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THE PUTATIVE ROLE OF FATTY ACIDS IN THE ACTIVATION OF TREHALOSE SYNTHESIS IN THE FAT BODY OF THE COCKROACH *Periplaneta americana* L.

by

Irshad Ali

Department of Zoology

submitted in partial fulfilment
of the requirement for the degree of
Doctor of Philosophy

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The University of Western Ontario
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Abstract

Hypertrehalosemic hormones produced by the corpus cardiacum increase the concentration of several fatty acids in cockroach fat body trophocytes. The phospholipase A_2 (PLA_2) activator melittin has a similar effect. The fatty acids include palmitic, stearic, oleic and linoleic acid. The hormone mediated release of these fatty acids is blocked by the PLA_2 inhibitors mepacrine and ρ-bromophenacyl bromide. These observations suggest that hormonal release of fatty acids, may be due to the activation of PLA_2.

The increase in non-esterified fatty acids appears to be linked to the increase in trehalose efflux since sugar efflux is stimulated by stearic, oleic, linoleic and arachidonic acid. The increase in intracellular free fatty acids is also correlated with the activation of phosphorylase, the rate limiting step in trehalose synthesis.

This study is the first to show that conversion of linoleic acid to arachidonic acid is stimulated by the hypertrehalosemic hormones. The data suggest that the effect of arachidonic acid and its metabolites are dependent on an increase in production of lincleic, and possibly other fatty acids, which are converted into arachidonic acid.

The cyclooxygenase inhibitors indomethacin and diclofenac are effective inhibitors of hormone activated

trehalose efflux. This indicates the importance of the arachidonic acid metabolite pathway in the activation of trehalose efflux. Stimulation of trehalose efflux by prostaglandin-like metabolites from trophocyte homogenate challenged with hormone and also by prostaglandin $F_{2\alpha}$ and A_2 also suggests that arachidonic acid metabolites are involved in hormonal stimulation of trehalose efflux from the trophocytes. This is one of the first studies to demonstrate a link between a specific physiological function, hormonal activation of trehalose efflux, and the prostglandins.

Trehalose efflux is inhibited by the lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA). Unlike the cyclooxygenase inhibitors, NDGA appears to have a potent inhibitory effect on PLA_2 since it also inhibits production of fatty acids. Inhibition of trehalose efflux by NDGA may be due to the inhibition of fatty acid production rather than inhibition of lipoxygenase.

A novel discovery in this study was the potent inhibitory effect of fatty acids, particularly linoleic and arachidonic acid, on the trehalose-6-phosphatase reaction. The concentration of fatty acid required to produce 50 percent inhibition is within the range expected to accur *in vivo*. The results suggest that trehalose-6-phosphatase may be a key step in the regulation of trehalose efflux from the trophocyte to the hemolymph.

TO MY FATHER

The most unselfish man I have ever known. Who has toiled unselfishly throughout his life so that his children may be educated and become better human beings. I hope that the completion of this thesis, in a small way, allows him a measure of satisfaction that indeed he was successful.

TO MY MOTHER

She was always there in the moment of need.

TO MY WIFE

For all her love and affection and most importantly her patience.

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I would like to express by enormous gratitude to Dr. Steele for allowing me the privilege of doing my Ph.D. thesis in his laboratory. I was also grateful for the generous time he spent in guiding me throughout my study and the tremendous editorial help during the preparation of this thesis.

I would also like to thank the Zoology department for not only giving me the opportunity to do my thesis but also for the experience of a lifetime. I had an enjoyable time meeting all the people that I encountered in the department.

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No department can run efficiently without great secretaries. I sincerely believe that the Zoology department has the greatest secretaries in the world. I would like to thank Jane Sexsmith, Melina Buragina, Sherri Waring and specially Mary Martin who was exceptionally helpful whenever the need arose.

I would also like to thank my advisory committee members, Dr. Tam and Dr. Podesta for their guidance.

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AA	arachidonic acid
ASA	acetylsalicylic acid
ADP	adenosine diphosphate
AMP	adenosine 5'-monophosphate
BPB	ρ-bromophenacyl bromide
CC I	synthetic hypertrehalosemic hormone I
CC II	synthetic hypertrehalosemic hormone II
CCE	crude natural corpora cardiaca hypertrehalosemic hormone
CPM	counts per minute
DICLO	diclofenac
DMSO	dimethyl sulfoxide
EDTA	ethylenediamine tetraacetic acid
FFA	free fatty acid
FID	flame ionization detector
G1P	glucose-1-phosphate
G6P	glucose-6-phosphate
G1,6P	glucose-1,6-diphosphate
GPE	gland pair equivalents
GC	gas chromatography
H7	1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride
HEPES	N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid
MEPA	mepacrine
NADH	nicotinamide adenine dinucleotide phosphate

NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NDGA	nordihydroguaiaretic acid
OAG	1-oleoyl-2-acetylglycerol
PEA	pent 4-enoic acid
PG	prostaglandins
PGA_2	prostaglandin A_2
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PI	phosphatidyl inositol
PKC	protein kinase C
PL	phospholipid
PLA_2	phospholipase A_2
PLC	phospholipase C
SPG	spingosine
T	trehalose
T6P	trehalose-6-phosphate
TES	N-tris (hydroxymethyl) methyl-2-aminoethasulfonic acid)

Part 1

INTRODUCTION

A comparison of sugar in insect hemolymph and vertebrate blood reveals that its concentration is much higher in insect hemolymph. The concentration is generally greater than 0.5% but may even exceed 8% (Wyatt, 1967). The principal carbohydrate in the hemolymph of most, if not all species of insects is trehalose (α-D-glucopyranosyl-α-D-glucopyranoside), a non-reducing disaccharide. In honey bees this sugar may represent as much as 95% of the total carbohydrate during postembryonic development (Tsao and Shuel, 1973). Trehalose may also increase in concentration during development as described for the leafworm (*Spodoptera littoralis*) larva (Boctor, 1974). Values for hemolymph trehalose range from 0 in non-feeding *Celerio euporibae* larvae to 65 mg ml^{-1} (189mM) in *Anthophora* larvae (Wyatt, 1967). However, in certain insects the sugar may be absent or present in very low concentration (Evans and Diethier, 1957; Wimer, 1969; Bedford, 1977).

Glucose is also present in the hemolymph of many insects, but generally at much lower levels than trehalose (Wyatt, 1967). This monosaccharide is present in trace amounts in *Anisolabis littorea* (Bedford, 1977) but ranges as high as 12.1 mM in *Locusta migratoria* (Jutsum and Goldsworthy, 1976).

Other sugars reported from insect hemolymph include arabinose, cellobiose, ribose and sucrose (Wyatt, 1967; Florkin and Jeauniaux, 1974; Phillipe *et al.*, 1976). In many instances, the type of sugar found in the hemolymph reflects that contained in the diet (Hansen, 1964; Maurizio, 1965). In addition to these sugars other carbohydrates or their derivatives have been reported to occur in hemolymph. These include: hexosamine (N-acetylgalactosamine and N-acetylglucosamine) in *Blaberus craniifer* (Philippe *et al.*, 1976) and other insects (Florkin and Jeauniaux, 1974), ß-glucosyl-O-tyrosine in silkworm pupae, *Leucania separata*, and *Drosophola buskii* larvae (Chen *et al.*, 1978; Isobe *et al.*, 1981), and glucuronic acid and inositol in *Anopheles stephensi* (Mack *et al.*, 1979). Glycogen may also be present in hemolymph but in low concentration (Wyatt, 1967; Chippendale, 1973).

The fat body is the major site of trehalose synthesis (Jungreis and Wyatt, 1972; Friedman, 1978). This organ removes ingested glucose from the hemolymph very rapidly. For example, radiolabelled glucose injected into *Periplaneta americana* is rapidly removed (50%, 20 min post-injection) and converted to trehalose and finally stored as glycogen in the fat body (Spring *et al.*, 1977). The glycogen stores are mobilized from the fat body under various physiological conditions (Matthews and Downer, 1974).

Hemolymph carbohydrate levels are subject to hormonal

control (Friedman, 1978). Despite this the amount of carbohydrate in the hemolymph has been shown to fluctuate under various conditions. Daily fluctuations in hemolymph sugar levels have been reported in *Periplaneta americana* (Hilliard and Butz, 1969), possibly as a result of diurnal changes in the titre of hormones. During flight certain insects draw upon the carbohydrate pool as an initial energy source; as a result the trehalose level declines (Bailey, 1975; Kammer and Heinrich, 1978; Jutsum and Goldsworthy, 1976; Lim and Lee, 1981). Starvation will also cause a significant reduction of hemolymph carbohydrate in *Manduca sexta*, *Locusta migratoria* and *Oxya japonica* (Dahlman, 1973; Mwangi and Goldsworthy, 1977; Lim and Lee, 1981). Hyperglycemia on the other hand can be the result of various forms of anaesthesia, saline injection, or handling (without anaesthesia) (Matthews and Downer, 1973; Hanaoka and Takahashi, 1976) and is induced by various amines, most notably octopamine (Downer, 1979).

The employment of a disaccharide by the insect as the principal sugar in the hemolymph serves two important functions. First the large size of the molecule relative to that of the monosaccharide, glucose, permits higher concentrations to be retained in the hemolymph by virtue of the slower rate of diffusion through cell membranes. The diffusion of trehalose through cell membranes has been determined to be significantly lower than the rate of

diffusion of glucose (Knowles, 1975). Secondly, trehalose synthesis facilitates the transfer of glucose from gut to fat body where glucose is converted to trehalose or glycogen. The trehalose synthesis pathway, because of its high activity, ensures that glucose is maintained at a low level in the hemolymph. A steep gradient, therefore, favours the uptake of glucose from the gut to the haemolymph (Treherne, 1958).

Trehalose in the haemolymph serves as a convenient vehicle for the transport of glucosyl residues from the fat body to the tissues where metabolism occurs. Trehalose is mobilized for various purposes such as resynthesis of the chitinous exoskeleton after each ecdysis. When ecdysis takes place, the chitin framework of the new cuticle, a polymer of acetylated glucosamine residues, must be resynthesized (Neville, 1975). The glucosamine residues incorporated into chitin are believed to originate mainly from the glucosyl residues of fat body glycogen via trehalose in the hemolymph. This conclusion is supported by the finding that glycogen reserves in the larval fat body of the silkmoth, *Bombyx mori*, decrease rapidly as the synthesis of new cuticle proceeds (Zaluska, 1959; Pant and Kumar, 1979). In other insects, for example, *Locusta migratoria*, carbohydrate is an important reserve of energy used during flight (Van Der Horst *et al.*, 1978) and that fat body glycogen is a major source of this carbohydrate (Van

Marrewjik *et al.*, 1980). This is also true for the cockroach (Downer and Parker, 1979).

At the site of utilization, for example muscle, trehalose is hydrolysed by the enzyme trehalase, giving rise to two glucose molecules which are then available for oxidation to provide energy. It is generally agreed that muscle and tissue trehalases are particulate and that the soluble enzyme is of hemolymph origin (Beenakkers *et al.*, 1985). Friedman and Alexander (1971) have shown the soluble enzyme from the flight muscle of *Phormia regina* to be electrophoretically distinct from hemolymph trehalase. Reed and Sacktor (1971) and Duve (1975) respectively found that particulate trehalase of *Phormia regina* and *Calliphora erythrocephala*, is concentrated in the mitochondrial fraction . In the fleshfly, *Sarcophaga barbata*, trehalase seems to be localized in the mitchondria (Sacktor, 1975) as in the honeybee (Brandt and Huber, 1979). Differential centrifugation of the flight muscles of the migratory locust, *Locusta migratoria*, showed trehalase to be present mainly in the microsomal fraction (Worm, 1981). A similar localization is also claimed for the silkmoth *Hyalophora cecropia* and the cockroach *Blaberus discoidalis*, as well as nearly every other tissue so far examined (Derr and Randall, 1966; Friedman, 1975; Gilby *et al.*, 1967) including the fat body of *Periplaneta americana* (Storey and Bailey, 1978).

