially more rapid in *P. sativum*.

DISCUSSION

The results obtained show that in both the species of pea studied there is a very rapid rise in the amount of membrane phospholipid during the imbibition phase. The initial amount of membranes is larger in the cultivated pea, but the rate of synthesis as determined by incorporation is larger in the wild pea. It is important to

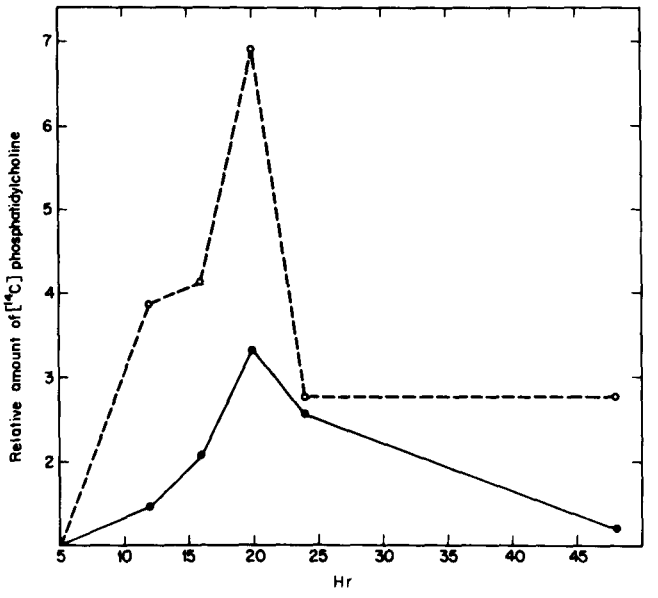

Fig. 4. Incorporation of [^{14}C]choline into ER of cotyledons of species of *Pisum* relative to incorporation after 5 hr. ●——●, *P. sativum*; ○----○, *P. elatius*.

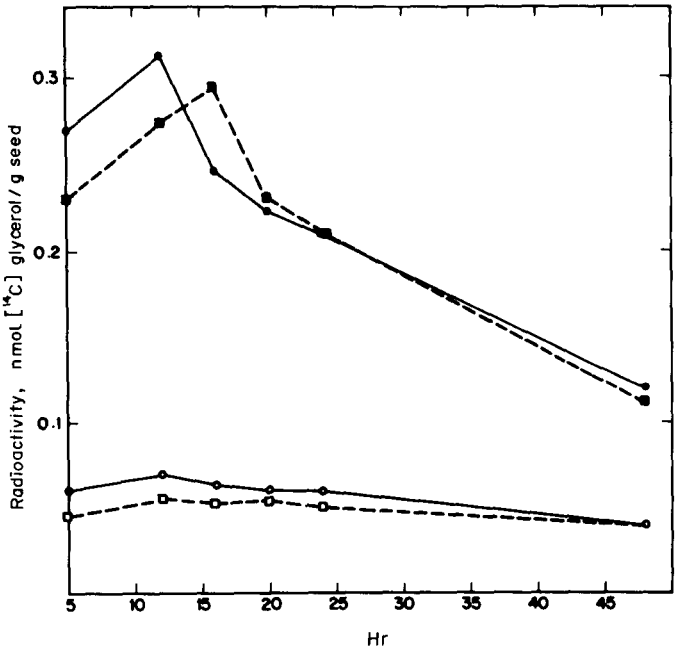

Fig. 5. Changes in incorporation of [U^{14}C]glycerol into phosphatidylcholine and phosphatidylethanolamine in membrane fractions of ER of germinating pea cotyledons. Results as nmol [^{14}C]glycerol/g seed. ●——●, Phosphatidylcholine—*P. sativum*; ■——■, phosphatidylcholine—*P. elatius*; ○——○, phosphatidylethanolamine—*P. sativum*; □----□, phosphatidylethanolamine—*P. elatius*.

note that phospholipid breakdown also begins earlier in the cultivated pea (Figs. 1 and 2). A drop in phospholipids has also been reported in mung beans [16]. In the endosperm of castor bean an initial rapid rise in the amount of ER has been reported [17]. Such rises in phospholipids are consistent with the very rapid increase in metabolic activity in the storage organs during the initial phases of germination. The subsequent drop in amount of phospholipid may be the result of the decreasing metabolic activity of the storage organs as the breakdown of reserve materials begins. Although the two membrane fractions which were isolated in this work were not pure, and indeed it is very difficult to obtain them pure, we were dealing with fractions relatively enriched in ER (zone 2) and plasma membranes (zone 1) as determined by the usual markers and from characterization of membrane fractions and also as determined by their position in a discontinuous sucrose gradient.