1.1 Synthesis of Trehalose

Figure 1 illustrates the biochemical pathway of trehalose synthesis in the fat body. The enzymatic reactions associated with the pathway were described first for the locust (*Schistocerca gregaria*) (Candy and Kilby, 1961) and subsequently in much greater detail for the larva of the silkmoth, *Hyalophora cecropia* (Murphy and Wyatt, 1965). The two major substrates for the synthesis of trehalose *in vivo* are glycogen stored in the fat body and glucose derived from the diet (Jungreis and Wyatt, 1972; Friedman, 1978; Spring *et al.*, 1977). As shown in Figure 1, the glucose moiety of uridine diphosphoglucose (UDPG) is transferred to glucose 6-phosphate (G6P) by trehalose 6-phosphate synthase (EC 2.4.1.15) to form trehalose 6-phosphate (T6P). The phosphate group of T6P is removed by trehalose 6-phosphatase (EC 3.1.3.12) to yield free trehalose (Candy and Kilby, 1961). Since the cell contains only catalytic quantities of UDPG, it must be regenerated from UDP following each glycosylation step in a reaction which requires ATP.

A detailed study of trehalose synthesis in *Hyalophora cecropia* fat body (Murphy and Wyatt, 1965) demonstrated that the relative rates of glycogen and trehalose biosynthesis are determined by the concentration of intermediates and the particular kinetic properties of glycogen synthase and T6P

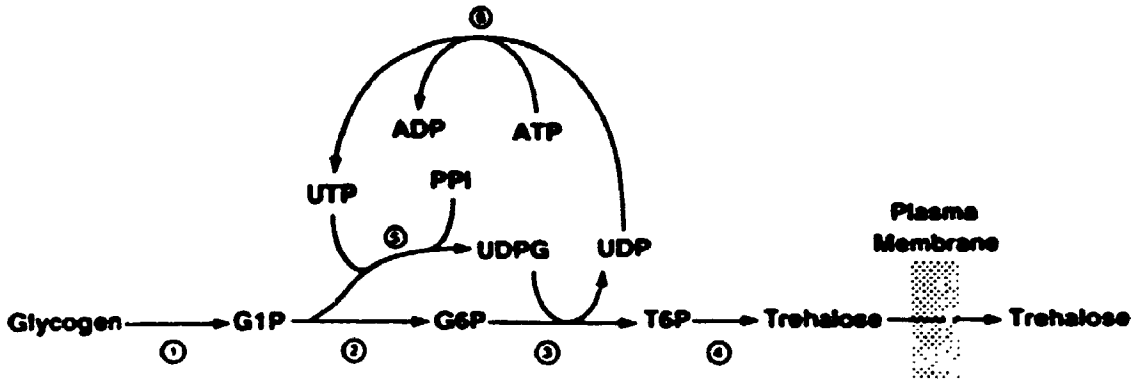


Figure 1. The trehalose biosynthetic pathway in *Periplaneta americana* fat body. The enzymes indicated by the circled numbers are, 1. Glycogen phosphorylase. 2. Phosphoglucomutase. 3. Trehalose 6-phosphate synthase. 4. Trehalose 6-phosphatase, 5. UDP-glucose phosphorylase. 6. Nucleoside diphosphokinase.

synthase which act in opposition to each other. G6P activates T6P synthase and also serves as the substrate for trehalose synthesis. This reaction is inhibited by free trehalose, a finding confirmed by Friedman (1967) who succeeded in demonstrating feedback inhibition of the T6P synthase reaction in *Phormia regina* fat body using physiological concentrations of trehalose. Interestingly, Friedman (1967) also demonstrated that trehalose increased the activity of trehalose 6-phosphatase, which in *Phormia regina* also exhibits significant hydrolytic activity towards G6P. Friedman (1971) found that the addition of 16.6 mM trehalose to the incubation medium containing intact fat body tissue not only increased the hydrolysis of G6P by 350 percent but also inhibited the synthesis of trehalose in *Phormia regina*. These results suggest that inhibition of trehalose synthesis is a complex interaction between T6P and the end product trehalose, in which there is activation of the G6P hydrolytic site. This allows the enzyme to remove one of the substrates of the trehalose synthesis reaction.

Murphy and Wyatt (1965), have suggested that normal trehalose levels in the haemolymph are probably regulated by feedback control of T6P synthase. This idea agrees with that of Friedman (1970) who showed that 8 mM trehalose inhibited T6P synthase by 70 percent. Moreover, this concentration of trehalose is well below that present in the

haemolymph. Those studies suggest that in fat body feedback inhibition of T6P synthase due to trehalose is overcome by an increase in G6P formed from glycogen. This idea is further supported by the finding that inhibition of T6P synthase is partially decreased by the presence of G6P (Murphy and Wyatt, 1965).

1.2 Hormonal Control of Trehalose Synthesis

The stimulatory effect of the *corpus cardiacum* on trehalose concentration in the haemolymph was first demonstrated in the cockroach, *Periplaneta americana* (Steele, 1961), an observation that has now been extended to include other species belonging to different taxonomic groups (Steele, 1983). These include the blowfly, *Phormia regina* (Friedman, 1967), the stick insect, *Carausius morosus* (Dutrieu and Gourdoux, 1967) and the tobacco hornworm, *Manduca sexta* (Ziegler, 1979). A hypertrehalosemic response to CC extract has also been demonstrated *in vitro* using the fat body of the cockroach, *Leucophaea maderae* (Wiens and Gilbert, 1967) and *Periplaneta americana* (McClure and Steele, 1981).

The hypertrehalosemic activity of CC extracts prepared from *Periplaneta americana* results from the action of two polypeptides contained in the glands which have been designated CC I and CC II (Scarborough *et al.*, 1984). Both polypeptides are blocked and consist of eight amino acids,

five of which are common to both. These peptides represent members of a family of invertebrate peptides that include locust adipokinetic hormone and crustacean red-pigment concentrating hormone. Both peptides are C- and N-terminally blocked, and exhibit a high degree of peptide sequence homology with other members of the adipokinetic/crustacean red-pigment concentrating hormone family (Scarborough *et al.*, 1984). All members of the family possess pGlu-1, Phe-4 and Trp-8, in addition to amidated C-terminals. In four members of the group (adipokenitic hormones I, II-S, II-L and the red-pigment concentrating hormone) the first four amino acids from the N-terminus are identical while the remaining members of the group display conservative changes. Most variability is seen in position 6 where one of four amino acids may occur. The sequence homology of these peptides especially that of CC II is remarkably similar to that of the NH_2-terminal portion of glucagon (Scarborough *et al.*, 1984; Ziegler *et al.*, 1985).

Hemolymph trehalose in the American cockroach, *Periplaneta americana*, increases by as much as 300% following injection of CC extract (Steele, 1961, 1963; Bowers and Friedman, 1963; Hanaoka and Takahashi, 1976). CC extracts elevate trehalose levels in the hemolymph of many species belonging to different orders. These include *Blaberus discoidalis* (Bowers, W. and Friedman, S., 1963),

Phormia regina (Friedman, 1967), *Carausius morosus* (Dutrieu and Gourdoux, 1967), *Calliphora erythrocephala* (Norman and Duve, 1969), *Locusta migratoria* (Goldsworthy, 1969), and *Manduca sexta* larvae (Ziegler, 1979). The CC factors are not species-specific since those prepared from *Locusta migratoria* and *Carausius morosus*, although having little or no effect in the donor species, are very active when assayed in *Periplaneta americana* (Gade, 1979,1980). Similarly, extracts prepared from the head of *Apis mellifera* cause hypertrehalosemia in *Periplaneta americana* (Van Norstrand *et al.*, 1980) but whether they have an effect in the bee itself is not known. These findings place in question the role of hypertrehalosemic factors in insects that respond weakly or not at all to the factor. The hypertrehalosemic response to CC extract can be elicited *in vitro* by incubating isolated fat body in Ringer solution (Wiens and Gilbert, 1967a, McClure and Steele, 1981). Although head ligation of adult *Periplaneta americana* (Spring *et al.*, 1977) and *Sarcophaga bullata* (Seligman *et al.*, 1969) as well as cardiacectomy of *Locusta migratoria* (Cazal, 1971) lowers trehalose in the haemolymph a definitive proof that normal levels of trehalose are maintained by the action of the CC is lacking. Nevertheless, the physiological role of the hypertrehalosemic factor seems to be associated with regulation of hemolymph trehalose.

1.3 Mode of Action of hypertrehalosemic hormone

The action of the hypertrehalosemic hormones in carbohydrate utilizing species such as the American cockroach, *Periplaneta americana*, results in the stimulation of trehalose efflux by the fat body (Steele, 1961). This effect has been shown in other species such as the blowfly, *Phormia regina* (Friedman, 1967), the stick insect, *Carausius morosus* (Dutrieu and Gourdoux, 1967) and the tobacco hornworm, *Manduca sexta* (Ziegler, 1979). In lipid utilizing species, the release and oxidation of lipid is stimulated by adipokinetic hormone (AKH) from the corpora cardiaca. In the adult locust, *Schistocerca gregaria*, AKH stimulates the release of diacylglyceride from the fat body and increases the oxidation of lipid by the flight muscles in preference to carbohydrates (Mayer and Candy, 1969; Robinson and Goldsworthy, 1974). CC from *Tenebrio molitor* and *Schistocerca gregaria* are interchangeable with respect to the stimulation of lipid mobilization in these species. Injection of CC extract from *Periplaneta americana* into *Locusta migratoria* causes lipid release but, interestingly, the fat body of *Periplaneta americana* does not release lipid in response to either its own CC or to gland extracts prepared from the locust (Goldsworthy *et al.*, 1972). In contrast to other insects the CC appear to stimulate the deposition of triacylglycerol in the fat body of the cockroach (Downer and Steele, 1972).

1.3.1 Activation of glycogenolysis

Numerous studies describing hormonal stimulation of trehalose synthesis in fat body have shown that the hypertrehalosemic factors activate phosphorylase (Steele, 1963; Wiens and Gilbert, 1967; Goldsworthy, 1970). The decrease in glycogen as trehalose increases (Steele, 1963; Hanaoka and Takahashi, 1976), supports this view. Trehalose released from the fat body to the external medium *in vitro* by the hypertrehalosemic hormone represents *de novo* synthesis (Steele and Hall, 1985). The synthesis of trehalose from exogenous glucose is not affected by the specific phosphorylase inhibitor 5-gluconolactone. Furthermore, the synthesis of trehalose in the presence of 5-gluconolactone is not stimulated by CC extract (Steele and Hall, 1985), clearly showing that the trehalose synthase reaction is not hormonally activated. These results support the hypothesis that the hormone initiates the release of glucosyl residues from glycogen by activation of phosphorylase.

Most, or all tissues of the insect contain glycogen but that in the fat body is quantitatively the most important because of the large tissue mass. Glycogen, a polymer of glucosyl residues derived from dietary glucose and other carbohydrates, is the principal form in which carbohydrate is stored when intake exceeds the metabolic demands of the insect. Unlike the remaining major glycogen reserve in the

musculature that in the fat body is readily depolymerized and distributed to other tissues (Steele, 1985). The glycogen content of fat body is extremely variable and depends on the nutritional state of the insect. In the cockroach *Periplaneta americana*, for example, the range is usually between 5 and 20 mg/g (Steele, 1963). The utilization of fat body glycogen, at least in certain insects, is under hormonal control since it is readily depleted by injection of CC extract and converted into trehalose which passes into the haemolymph. In *Periplaneta americana* the additional trehalose that enters the haemolymph following injection of CC extract can be accounted for by the loss of glycogen from the tissue (Steele, 1963; Bowers and Friedman, 1963; Hanaoka and Takahashi, 1976). Both hemolymph trehalose and fat body glycogen eventually return to the original levels, thus giving further evidence that the hormone stimulated increment in trehalose is derived entirely from glycogen.

The CC factor acts directly on the fat body since the glycogenolytic effect and accompanying trehalose efflux can be demonstrated *in vitro* (Wiens and Gilbert, 1967a). The studies cited above give no proof that the CC factor is actually released *in vivo*. Nevertheless, the study by Downer and Parker (1979) showing that glycogen in the thoracic fat body is reduced from 8.36g/mg to 2.3g/mg during 10 min flight suggests that release of the factor may occur.