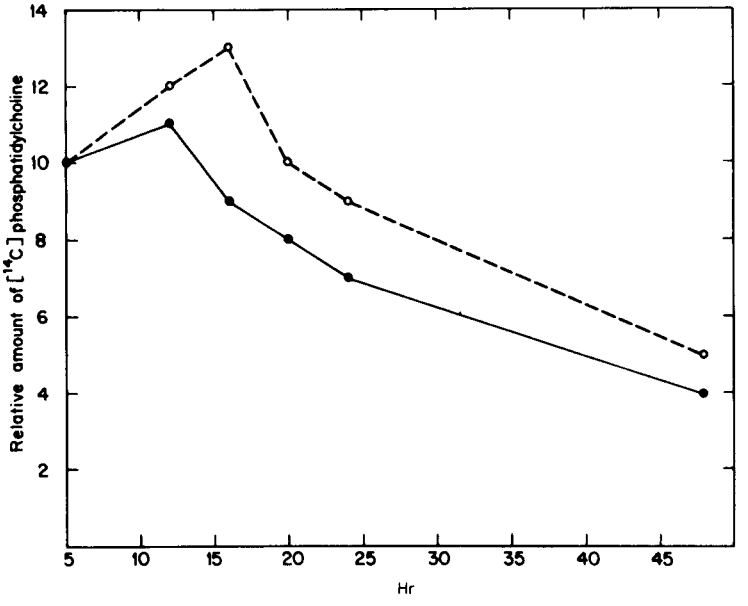

Fig. 6. Changes in incorporation of glycerol into phosphatidylcholine in cotyledons of species of *Pisum*. ●———●, *P. sativum*; ○- - - -○, *P. elatius*.

The experiments on choline incorporation present two difficulties in interpretation. The relative initial amounts of membranes in the two species is not the same (Fig. 1). Furthermore, the endogenous pool of choline in the two species is not the same initially, and it changes at different rates in the two species. As a result it is impossible to compare absolute rates of incorporation between the two species. However, it is quite clear, from the use of two different precursors, choline and glycerol, that the cultivated pea has an initially more rapid rate in synthesis and/or turnover of membranes than *P. elatius*. This is in accord with other aspects of metabolism of the two species [2–4]. The problem of pool size has been mentioned by previous workers [16, 18], but in a recent work on membrane metabolism, pool sizes were not determined [10, 11]. This is clearly a crucial problem in any comparative study. If an approximate allowance is made for pool size in the two species, the amounts of choline incorporated/g cotyledons are found to be quite similar. The time at which the peak in labelling is obtained is not appreciably altered by such correction. It is impossible from the experiments described here to calculate rates of synthesis of phospholipids. The amount of label appearing in any given fraction at a given time will be a function of the rate of synthesis, the rate of exchange, the rate of breakdown and the pool size of precursor, and the amount of radioactive precursor available for synthesis. All these are changing throughout germination and there is good reason to believe that they change at different rates in the two species.

Nevertheless, three clear conclusions can be drawn from this work. In *Pisum* species membranes are synthesized in large quantities during imbibition. Both the backbone of the phospholipids and the choline tail are involved in synthesis and/or turnover, although at different rates. The rate of metabolism in the wild pea is initially slower but is maintained for longer than that of the cultivated peas. This is consistent with the overall germination behaviour and ecological behaviour of the two species as found in other investigations on them. It seems, therefore, that synthesis of membrane components is an important early event in germination. A study of its control may, therefore, provide indications of what controls germination behaviour.

EXPERIMENTAL

Untreated seeds of *P. sativum* L. cv. Alaska were obtained from the Ferry Morse Seed Co. and seeds of *P. elatius* M.B. were harvested from plants grown in the gardens of the Department.

Germination. Seeds were surface-sterilized with 1% $HgCl_2$ prior to germination, washed and germinated at 26° in the dark. The seeds were imbibed for 5 hr in a soln containing 50 μg/ml Penicillin G, 50 μg/ml Streptomycin sulphate and 25 μg/ml chloramphenicol and then placed in Petri dishes on cotton wool with 17 ml H_2O/dish. For expts with radioactive precursors the seed coat was removed prior to imbibition, and the radioactive material supplied for 5 hr together with the antibiotics. [^{14}C-Methyl]choline chloride (54 mCi/mmol), 5 μCi, or [U-^{14}C]glycerol (37 mCi/mmol), 5 μCi, was applied in 7 ml soln/2–3 g dry seeds. After suitable periods of germination, the cotyledons were removed and then analysed.