The rate limiting step in the pathway between glycogen and trehalose stimulated by CC extract is catalysed by phosphorylase (Steele and Hall, 1985) although activation of this enzyme does not always cause hemolymph trehalose to increase.

1.3.2 Second messenger system

Several studies have been carried out to determine the mode of action of CC hormones on the fat body. Various studies (Hanaoka and Takahashi, 1977; Gade, 1977; Orr *et al.*, 1985) have shown that CC extract increases cAMP in fat body. Although exogenous cAMP stimulates phosphorylase in intact tissue (Steele, 1964; McClure and Steele, 1981), no study has yet succeeded in showing a stimulatory effect of cAMP on phosphorylase or phosphorylase kinase in cell free extract of insect fat body (Ashida and Wyatt, 1979). And since Orr *et al.* (1988) showed that neither of the hypertrehalosemic peptides elevate cyclic AMP levels, thus the action of these hormones is not mediated by this cyclic nucleotide.

The stimulation of trehalose efflux by CC extract is absolutely dependent on extracellular Ca^{2+} (Steele and Paul, 1985). The removal of Ca^{2+} from the incubation medium prevents stimulation of trehalose efflux. These studies led Steele and Paul (1985), to conclude that the hypertrehalosemic hormones initiate an influx of Ca^{2+} into

the cell.

The majority of Ca^{2+} mediated processes are activated by phosphotidylinositol second messenger systems (Berridge, 1987). Phosphotidylinositol (PIP_2) is a trace membrane phospholipid which can be hydrolysed to generate two second messengers, inositol trisphosphate (IP_3), and diacylglycerol (DG). Inositol phosphates (IP_3, IP_4, and other species) raise intracellular Ca^{2+} levels (Berridge, 1987) whereas DG activates protein kinase C (PKC) which modulates the activity of several enzymes (Kikkawa and Nishizuka, 1986).

There is much interest in receptor-stimulated PIP_2-specific PLC (phospholipase C) because it provides a link between the occupation of cell-surface receptors and formation of the intracellular messengers IP_3 and diacylglycerol which regulate intracellular Ca^{2+} and the activity of protein kinase C (Berridge, 1987; Nishizuka, 1988). Although several mammalian PLCs have now been identified and purified (Ryu *et al.*, 1987), the relationship between these enzymes and the reaction stimulated by the G proteins is unknown. In insects, in contrast to vertebrates, G proteins have been shown to couple activated receptors to PLC for both hormone and light-regulated phosphoinositide hydrolysis (Payne, 19' Indeed, the first convincing demonstration of an interaction between a receptor and a G protein in any animal was in blow fly salivary gland where serotonin and a stable analogue of GTP

were shown to synergistically stimulate PLC activity (Litosch *et al.*, 1985). Subsequent studies have provided further evidence: Light stimulates GTPase activity in *Musca domestica* and *Manduca sexta* photoreceptors (Blumenfeld *et al.*, 1985); AlF_4 and stable analogues of GTP have effects similar to light on the photoreceptor potential (Minke and Stephenson, 1985); and light stimulates covalent binding of a GTP analogue to a 41-kd protein in *Musca* photoreceptors (Bentrop and Paulsen, 1986; Devary *et al.*, 1987). Vroemen *et al.* (1995) have found that AKH receptor(s) are coupled to cAMP formation and glycogen phosphorylase activation via the stimulatory guanine nucleotide-binding protein (G(s)). The G proteins, therefore, may be linked to the activity of several enzymes in the invertebrates.

Blowfly salivary gland has provided a model system from which important results have been obtained. The increase in intracellular Ca^{2+} that follows activation of this class of receptors typically involves the release of Ca^{2+} from intracellular stores as well as entry at the plasma membrane. The first demonstration that Ca^{2+} entry requires phosphoinositide hydrolysis came from experiments that exploited two unusual features of blowfly salivary gland- the small size of the intracellular free inositol pool, and the depletion of that pool during hormonal stimulation. During prolonged stimulation of the glands with serotonin, Ca^{2+} entry eventually wanes as inositol is lost and can

presumably no longer maintain the pool of PIP_2. When exogenous inositol is provided, the cells resynthesize PIP_2, and serotonin-stimulated Ca^{2+} entry is restored (Berridge and Fain, 1979; Fain and Berridge, 1979a; Fain and Berridge, 1979b).

The precise role of the various inositol phosphates in regulating Ca^{2+} entry is not clear (Irvine, 1989). Blowfly salivary gland was one of the first tissues in which hydrolysis of PIP_2 was shown to be the initial hormone-stimulated reaction (Berridge, 1983; Berridge *et al.*, 1984), thus prompting the experiments that demonstrated the mobilization of intracellular Ca^{2+} stores by $Ins(1,4,5)P_2$. This and other evidence suggests that hormones stimulate formation of $Ins(1,4,5)P_3$ in insects (Trimmer and Berridge, 1985; Tublitz, 1988). Hormones stimulate the mobilization of intracellular Ca^{2+} (Galione and Berridge, 1988; House and Ginsborg, 1982; Prince and Berridge, 1973) following formation of $Ins(1,4,5)P_3$ (Berridge *et al.*, 1984), so that the Ca^{2+} signal depends on phosphoinositide hydrolysis (Berridge and Fain, 1979; Fain and Berridge, 1979a; Fain and Berridge, 1979b). However, direct demonstration of the effects of $Ins(1,4,5)P_3$ on the intracellular Ca^{2+} stores of insects is so far restricted to reports that $Ins(1,4,5)P_3$, increases heart rate in transiently permeablized cardiac muscle cells of *Manduca sexta* (Tublitz, 1988) and mimics the effect of light *Musca domestica* photoreceptors (Devary *et*

al., 1987).

Involvement of the IP_3 second messenger system in the action of the hypertrehalosemic hormones is suggested by the need for extracellular Ca^{2+} in the stimulation of trehalose efflux. This hypothesis is further strengthened by the recent finding that CC hormones substantially increase intracellular IP_3 levels (Brown, 1990). Thus it appears that the elevation of intracellular Ca^{2+} may be linked to an increase in inositol phosphates. The finding by Jahagirdar *et al.* (1987) that the hypertrehalosemic peptides significantly increase intracellular Ca^{2+} in haemocytes further strengthens this hypothesis. The elevation of intracellular Ca^{2+} appears necessary for the stimulation of trehalose synthesis by the hormone but elevation of Ca^{2+} alone is not sufficient. The Ca^{2+} ionophore A23187 (Reed and Lardy, 1972), causes substantial activation of phosphorylase but there is little or no trehalose efflux (McClure and Steele, 1981). Thus the release of trehalose appears to be calcium mediated although other factors may also be necessary.

It is possible that hormonal activation of trehalose synthesis may require activation of the other limb of the phosphoinositide signalling pathway. Evidence, however, for the participation of other limb of the phosphoinositide signalling pathway, which involves activation of protein kinase C by diacylglycerol (Nishizuka, 1988) is scant in

insects. Formation of diacylglycerol has been demonstrated in blowfly salivary glands (Litosch *et al.*, 1982). Stable analogues of diacylglycerol, the phorbol diesters, specifically bind to locust and cockroach nervous systems (Knipper and Beer, 1987; Kuo *et al.*, 1980) and, in common with the mammalian systems, they stabilize the association of protein kinase C with the plasma membrane (Orr *et al.*, 1988). However, stimulation of protein kinase C activity has not been demonstrated. The only reported physiological effect of phorbol esters in an insect is its enhancement of basal and octopamine-stimulated cAMP formation in an insect cell line of *Choristoneura fumiferana* (IBRI-CF1)(Orr *et al.*, 1988).

1.3.3 Arachidonic acid metabolism

In many tissues DG is hydrolysed to yield unsaturated fatty acids which may be further metabolized to numerous active species. The activated cell not only releases unsaturated fatty acids from phosphatidylinositol, but also from phosphatidylcholine, phosphatidylethanolamine, and phosphatidic acid (Broekman, 1986) by the action of the Ca^{2+} activated phospholipase A_2 (Irvine, 1982). In mammalian systems the most prevalent unsaturated fatty acid released by the activated phospholipase is arachidonic acid. Arachidonic acid, however, is not the only fatty acid released from phospholipids. Stearic and oleic acid

(Broekman, 1986) and other fatty acids may also be released concomitantly by the activated cell.

Some studies suggest that fatty acids may directly modulate enzymatic activity or other cellular processes (Ordway *et al.*, 1991). A plethora of studies however indicate that it is not the fatty acids themselves but their metabolites which are potent mediators of cellular activity (Chang *et al.*, 1987; Needleman *et al.*, 1986; Irvine, 1982). The release of the fatty acid is the rate-limiting step in the formation of several metabolites (Chang *et al.*, 1987), either by the cyclooxygenase or lipoxygenase pathways, to produce a series of prostaglandins and thromboxanes or leukotrienes respectively (Needleman *et al.*, 1986). Arachidonic acid is present in low concentration in *Musca* (Wakayama *et al.*, 1986a) and is formed after serotonin stimulation of blowfly salivary gland (Litosch, *et al.*, 1982). Both prostaglandin synthase (Wakayama *et al.*, 1986b) and the prostaglandins (Brady, 1983) are present in insects, but the physiological role of these potential messengers is largely unexplored.

Destaphano *et al.* (1974) were the first to show synthesis of PGE_2 in insects. More recently Stanley-Samuelson and Ogg (1994) have shown that tobacco hornworm fat body microsomal-enriched preparations synthesize four prostaglandins, PGA_2, PGE_2, $PGF_{2\alpha}$ and PGD_2, of which prostaglandin A^2 was the major product. $PGF_{2\alpha}$ is synthesized

by the contents of the spermatophore of the cricket, *Teleogryllus commodus* (Loher *et al.*, 1981; Tobe and Loher, 1983). Lange (1984) observed biosynthesis of PGE_2 and $PGF_{2\alpha}$ by opalescent glands, seminal vesicles and spermatophore contents of the locust *Locusta migratoria*. Wakayama *et al.* (1986c), also characterized PG synthesis in reproductive tracts and whole adults of the housefly *Musca domestica* as did Brenner and Bernasconi (1989) in the gonads of the blood sucking insect *Triatoma infestans*. Knowledge of these systems has been extended by the demonstration that lipoxygenase activity and low cyclooxygenase activity has been detected in reproductive tissues of the firebrat *Thermobia domestica* (Ragab *et al.*, 1987). Prostaglandins have also been detected in other insect species, albeit, without studies of PG biosynthesis (Stanley-Samuelson and Loher, 1986; Stanley-Samuelson, 1993,1994). It is reasonable therefore to assume that most, if not all insects, are competent to synthesize prostaglandins and other eicosanoids.

The insect enzymes that synthesize eicosanoids appear similar to their mammalian counterparts because they are inhibited by similar compounds. Tobe and Loher (1983) found that 0.5 mM aspirin decreased PG biosynthesis in the spermatophore by more than 90%. Wakayama *et al.* (1986c) also found that several anti-inflammatory drugs inhibited prostaglandin synthesis in housefly. Naproxen,

acetaminophen, aspirin and indomethacin are all effective prostaglandin synthase inhibitors but exhibit tissue-dependent potencies (Wakawama *et al.*, 1986c). For example, indomethacin did not inhibit prostaglandin synthesis in preparations of male reproductive tract of the house cricket (Destaphano *et al.*, 1974). Variation in sensitivity to the same inhibitor indicates that prostaglandin synthesis differs substantially among animals (Stanley-Samuelson and Ogg, 1994). The evidence suggests that the activity of the lipoxygenase pathway is influenced by that of the cyclooxygenase pathway and vice versa. Incubation of fat body *in vitro* with naproxen results in the synthesis of hydroxyeicosatetraenoic acids (HETEs) which are lipoxygenase metabolites of arachidonic acid (Stanley-Samuelson and Ogg, 1994). In the absence of naproxen very little HETEs are produced whereas at high concentrations of naproxen as much as 4000pmole/mg/ml are found. Fat body also produces HETEs in the presence of indomethacin but less than that found with naproxen (Stanley-Samuelson and Ogg, 1994). These results are consistent with the fact that indomethacin inhibits cyclooxygenase as well as lipoxygenase in the fat body. Increased production of lipoxygenase products in the presence of cyclooxygenase inhibitors is also a characteristic of the mammalian models (Salari *et al.*, 1984). This allows one to conclude that it is substrate availability rather than stimulation of lipoxygenase

activity that gives rise to lipoxygenase products in the presence of cyclooxygenase inhibitors.

1.4 Lipid mobilising action of hypertrehalosemic hormones

The release and oxidation of lipid in certain insects is stimulated by adipokinetic hormone (AKH) from the corpora cardiaca. Mayer and Candy (1969) showed that CC extracts from *Schistocerca gregaria* elevate haemolymph lipid. The increase in lipid is mainly in the diacylglycerol fraction which is bound to a hemolymph protein. Interestingly, injection of *Periplaneta americana* CC extract which increases hemolymph trehalose in the same species stimulates lipid release from the fat body of *Locusta migratoria*. The fat body of the cockroach does not release lipids in response to either its own CC or to CC taken from the locust (Downer, 1972).

There is some data in the literature to suggest that lipid is mobilized in the fat body by hormones for use within that tissue as a source of energy. The synthesis of trehalose is an energy dependent process because UDPG is required as an intermediate precursor (Candy and Kilby, 1961). Since the cell contains only catalytic quantities of UDPG, it must be regenerated following each glycosylation step for which it requires ATP. The energy requirement for trehalose synthesis is supported by the observation that there is a strong correlation between trehalose production

and O_2 consumption in the fat body (Wiens and Gilbert, 1965). The expenditure of energy for the synthesis of trehalose is also described by McDougall and Steele (1988) who found both trehalose synthesis and O_2 consumption of *Periplaneta americana* fat body increased by 120% & 70% respectively.

Important questions regarding the source of energy for the synthesis of trehalose remain unanswered. A significant step in this direction was the finding by Wiens and Gilbert (1965) that CC stimulation of *Leucophaea maderae* fat body causes a shift in metabolism towards greater oxidation of fatty acids and a decrease in that of carbohydrate which is reflected in a lower value for the R.Q. (Wiens and Gilbert, 1965). Gourdoux (1980) found that CC extracts strongly divert glucose from the pentose phosphate pathway, which is very active in *Tenebrio molitor*. Gourdoux *et al.*, (1985) also found that CC extracts divert glucose from the pentose phosphate pathway and that glucose oxidation is reduced. The finding by McDougall and Steele (1988) that stimulation of trehalose synthesis is accompanied by a decrease in the oxidation of [6-^{14}C]glucose (22%) and an increase in the oxidation of [1-^{14}C]oleic acid (106%) indicates that lipids are a potential source of energy.

There are no studies that show directly the release of fatty acids by the hypertrehalosemic hormones. However, indirect evidence as described above suggest that the

generation of energy from free fatty acids may be very important. This view is supported by the finding that pent-4-enoic acid (PEA), an inhibitor of ß-oxidation owing to its action on the ß-ketothiolase reaction (DeRenzo *et al.*, 1958), inhibited trehalose production by fat body *in vitro* by 32% (McDougall and Steele, 1988). Trehalose synthesis was stimulated 113% by CC extract but only by 58% if 1 mM PEA was included with the extract. This strengthens the hypothesis that fatty acids are mobilized and oxidized by the hypertrehalosemic hormones. ATP production (estimated from O_2 consumption) however was 3-fold greater than that required to account for the synthesis of trehalose (McDougall and Steele, 1988) . The data suggest that CC extract stimulates energy dependent reactions in addition to those required for trehalose synthesis. It also supports the proposal that fatty acids are an important source of energy for CC stimulated trehalose synthesis.

1.4 Unsolved problems

One of the enzymes that the corpora cardiaca hormones activate is glycogen phosphorylase (Steele, 1963; Wiens and Gilbert, 1967; Goldsworthy, 1970). The activation of this enzyme is critical to the stimulation of trehalose synthesis and efflux since it provides the glucosyl residues necessary to synthesize trehalose. Nevertheless, the fact that activation of glycogen phosphorylase by cAMP (Steele *et al.*,

1988), the calcium ionophore, A23187 (McClure and Steele, 1981), and methylxanthines (Steele *et al.*, 1988) results in little or no trehalose efflux suggests that sites other than phosphorylase may be regulated during trehalose synthesis.

1.5 Proposed work

It is intended that this study extend our knowledge of the mode of action of the hypertrehalosemic hormones. This will be done by asking the following questions:

1. Is the protein kinase C pathway involved in the mode of action of the hypertrehalosemic hormones?
2. Do free fatty acids play a role in the stimulation of trehalose production by the hypertrehalosemic hormones.
3. Are arachidonic acid metabolites associated with the signal transduction mechanism employed by the hypertrehalosemic hormones?

Part 2

MATERIALS AND METHODS

2.1 INSECTS

Young adult male American cockroaches, *Periplaneta americana*, were obtained from a colony which has been maintained for many years in the laboratory. The cockroaches were reared in an insectary maintained at 30±2°C and a relative humidity of 60±10%. The lights in the insectary were turned on automatically each morning at 7:00 am and remained on for 12h. Water and ground Purina Dog Chow ® supplemented with 10% sucrose (w/w) were provided *ad libitum*.

The cockroaches were neck-ligated in late afternoon for use the following morning and held in an atmosphere of high humidity until required in order to prevent desiccation.

2.2 MATERIALS

The inhibitors, indomethacin, acetylsalicylic acid, nordihydroguaiaretic acid, diclofenac, ρ-bromophenacyl bromide, mepacrine, sphingosine, H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride and the phospholipase A_2 activator melittin were purchased from Sigma Chemical Co., St. Louis, Missouri.

Fatty acids, fatty acid methyl esters and prostaglandin standards were purchased from Sigma Chemical Co., St. Louis,

Missouri and BIOMOL Research Laboratories, Inc., Plymouth Meeting, Pensylvania.

The enzymes phosphoglucomutase, glucose-6-phosphate dehydrogenase and collagenase A were purchased from Boehringer Mannheim Canada Ltd., Dorval, Quebec.

α,α,α-Trifluoro-m-toluidine, methyl iodide, silver oxide, methyl propionate and 1-methyl-3-nitro-1-nitrosoguanidine were purchased from Aldrich. 4',5'-Dibromofluorescein was purchased from Eastman Kodak Co., Rochester, N.Y.

All solvents, including chloroform, methanol and acetonitrile, were purchased from Fisher Scientific Co., Toronto. The scintillation fluid, BCS (Biodegradable Counting Scintillant) was purchased from Amersham Canada Ltd., Oakville, Ontario.

2.3 Hypertrehalosemic Hormones

Natural hormone was prepared by making a crude extract of the *corpora cardiaca*. The glands were dissected free from the brain and homogenized in 0.5 ml of 0.9% NaCl at 0°C with 15 strokes of the pestle of a small Potter-Elvehjem homogenizer. The homogenate was then transferred to a centrifuge tube and both the homogenizer tube and pestle rinsed with 2 x 0.5 ml of 0.9% NaCl. The washings were combined with the original sample and the tube placed in a boiling water bath for 1 min to denature any proteases

present. After centrifugation at 1200g x 5 min to remove denatured protein the extract was diluted with 0.9% NaCl to yield a concentration of 10 gland pairs/ml and stored at -20°C. Since the exact quantity of each hypertrehalosemic peptide in the crude preparation was not known, the amount of CC extract used was expressed as gland pair equivalents (GPE).

The synthetic hormones CC I and CC II were a gift, obtained from Dr. D.A. Schooley, Zoecon Corporation, California. A 1 mM stock solution in 50% ethanol was prepared and stored at -20°C in a polyethylene tube.

2.4 Salines

During the course of this investigation there was a need to use physiological salines of different compositions. The need arose due to the fact that both fat body and dispersed cells were used in this study. The physiological salines have been optimized for each of the tissues, and since each consisted of different compositions, different salines were prepared. Salines I and II were used for fat body incubations, whereas, salines III and IV were utilized for the dispersed cells. Saline II was used when fat body lobes were pre-incubated with various agents, whereas, saline I was employed to determine trehalose efflux from fat body under various treatments. The addition of trehalose to the saline has a sparing effect on the fat body glycogen as

indicated by the significantly higher trehalose efflux by the fat body lobes pre-incubated with trehalose in the incubation medium. Saline III was used to perform most experiments using dispersed cells except those involving determination of trehalose efflux where saline IV was used. The composition of these salines is described below.

1) Physiological Saline I. This saline contained 154 mM NaCl; 8 mM KCl; 2 mM $CaCl_2$; 3 mM $MgCl_2$; and 5 mM HEPES buffer. All components were combined in a volume equal to approximately 90 percent of the final volume and the pH adjusted to 7.4 with 1N NaOH. Distilled water was then added to give the desired final volume.

2) Physiological saline II. This saline contained 154 mM NaCl; 8 mM KCl; 2 mM $CaCl_2$; 3 mM $MgCl_2$; 40 mM trehalose; and 5 mM HEPES buffer. All components were combined in a volume equal to approximately 90 percent of the final volume and the pH adjusted to 7.4 with 1N NaOH. Distilled water was then added to give the desired final volume.

3) Physiological saline III. This saline contained 215 mM NaCl; 4.8 mM KCl; 1 mM $CaCl_2$; 1 mM $MgCl_2$; 40 mM trehalose; 5 mM glucose; and 40 mM HEPES buffer. All components were combined in a volume equal to approximately 90 percent of the final volume and the pH adjusted to 7.4 with 1N NaOH. Distilled water was then added to give the desired final volume.

4) Physiological saline IV. This saline contained 215

mM NaCl; 4.8 mM KCl; 1 mM $CaCl_2$; 1 mM $MgCl_2$; 5 mM glucose; and 40 mM HEPES buffer. All components were combined in a volume equal to approximately 90 percent of the final volume and the pH adjusted to 7.4 with 1N NaOH. Distilled water was then added to give the desired final volume.

Each of the salines, was aerated prior to use. The air, drawn from the compressed air lines, was filtered through a tube (i.d. 4.5 mm) containing a 5 cm filter of loosely packed absorbent cotton to remove any oil or particulate matter that might be present in the air line.

2.5 Preparation of inhibitors and agonists

The phospholipase A_2 inhibitor, ρ-bromophenacyl bromide; the cyclooxygenase inhibitors, indomethacin, diclofenac and acetylsalicylic acid; and the lipoxygenase inhibitor, nordihydroguaiaretic acid; were prepared in a solution of dimethyl sulfoxide (DMSO)/95% ethanol (50:50, V/V). The phospholipase A_2 inhibitor, mepacrine was prepared in a solution of DMSO/H_2O (50:50, V/V).

The protein kinase C inhibitors, H7 and sphingosine, and the phospholipase A_2 activator, melittin, were prepared in DMSO.

The free fatty acids and prostaglandins were dissolved in 95 % ethanol.

The vehicle solutions used were not only instrumental in maintaining the inhibitors and agonists in solution but

also in aiding their entry into cells. The chemical agents were prepared in such a fashion so that the desired final concentration of the agent was obtained without having the vehicle solution exceed 0.5 % (final concentration) in the incubation medium.

The fatty acid methyl ester standards used for gas chromatography were prepared in hexane.

2.6 Dissection of Fat Body

The wings and legs of the cockroach were removed and the insect pinned dorsal side up in a wax dissecting dish. A longitudinal incision was made along the dorsal midline and the terga reflected laterally with the aid of pins. A few drops of physiological saline (I) was added to keep the abdominal cavity of the insect moist. Iridectomy scissors and No. 5 watchmakers forceps were used to remove the digestive tract, care being taken to avoid punctures that could result in the release of digestive enzymes which could cause possible damage to the fat body. The fat body was carefully cut away from the inner wall of the body cavity. Usually, the tissue was used immediately after removal but, in some instances, was held in fresh saline for a period not exceeding 30 min before use.