Isolation of membrane fraction. Cotyledons were ground with Tris–Mes buffer, 25 mM each, pH 7.5, containing 3 mM EDTA and 0.2 M sucrose (4 ml/g cotyledons) [14]. The homogenate was filtered through gauze and centrifuged at 3000 *g* for 5 min. The supernatant was centrifuged at 13 000 *g* (15 min) and the resultant supernatant centrifuged for 30 min at 80 000 *g* in a MSE SW rotor. The ppt. was resuspended in Tris–Mes buffer as above without EDTA and recentrifuged at 80 000 *g*. The ppt. was suspended in 0.7 ml buffer and applied to a nonlinear 45% w/w sucrose gradient in 25 mM Tris–Mes, pH 7.3, layered under 34% w/w sucrose in the same buffer. The gradient was centrifuged for 2 hr at 82 000 *g*. Two bands were recovered, one at the interphase between 45 and 34% sucrose (density 1.17–1.19, zone 1) and one at the top of the 34% sucrose layer (density 1.11–1.13, zone 2). The bands were collected and suspended in the Tris–Mes buffer, and the membranes precipitated by centrifugation at 100 000 *g* (15 min).

Phospholipid extraction. For determination of total phospholipids the ppt. obtained after 80 000 *g* (30 min) was used.

For studies of incorporation into defined membrane fractions, the ppts after 100000 g (15 min) were used. The phospholipids were extracted from this fraction as follows. The appropriate fraction was extracted with *iso*-PrOH for 3–4 min at room temp. [19]. The suspension was precipitated at 13000 g (5 min) and the supernatant retained. The ppt. was re-extracted with *iso*-PrOH and the extracts combined and evapd *in vacuo* at 50°. The extract together with the 13000 g ppt. was combined and dissolved in MeOH. After shaking with MeOH at room temp. for 30 min, $CHCl_3$ was added to give a ratio of $CHCl_3$–MeOH (2:1) [20]. After a further 15 hr the soln was centrifuged at 13000 g (10 min). The supernatant was retained and the extract re-extracted with $CHCl_3$–MeOH for 30 min, as before. The extracts were combined and the soln washed with 0.1% $MgCl_2$, one part for each 5 parts of soln. The soln was centrifuged to separate the phases and the organic phase retained and evapd *in vacuo*, redissolved in $CHCl_3$ and evapd to a vol. of 0.3 ml in a stream of N_2.

Separation of phospholipids and quantitative determination. For separation into the various phospholipid compounds the extract obtained above was applied to TLC plastic sheets coated with Si gel 60 and the plates developed with a Me_2CO–petrol (bp 60–80°) 3:1 [19] to remove neutral lipids. The plates were then developed in the same direction with $CHCl_3$–MeOH–HOAc–H_2O (137:21:21:6) [21] and the different phospholipids located by exposure to I_2 and spraying with ninhydrin or Dragendorff's reagent [19].

For quantitative determination of total phospholipids, the TLC plates were developed with Me_2CO–petrol only, the zone remaining at the origin scraped off and dissolved in $CHCl_3$–MeOH (2:1). The soln was made up to 1 ml with H_2O and digested with 0.5 ml 70% $HClO_4$ and using H_2O_2 to clarify the soln. The clear final soln was made up to 1.5 ml with H_2O and phosphate determined according to ref. [22].

For determination of radioactive precursors, incorporated into defined phospholipids, the zones of the chromatogram containing phosphatidylcholine or phosphatidylethanolamine were scraped off the TLC plates and their radioactivity was determined by scintillation counting.

Determination of free choline. Cotyledons (3 g) were boiled with 25 ml acetate buffer 0.1 M, pH 4.6, for 3 min and then ground and extracted for 15 min at 70°. After centrifugation, protein was removed with TCA, 7.5% final concn. After removal of the protein ppt. the soln was brought to pH 8.5 with ammonia and 2% Reineckes salt in MeOH added (1 ml/7.5 ml soln) and allowed to stand for 2 hr at 4°. This procedure differentiates between tertiary amines with and without a carboxyl group [23]. The concn of choline in the Reinecke salts of choline was determined spectrophotometrically [24]. Purity of the Reinecke salt of choline was determined by TLC [23, 25].

Assay of marker enzymes. Cytochrome *c* oxidase was determined by following the oxidation of reduced cytochrome *c* spectrophotometrically and cytochrome *c*–NADPH reductase by reduction of cytochrome *c* in the presence of NADPH and KCN [26]. ATPase was determined according to ref. [27] and the phosphate liberated estimated according to ref. [28].

Acknowledgement—This work was supported by a grant from the United States—Israel Binational Science Foundation, Jerusalem, Israel.

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