2.7 Disaggregation of Fat Body

The disaggregation of the cockroach fat body followed

the method devised by Steele and Ireland (1994). Eight fat body lobes (four fat bodies) were pooled and minced on the wax surface of a dissecting dish using iridectomy scissors. The minced tissue was then transferred to a 10 ml beaker containing 2 ml saline III and 1.75 mg of collagenase. The mixture was incubated for 1 h at 30°C in a shaker water bath. During this time the tissue was drawn into a 5 ml pipette and expelled 3 times at each of 24 min, 40 min and 55 min intervals to facilitate separation of the cells. After the final pipetting, the dispersed cells were filtered through a single layer of cheese cloth into a plastic conical centrifuge tube. The cheese cloth was rinsed with fresh saline III and the cells, in a final total volume of 9 ml of saline III, were centrifuged in a IEC clinical centrifuge at 27 x g for 5 min. The resulting upper layer of approximately 8.5 ml which contained the trophocytes was gently swirled to facilitate transferral of these cells to the cell harvester. This procedure separated the upper layer containing the trophocytes from the undisturbed pellet consisting of urate cells and mycetocytes. A small sample of the dispersed cells was removed at this time to determine the yield using a haemocytometer. For some experiments the cells in the pellet were recovered in saline III for further use.

The cell harvester consisted of the barrel of a 10 ml plastic syringe fitted with a 20 gauge needle trimmed to a

length of 1 cm and the end filed smooth. Fifteen cm of INTRAMEDIC® polyethylene tubing (0.864 mm i.d.) was attached to the needle. During centrifugation the tubing was taped to the side of the syringe so that the end of the tubing was held above the level of the mixture in the syringe to prevent loss of the dispersed trophocyte preparation. After centrifugation, the infranatant was drained from below so that the packed trophocytes at the surface were retained within the harvester. The cells in the harvester were washed three times with fresh saline III to remove the collagenase and were retained in this saline for immediate use.

2.8 Protein determination

Protein was determined using the Bio-Rad® assay which is based on the method of Bradford (1976). The fat body lobes were homogenized in 1 ml of 0.9% NaCl and centrifuged at 1300g x 5 min. Ten μl of the infranatant was added to 790 μl H_2O to which 200 μl of Bio-rad reagent was added. The samples were vortex mixed and allowed to stand for 10 min at room temperature. The absorbance of the samples was measured at 595 nm. Protein standards (BSA) and blanks were prepared in a similar manner. The quantity of protein in the unknown standards was determined by interpolation from a standard curve constructed from a series of standards of known protein concentration.

2.9 Measurement of ^{14}C-radioactivity

The scrapings from the TLC plates (section 2.11.2.3) were placed in scintlllation vials to which 5 ml scintillation fluid (BCS Biodegradable Counting Scintillant, Amersham Canada Ltd., Oakville, Ontario, Canada) was added. The samples were thoroughly mixed and the radioactivity determined on a Canberra Packard 1900 TR liquid scintillation counter. The radioactivity measurements are expressed as counts per minute (CPM). Quenching was found to be very low and for this reason the data were uncorrected.

2.10 *In vitro* tissue studies

2.10.1 Intact fat body

Paired fat body lobes were removed from the cockroaches, rinsed in saline I, and incubated individually in 2.0 ml of the same saline in 10 ml beakers at 30°C in a shaker water bath. Usually, the tissue was pre-incubated in saline II, with or without inhibitors or activators, but not hormones, for 1 h. The lobes were then transferred to saline I containing the appropriate inhibitors, activators, or agonists. The fat body was generally incubated for an additional 1 h following which samples of the medium (usually 50 μl) were taken for analysis.

2.10.2 Disaggregrated cells

2.10.2.1 Intracellular trehalose

Dispersed cells were usually incubated in a final volume of 350 µl of saline IV in 10 ml round-bottom polyethylene tubes. For inhibitor studies the cells were pre-incubated with the inhibitors for 15 min. This was followed by further incubation with the appropriate agonist for an additional 15 minutes. Following incubation, 250 µl of well mixed cell suspension was transferred to a syringe containing 4.0 ml saline IV. The syringe was fitted with a 20 gauge needle to which plastic tubing was attached and secured to the side of the syringe with masking tape. The bottom of the barrel of the syringe held a tightly fitting circular glass microfiber filter disc (Whatman GF/A) which served as a sieve to separate the cells from the incubation medium. The tubing secured to the side of the syringe could then be removed and lowered to allow the medium to drain so that the cells were retained by the filter. The cells resting on the filter were then washed rapidly with three 1.0 ml aliquots of saline IV. The glass microfiber filter was removed and placed in a round-bottom centrifuge tube containing 2.0 ml chloroform/methanol (2/1; V/V). After extraction the trehalose was extracted and determined by gas chromatography as described in section 2.9.1.2.

2.10.2.2 Trehalose efflux

The measurement of trehalose released from dispersed cells was determined by incubating the trophocytes in 10 ml round-bottom polyethylene tubes in 0.5 ml of saline IV. At pre-determined times 100 µl was removed and added to a centrifuge tube containing 2.0 ml chloroform/methanol (2/1; V/V). Trehalose was then extracted and determined by GC as described in section 2.11.1.2.

2.11 Metabolite assays

2.11.1 Determination of Trehalose

2.11.1.1 Spectrophotometric assay

The spectrophotometric assay of trehalose was performed using anthrone reagent (Carroll *et al.*, 1956). The anthrone reagent was prepared by the addition of 10 g of thiourea and 500 mg of anthrone to 1 litre of 72% sulfuric acid. The reagent was chilled before use and stored at 4°C. Trehalose released into the incubation medium by fat body lobes was determined by adding 50 µl of incubation medium to 450 µl H_2O. The samples were chilled to 0°C and 2.5 ml of cold anthrone reagent added. The tubes containing the samples were shaken in an ice bath followed by thorough mixing of the samples on a vortex mixer. The tubes were then placed in a boiling water bath for 15 min, cooled, and the absorbance of the samples determined at 620 nm.

2.11.1.2 Gas chromatography assay

Trehalose in cells, or cells plus medium, was separated from lipid which interferes with the assay using a modified Bligh and Dyer (1959) extraction method. The samples, in 2.0 ml chloroform:methanol (2:1, V\V) were extracted with 2.0 ml of methanol:H_2O:chloroform (48:47:3, V\V\V) followed by extraction with 1.0 ml of the same solution. The aqueous layers (top phase) from each extraction were combined and dried using a rotary evaporator. The residue was taken up in 1.0 ml of H_2O and of this 0.85 ml was transferred to a conical centrifuge tube fitted with a teflon-lined screw cap and dried overnight in a vacuum oven at 70°C. To the dry sample 0.1 ml of TMSI (N-trimethylsilylimidazole) in pyridine (1:1, V\V) was added and the samples placed in a water bath at 75°C for 40 min. The sample was mixed by vortexing at 10 min intervals. To the tubes 0.2 ml of H_2O and 0.2 ml of hexane (containing 2.0 μg tetracosane as internal standard) were added and vortex mixed. The lower aqueous phase was removed and discarded and an additional 100 μl of H_2O was added, mixed and then removed to wash the hexane solution. The organic phase containing the derivatized sample was then ready for gas chromatography.

Samples were analyzed on a Hewlett-Packard 5711A gas chromatograph with a flame inoization detector (FID) and 3380A integrator. The GC was fitted with a 50 cm x 4 mm i.d. glass column packed with 3% (W/W) OV-17 on Chromosorb

G-HP (80-100 mesh). Five µl of the 200 µl sample was injected into the column.

2.11.2 Determination and measurement of free fatty acids

2.11.2.1 Preparation of cell samples

The cells were separated from the incubation medium by pipetting the 1.0 ml sample of incubation medium into a syringe which had been fitted with a circular glass microfiber filter (Whatman GF/A) disc at the bottom. The syringe was attached to a 20G needle which pierced a rubber stopper sitting on a filter flask. The flask contained ice which supported a centrifuge tube containing 1.5 ml of methanol placed within the flask in such a way so that the end of the needle was positioned within the centrifuge tube. When the sample was transferred to the syringe mild vacuum caused the medium to collect in the centrifuge tube within the flask while the cells were retained on the filter disc. To allow easy access to the filter disc, the 10 ml syringe barrel was severed at the 4 ml mark. The filter was removed with forceps and transferred to a centrifuge tube containing 1.5 ml of ice cold methanol. The centrifuge tubes containing the samples were then flushed with N_2 and capped with a teflon lined screw cap. Each sample was mixed thoroughly by vortexing and allowed to stand for 20 minutes at 4°C after which they were centrifuged at 450g x 20 min. The sample supernatants were now ready for the extraction of

free fatty acids.

2.11.2.2 G.C. analysis

Free fatty acids were separated and quantified by GC using "on column" methylation with trimethyl-(α,α,α-trifluoro-m-tolyl) ammonium hydroxide (TMTFTH) (MacGee and Allen, 1974).

TMTFTH is not available commercially and was prepared from trimethyl-(α,α,α-trifluoro-m-tolyl) iodide (TMTFTI) (MacGee and Allen, 1974) as follows. A mixture of 10 ml of α,α,α-trifluoro-m-toluidine and 20 ml of methyl iodide was placed in a stoppered 250 ml Erlenmeyer flask for 24 h in the dark at room temperature. Fifty ml of methanol and two boiling stones were added to the mixture and the suspension heated in a fumehood until the vapours in the neck of the flask attained a temperature between 60 and 65°C. The flask was then immersed in an ice-bath for 10 min and the crystals of TMTFTI collected on a Buchner funnel. The crystals were washed on the funnel with a small volume of ice-cold methanol which was removed by vacuum filtration. The damp product was transferred to an Erlenmeyer flask and left overnight to recrystallize from 25 ml of methanol. The crystals of TMTFTI were collected and washed as before and then dried in a vacuum oven at 50°C.

Aqueous TMTFTH (0.5 M) was prepared by mixing 0.66 g of TMTFTI, 0.35 g of silver oxide, and 4.0 ml distilled water

in a test tube until the TMTFTI had dissolved. After centrifugation at 600g x 1 min, a sample of the clear supernatant fluid was diluted ten-fold with distilled water and tested for iodide using 0.1 M silver nitrate in 6 M nitric acid. This usually resulted in a positive test for halide. If positive, the solution was held overnight in the refrigerator. This produced a solution of TMTFTH which was free of halide. The reagent solution was stored in the refrigerator.

Methyl propionate-methanol (1:2) was prepared by mixing one volume of methyl propionate, previously dried with anhydrous sodium carbonate, with two volumes of methanol.

The fatty acids were extracted from the aqueous medium by the addition of 1.5 ml of methanol and 5 ml of hexane to the sample in a glass stoppered conical centrifuge tube followed by vigorous shaking. The mixture was then acidified by adding 0.5 ml of 1M phosphoric acid which drives the fatty acids into the hexane phase. After shaking vigorously for 1 min followed by centrifugation at 450g x 2 min the lower methanol phase was removed with a syringe. The remaining hexane phase was then washed with two successive 2 ml aliquots of 0.1 M phosphoric acid. The complete removal of this lower aqueous phase is essential for efficient recovery of the fatty acid methyl esters. Any aqueous solution remaining associated with the hexane phase significantly reduces the recovery of the fatty acid methyl

esters. As a precaution, after the removal of the aqueous washings, the hexane solution was centrifuged (450g x 2 min) and any residual aqueous solution was removed, together with a small amount of the hexane at the bottom of the centrifuge tube to ensure total removal of any remaining water. This procedure was then repeated. Ten μl of TMTFTH was added to the hexane containing the fatty acids. TMTFTH forms quaternary salts of the fatty acids which are no longer soluble in the hexane but are retained in the small volume of TMTFTH. After addition of the TMTFTH the sample was shaken vigorously for 1 min and centrifuged at 450g x 2 min. The hexane layer was discarded leaving the quaternary salts of the fatty acids in the TMTFTH ready for determination by GC.

The quaternary salts of the fatty acids formed with TMTFTH react with methyl propionate-methanol (1:2, V/V) in the injection port of the GC resulting in the formation of methyl esters. Methyl propionate-methanol was taken up into a 10 μl Hamilton syringe and expelled so that the needle of the syringe held a volume of approximately 0.5 μl of methyl propionate-methanol. One ul of TMTFTH containing the derivatized fatty acids was taken up followed by 0.5 μl of methyl propionate-methanol. The TMTFTH solution sandwiched between the methyl propionate-methanol solutions was mixed by drawing the plunger up to the 5.0 μl mark and then returned to the 2.0 μl mark. The needle was then inserted

into the injection port of the GC and the contents of the syringe expelled rapidly.

Fatty acid methyl esters were analyzed on a Hewlett-Packard 5711A gas chromatograph with a flame ionization detector (FID) and the data recorded using a Hewlett Packard 3380A integrator. The GC was fitted with a J.& W. DB-Wax capillary column (30m x 0.53 i.d.). The splitless injection port and flame ionization detector were set at 200°C and the oven programmed to operate from 190°C-230°C at a rate of 8°C/min. Helium at 5 ml/min was used as the carrier gas. Myristic acid served as the internal standard. Authentic fatty acid methyl ester standards were used to construct standard curves from which the fatty acid concentration of was determined. The recovery of palmitic, stearic, oleic and linoleic acids was found to be 95, 86, 85 and 90% respectively.

2.11.2.3 Thin Layer Chromatography

The thin layer chromatography procedures used for the analysis of fatty acids are based on methods described by Wilson and Sargent (1992) and Dudley and Anderson (1975). The sample in 1.5 ml ice-cold methanol was transferred to a centrifuge tube fitted with a teflon-lined screw cap, flushed with N_2 and allowed to stand for 20 minutes at 5°C. After centrifugation at 4 30g x 20 min the supernatant was transferred to a centrifuge tube containing 5 ml of hexane

and vortex mixed followed by the addition of 0.5 ml of 1M phosphoric acid. The samples were then mixed vigorously for 30 s and centrifuged at 450 g x 2 min. The aqueous layer was removed and discarded, and the hexane phase washed with 2.0 ml of H_2O. The hexane was then dried at 0°C under a stream of N_2, and the sample derivatized with diazomethane to form methyl esters of the fatty acids (Schlenk and Gellerman, 1960).

Diazomethane was generated using a micromole capacity generator. Approximately 33 mg of 1-methyl-3-nitro-1-nitrosoguanidine was placed in the reservoir of the generator together with 125 µl of water. The water is present to assist in absorbing heat which may be generated by the reaction. One ml of diethyl ether is placed in the outer vessel and the apparatus assembled so that it is air-tight. The lower part of the apparatus is then immersed in an ice bath. When equilibrated, 150 µl of 5 N NaOH is injected slowly through the teflon rubber septum using a syringe with a narrow gauge needle (No. 22) to prevent leakage around the shank. The syringe is removed and the reaction allowed to proceed in the ice bath for at least 45 minutes or until the diazomethane is required. The diazomethane generated collects in the ether. Sufficient diazomethane was generated by this procedure to enable as many as eight samples to be derivatized.

Diazomethane reacts very slowly in absolute ether

therefore 2 M methanol (final concentration) was added to the samples which acted as a catalyst (Schlenk and Gellerman, 1960). The samples were kept in a water bath at room temperature for 15 minutes. The derivatized sample was then applied to TLC plates with a 10 μl Hamilton syringe.

Silica Gel G or silica Gel 60 TLC plates (20 cm x 20 cm x 0.25 mm) (Fisher Scientific Co., Toronto) were prepared by dipping in acetonitrile containing silver nitrate (1 g silver nitrate/10 ml acetonitrile). The plates were then dried in the dark and activated for 30 minutes at 110°C. The plates were cooled to room temperature and used within one hour of activation.

The samples were applied to the TLC plates as a narrow band 2 cm in width. To locate ^{14}C labelled arachidonic and γ-linolenic acids, 100 μg of the unlabelled fatty acid methyl esters were added to the labelled samples. Unlabelled fatty acids were also chromatographed separately on the same plate.

Prior to use the atmosphere in the TLC chamber was saturated with the solvents used for the separation. The mixture containing the fatty acid methyl esters was resolved using toluene-acetonitrile (97:3, V/V) (Wilson and Sargent, 1992). The separation was performed at room temperature and was usually complete in 45 min. Separation was considered to be complete after the solvent front had moved 16 cm. The plates were dried under a stream of N_2, sprayed lightly

with 0.1% solution of 4',5'-dibromofluorescein in isopropanol, dried again under N_2, and placed in a tank saturated with NH_4OH vapours. After 5 minutes, pink spots appeared on a yellow background which became more pronounced when viewed under UV light. The individual bands viewed under UV were marked and subsequently scraped from the plate into scintillation vials to which 5 ml of scintillation fluid was added and the radioactivity determined.

2.12 Enzymatic assays

2.12.1 Glycogen phosphorylase assay

Phosphorylase was assayed in the direction of glycogen hydrolysis with an enzymatic assay based on that described by Childress and Sacktor (1970) and modified slightly by Ziegler *et al.* (1979). For the assay of phosphorylase *a* the final volume of the reaction mixture was 1.0 ml. The mixture contained 40 mM triethanolamine buffer; 5 mM imidazole; 1.9 mM EDTA; 1.3 mM dithiothrietol; 76 mM KH_2PO_4; 4 mM $MgSO_4$; 0.57 mM NADP; 1.2 μM glucose 1,6-diphosphate and 10 mg/ml glycogen. The glycoger was treated with charcoal to remove AMP (Stevenson and Wyatt, 1964). The components listed above, with the exception of NADP and glucose 1,6-diphosphate, were dissolved in water at double the final concentration. The pH was titrated to 7.4 with 0.1 N NaOH and the solution diluted to 60 percent of the final volume. This stock solution was stored at -20°C. The NADP and

glucose 1,6-diphosphate were prepared fresh in distilled water as 11.4 mM and 24 μM solutions respectively.

The analytical enzymes were dissolved in an enzyme dilution medium consisting of 40 mM triethanolamine.HCL; 5 mM imidazole; 1 mM $MgSO_4$ and 0.0062 percent bovine serum albumin. The solution was titrated to pH 7.0 with 0.1 N NaOH before dilution to the final volume. The solution was stored frozen at -20°C. Glucose 6-phosphatase and phosphoglucomutase were added prior to use to yield 0.85 units and 0.24 units respectively per 100 μl of solution.

Dispersed cells in 250 μl saline III solution were distributed among 15 ml round bottomed centrifuge tubes and allowed to equilibrate in the water bath for 30 minutes at 30°C. In the phosphorylase activation studies the cells were incubated with hormones for 5 minutes. In the inhibitor studies the cells were preincubated with the inhibitors for 15 minutes prior to addition of the hormones. The reactions were terminated by the addition of 750 μl of ice cold NaF/EDTA (NaF, 0.1M; EDTA, 0.01M) followed immediately by sonification. The tubes were kept chilled and centrifuged for 15 minutes at 10,000 rpm (Sorvall centrifuge).

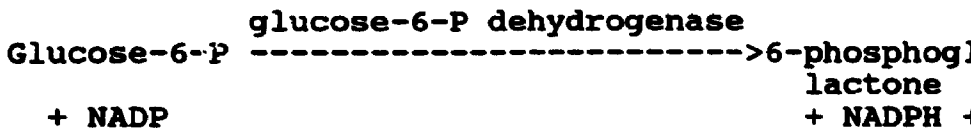
The phosphorylase assay was carried out in 1.0 ml cuvettes in which the enzymatic conversion of glycogen to glucose 1-phosphate was determined. The cuvette contained 600 ul of substrate mixture together with 50 μl of each of

the freshly prepared NADP and glucose 1,6-diP solutions and 200 µl of the sample to be assayed. One hundred µl of enzyme solution was added to start the reaction and the solution quickly mixed by inverting the capped cuvette. The blank against which the sample was read was identically prepared except that 200 µl of the NaF/EDTA homogenizing solution was added in place of the sample solution.

Once the NaF/EDTA homogenizing solution had been added to the cell samples and the preparation sonicated, the phosphorylase freed from the cells was no longer interconvertible between the active *a* form and the relatively inactive *b* form. The enzymes phosphorylase kinase and phosphorylase phosphatase which are responsible for the interconversion of the active and inactive forms are effectively inhibited by NaF and EDTA respectively. The percentage of phosphorylase *a* is therefore "fixed" at the level that existed in the tissue when the reaction was terminated. The assay measures the progressive accumulation of NADPH at 340 nm as a result of the following coupled reactions:

$$\text{Glycogen}_{(n)} + \text{Pi} \xrightarrow{\text{phosphorylase "a"}} \text{Glycogen}_{(n-1)} + \text{Glucose-1-P}$$

$$\text{Glucose-1-P} \xrightarrow[\text{glucose-1-6-diP}]{\text{phosphoglucomutase}} \text{Glucose-6-P}$$



The progression of the reaction was followed on the video display monitor on the spectrophotometer. When the accumulation of NADPH became linear (2-3 min), the change in absorbance was recorded for 5 min. At this point, 50 µl of 40 mM 5'-AMP was added to the experimental cuvette and mixed thoroughly. The addition of the 5'-AMP caused the phosphorylase to establish a new rate of NADPH synthesis. The reaction was again followed for an additional 5 min.

The change in absorbance per unit time could be used to determine the production of NADPH both in the presence and absence of 5'-AMP and hence the synthesis of glucose 1-phosphate. This information, which reflects the activity of phosphorylase, can be used to determine the proportion of the enzyme in the active or a form. The concentration of NADPH in the cuvette is determined by the formula:

$$c = A/\epsilon$$

where c is concentration in moles/liter; A is the absorbance of NADPH and ϵ is the extinction coefficient for NADPH with a light path of 1 cm at 340 nm.

2.12.2 Trehalose-6-Phosphatase Assay

The protocol employed for the preparation of trehalose-6-phosphatase is based on the description by Friedman and

Hsueh (1979), with the exception that the enzyme was prepared from dispersed cells rather than whole fat body. Dispersed cells were homogenized in a buffer containing 0.5 M TES (N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid) and 1.0 mM EDTA (Ethylenediamine tetraacethic acid) adjusted to pH 8.0. The cells were homogenized in a round-bottomed centrifuged tube with a hand-held homogenizer (OMNI 2000 fitted with a 5 mm generator) at setting #3 for 30 seconds. The homogenate was centrifuged at 10,000g x 5 min and the supernatant decanted and recentrifuged at 33,000g x 15 min. The supernatant from the second centrifugation was used as the source of enzyme.

The trehalose-6-phosphatase reaction was carried out in a medium containing 50 mM TES, 1 mM EDTA, 12 mM $MgCl_2$, and 3.3 mM T6P titrated to pH 7.0 with 1 N NaOH plus enzyme preparation in a total volume of 0.5 ml. The mixture was incubated for 20 minutes at 32°C and the reaction halted by the addition of 0.5 ml of 10% trichloroacetic acid (W/V). The samples were centrifuged and 0.5 ml of the supernatant used to determine the inorganic phosphate.

Inorganic phosphate was determined by the method of Zakim and Vassey (1973). One half ml of the centrifuged sample was transferred to a test tube and 5.0 ml of ammonium molybdate solution (1.6% (W/V) in 1N H_2SO_4) was added. This was followed by the addition of 0.8 ml of ferrous sulphate solution (2.5 g in 25 ml 0.15 N H_2SO_4). The sample was

mixed and the absorbance read immediately at 660 nm.

2.13 Statistical analysis

Paired Student t-tests, ANOVA, and Duncan's multiple comparison test were employed (de Cartanzaro, 1989; Zar, 1984).

Part 3

RESULTS

3.1 Hormonal Activation of Trehalose Synthesis

3.1.1 The hypertrehalosemic effect

The stimulation of trehalose synthesis by the natural and synthetic hormones as estimated by their effect on trehalose efflux from fat body *in vitro* is shown in Fig. 2. Each hormone was used at a concentration sufficient to elicit maximal response. The percentage increment in trehalose efflux owing to the hormonal agent is 120, 148 and 135 percent respectively for CCE, CC I and CC II. The maximal response obtained for each group as a percent of the control value is similar but there is variation in the amount of trehalose released from the control tissue for each group. Although these values do not differ in a statistical sense they indicate the variation that occurs when using isolated fat bodies for *in vitro* studies. The variation arises because of the difficulty in obtaining adults of nearly identical age and nutritional status.

Fig. 3. illustrates the effect of the synthetic hormones CC I and CC II on trehalose efflux from dispersed fat body cells. The results show that the "pellet cells", consisting of urocytes and mycetocytes, have lower basal production of trehalose than do the trophocytes. Interestingly, the mixture of urocytes and mycetocytes does

Figure 2. The stimulatory effect of natural and synthetic hypertrehalosemic hormones on trehalose efflux from fat body *in vitro*. Paired fat body lobes were incubated individually in 2 ml of physiological saline I at 30°C in a shaker water bath. Hormone (crude natural hormone, CCE, 0.2 GPE/ml or synthetic hormone, CC I or CC II, 10 pmol per ml, final concentration) was added to the medium (saline I) containing one lobe of each pair of fat body lobes. After incubation for 1h 50 µl of medium was withdrawn for measurement of trehalose with anthrone reagent. The protein content of the tissue was determined as described in the materials and methods. The data show that the hypertrehalosemic hormones had a significant stimulatory effect on trehalose efflux ($p < 0.005$ for CC extract, CC I and CC II, $n = 8$). Abbreviations used are CC I, synthetic hypertrehalosemic hormone I; CC II, synthetic hypertrehalosemic hormone II; CCE, Crude natural hypertrehlosemic hormone.

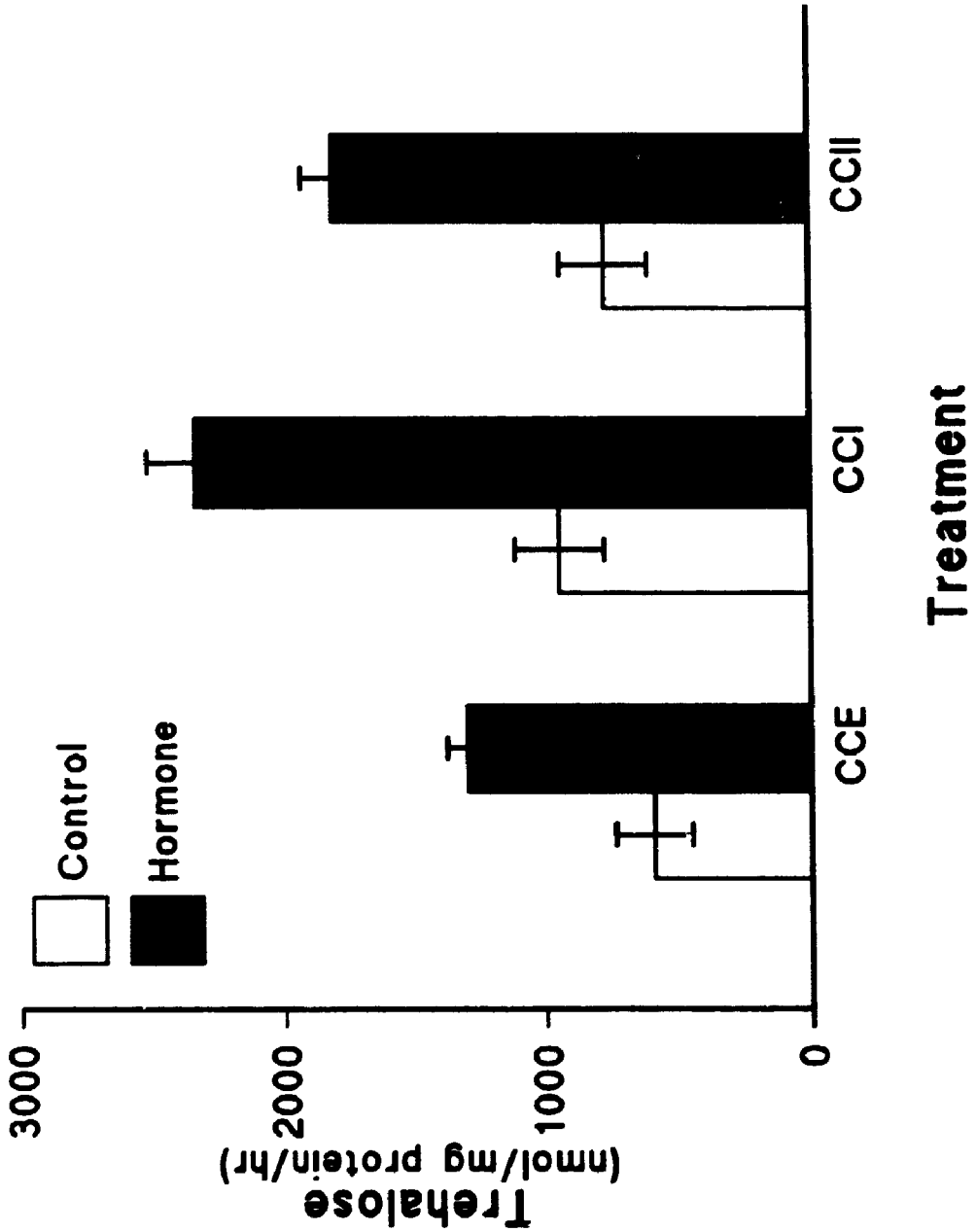
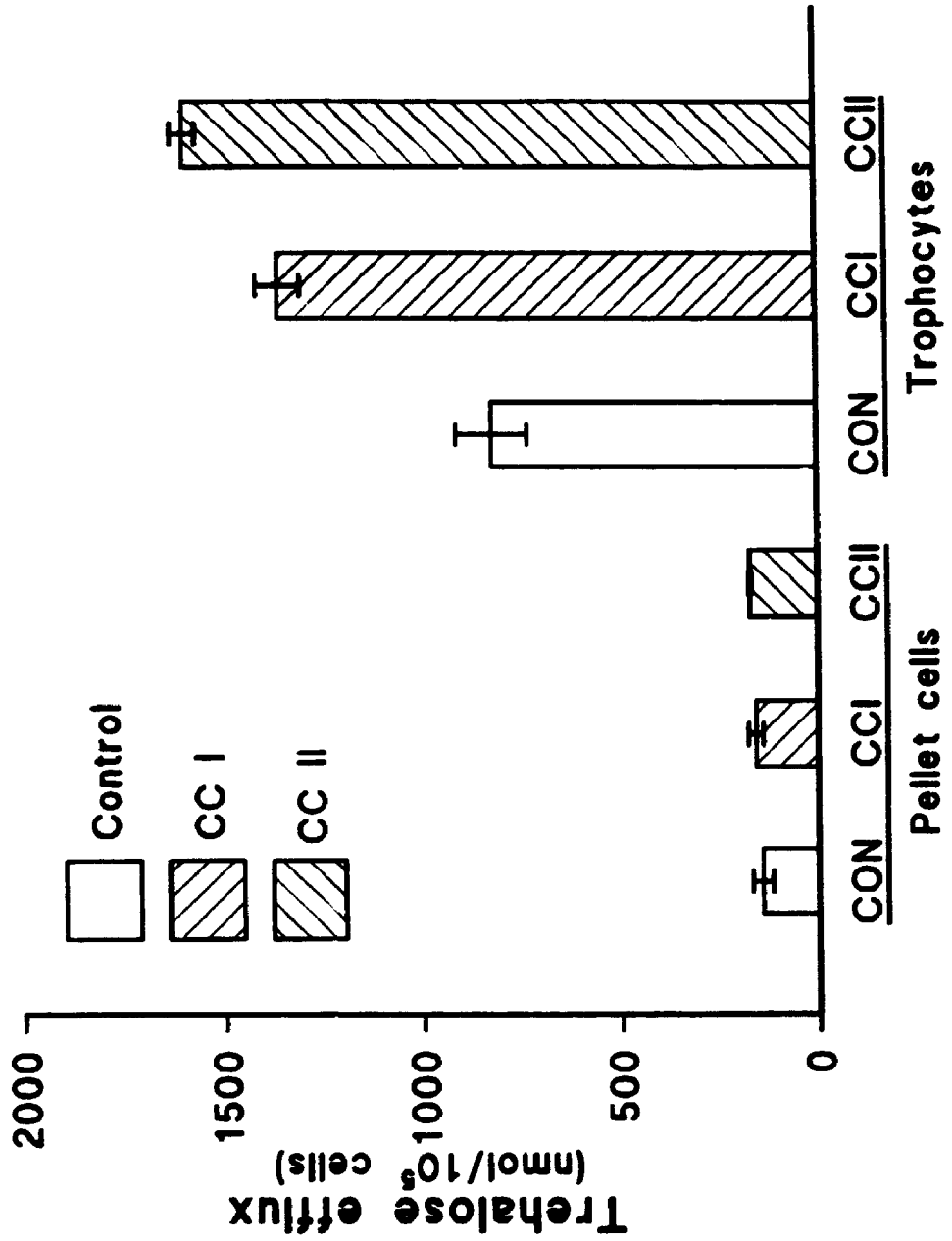

Trehalose
(nmol/mg protein/hr)
3000
2000
1000
0
Control
Hormone
CCE
CCI
CCII
Treatment

Figure 3. Stimulation of trehalose production by dispersed cells. Trehalose production was determined by incubating trophocytes (~15,000/sample) and pellet cells containing urocytes and mycetocytes (~75,000/sample) in 0.5 ml physiological saline IV in 10 ml polyethylene tubes at 30°C in a shaker water bath. Following incubation of the cells for 1h, either in the absence of hormone (CON) or in the presence of CC I or CC II (10 pmol/0.5 ml), 100 µl of medium was removed and placed in a centrifuge tube containing 2 ml chloroform/methanol (2/1:V/V). The sample was derivatized for the estimation of trehalose as described in the materials and methods. Analysis of the data reveals that the effect of the hormones on the trophocytes is significant ($p < 0.001$, $n = 3$). Abbreviations used are CON, control; CC I, synthetic hypertrehalosemic hormone I; CC II, synthetic hypertrehalosemic hormone II.


Trehalose efflux
(nmol/10⁵ cells)
2000
1500
1000
500
0
Control
CC I
CC II
CON CCI CCII
Pellet cells
CON CCI CCII
Trophocytes

not respond to either of the synthetic hormones. In contrast, trehalose efflux from the trophocytes is significantly increased when the cells are challenged with either CC I or CC II. The higher rate of trehalose efflux from the trophocytes than from the urocytes and mycetocytes possibly reflects a higher concentration of glycogen in the trophocytes although this has not been confirmed. The results show that disaggregation of the fat body with collagenase does not damage or otherwise interfere with the hormone/receptor mechanism in the membrane.

Figure 4. shows that the natural hormone exerts a marked time-dependent stimulatory effect on trehalose production in the trophocytes. This effect is sustained for 30 min after which the rate of production appears to be constant for both populations of cells. In these experiments no effort has been made to distinguish between the intra- and extracellular pools of trehalose.

3.1.2 Activation of phosphorylase

The data in Table 1 show that both natural and synthetic hormones activate phosphorylase. Activation occurs promptly and is consistent with the increase in trehalose efflux produced by the same hormones in intact fat body.

Figure 4. The relationship between time and production of trehalose in dispersed trophocytes. Trehalose production by untreated and hormone (CCE) challenged trophocytes (~15,000/sample) is illustrated using cells which have all been drawn from the same population. The procedure for incubating and sampling the cells is described in the legend to Fig. 3. The concentration crude of natural hypertrehalosemic hormone used was 0.2 gland pair equivalents of corpora cardiaca per 0.5 ml. Analysis of the data (ANOVA) shows that the crude natural hormone has a significant ($p < 0.01$, $n = 3$) time-dependent stimulatory effect on trehalose production by the trophocytes.

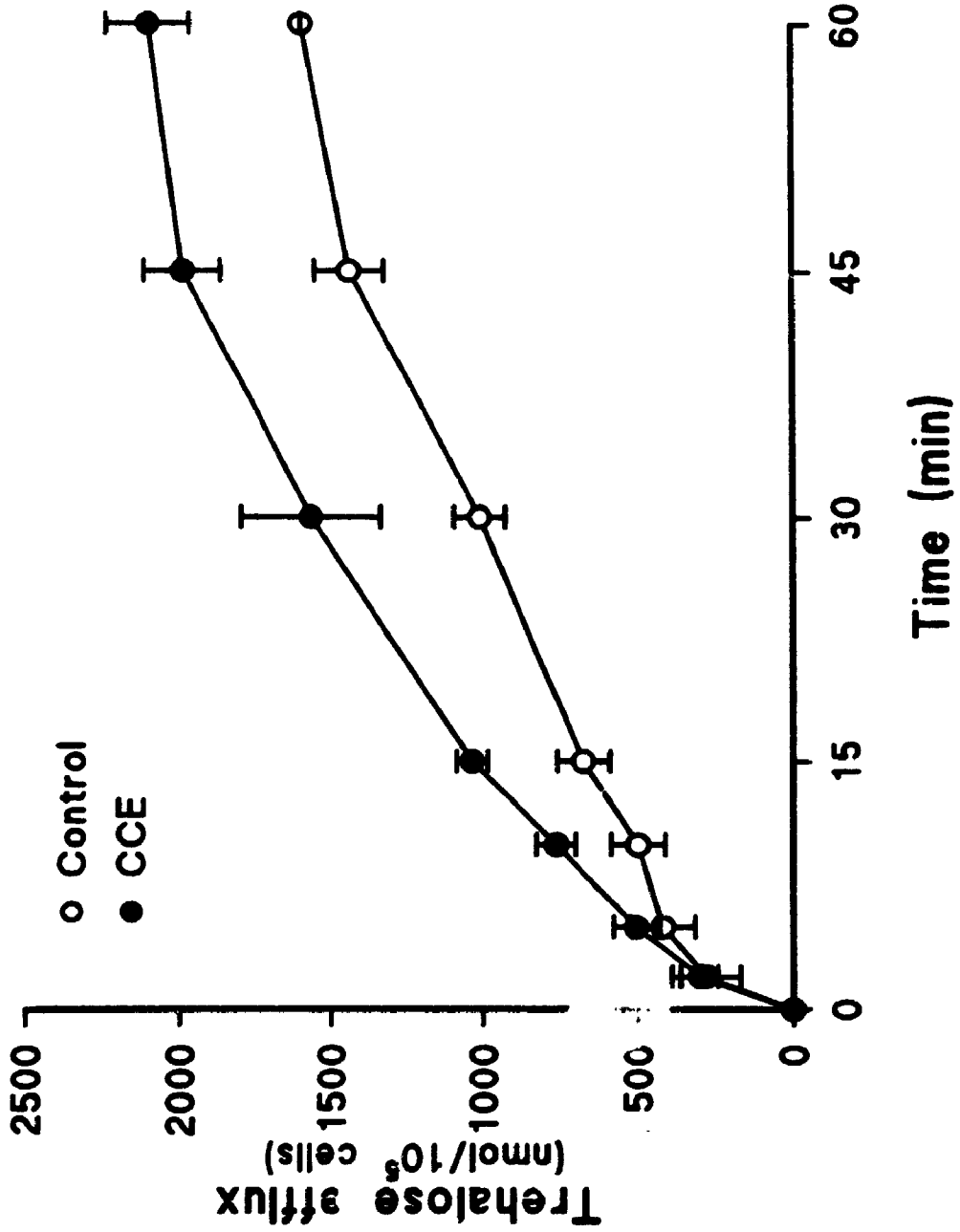

Trehalose efflux
(nmol/10^5 cells)
2500
2000
1500
1000
500
0
0
15
30
45
60
Time (min)
o Control
● CCE

Table 1. Activation of glycogen phosphorylase in trophocytes by hypertrehalosemic hormones.

	Phosphorylase activity (nmol NADPH.min^{-1}.mg^{-1} protein)		Percent *a*
	a	Total	
Control	28.8±1.9	102.5±7.0	28.5±1.5
CCE	65.6±8.6	93.3±7.6	69.2±2.3
CC I	59.6±8.8	88.9±13.2	67.2±2.3
CC II	58.0±12.4	89.3±19.1	64.9±1.0

Trophocytes (~30,000/sample) in 250 µl of saline III in 15 ml round-bottomed glass tubes were allowed to equilibrate with gentle shaking for 30 min at 30°C. The cell samples were treated with hypertrehalosemic hormones (CCE, 0.2 gland pair equivalents/sample; CC I and CC II, 10 pmol/sample). Some cell samples were left untreated to serve as controls. Incubation was continued for 5.0 minutes when the reaction was stopped by the addition of 750 µl ice-cold NaF/EDTA solution followed by sonification. The samples were then processed and phosphorylase activity determined as described in the materials and methods.
The data are expressed as means ± s.e.m.
ANOVA performed on the data revealed that the differences between the Control (n=13) and CCE(n=17), CC I(n=5), and CC II(n=4) to be statistically significant ($p < 0.001$).
Abbreviations used are CCE, CC extract containing the natural hypertrehalosemic hormones; CC I, synthetic hypertrehalosemic hormone I; CC II, synthetic hypertrehalosemic hormone II.

3.1.3 Evidence for second messenger systems

3.1.3.1 Putative role of protein kinase C

To determine whether protein kinase C may be involved in hormonal stimulation of trehalose efflux, inhibitors and activators of the enzyme were employed. Fat body lobes were incubated with inhibitors and activators of PKC in conjunction with crude natural hypertrehalosemic hormone to determine whether the normal hormonal response was affected.

Fig. 5A shows that H7 (1-(5-isoquinolinesulfonyl)-2-methyl piperazine dihydrochloride) had no effect on the hypertrehalosemic response induced by CC I. Since the concentration of H7 used would be expected to inhibit protein kinase C the absence of an effect on trehalose efflux suggests no involvement of protein kinase C in the stimulation of trehalose efflux by CC I.

The likelihood that protein kinase C is not involved in hormonal stimulation of trehalose efflux is also supported by the finding that sphingosine, a competitive inhibitor for protein kinase C, also has no effect on CC stimulated trehalose efflux (Fig. 5B). These results suggest that protein kinase C may not be involved in hormonal stimulation of trehalose efflux.

Protein kinase C can be activated in some tissues by OAG (1-oleoyl-2-acetylglycerol) which mimics the diacylglycerol derived from the hydrolysis of phosphatidylinositol (Kroll *et al.*, 1988). Fig. 6

Figure 5. Failure of protein kinase C inhibitors to block stimulated trehalose efflux from fat body *in vitro*. A. Paired fat body lobes were incubated individually in 2 ml of physiological saline I at 30°C in a shaker water bath. One lobe of each pair was incubated with 86 μM H7 (1-(5-isoquinolinesulfonyl)-2-methyl-piperizine dihydrochloride) and both lobes treated with synthetic hypertrehalosemic hormone CC I (10 pmol/ml). B. Paired fat body lobes were incubated individually in 2 ml of physiological saline I at 30°C in a shaker water bath. One lobe of each pair was incubated with 83 μM sphingosine (SPG) and both lobes challenged with natural hormone (CCE, 0.2 GPE/ml). In both experiments 50μl of medium was removed at zero time for trehalose determination with anthrone reagent. The protein content of the tissue was determined as described in the materials and methods. A paired t-test showed that neither H7 nor SPG affected trehalose efflux (n = 8). Abbreviations used are CC I, synthetic hypertrehalosemic hormone I; CCE, Crude natural hypertrehlosemic hormones.

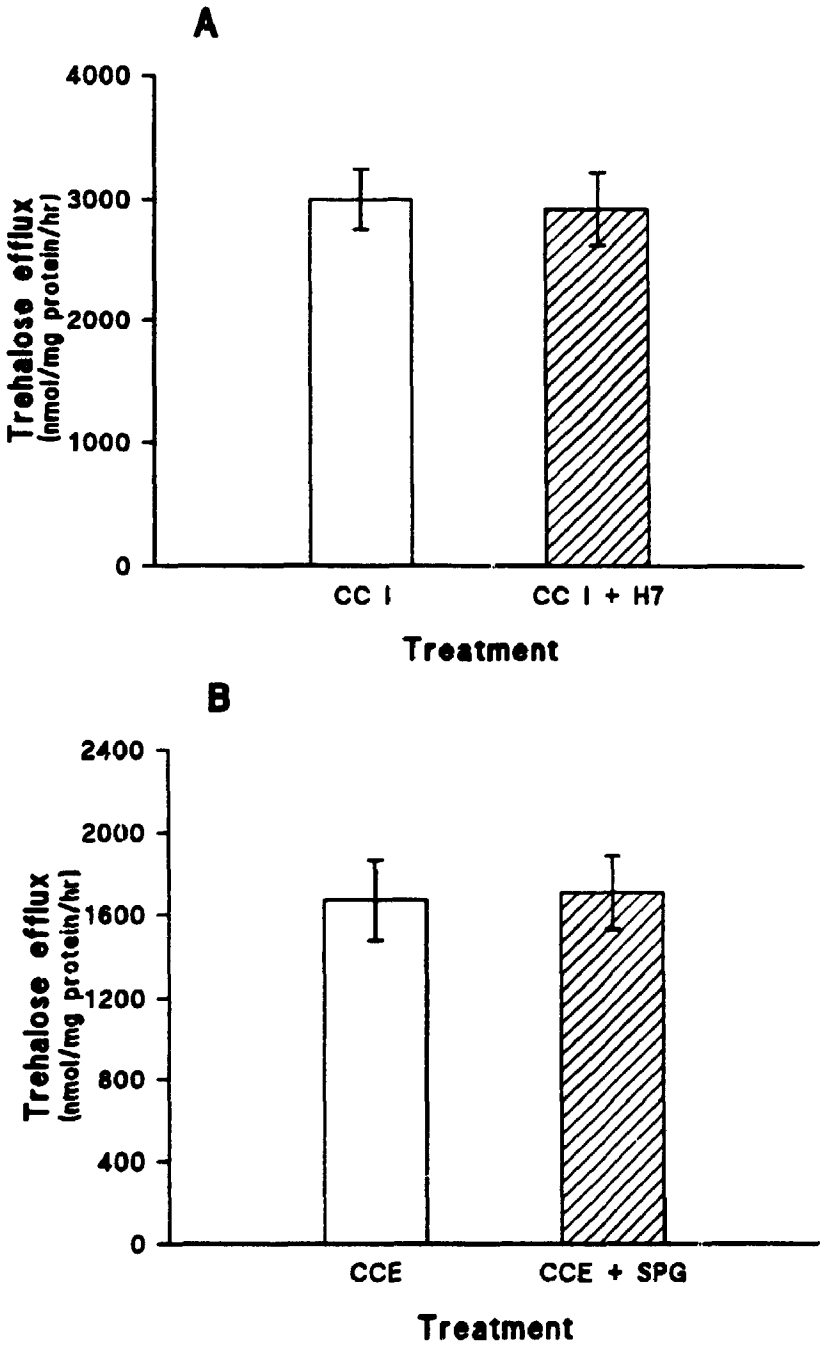
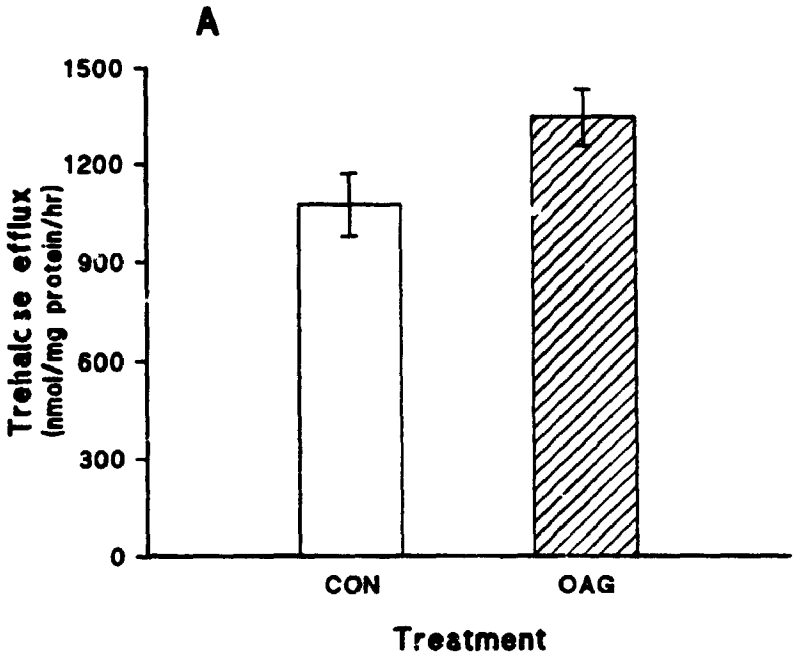
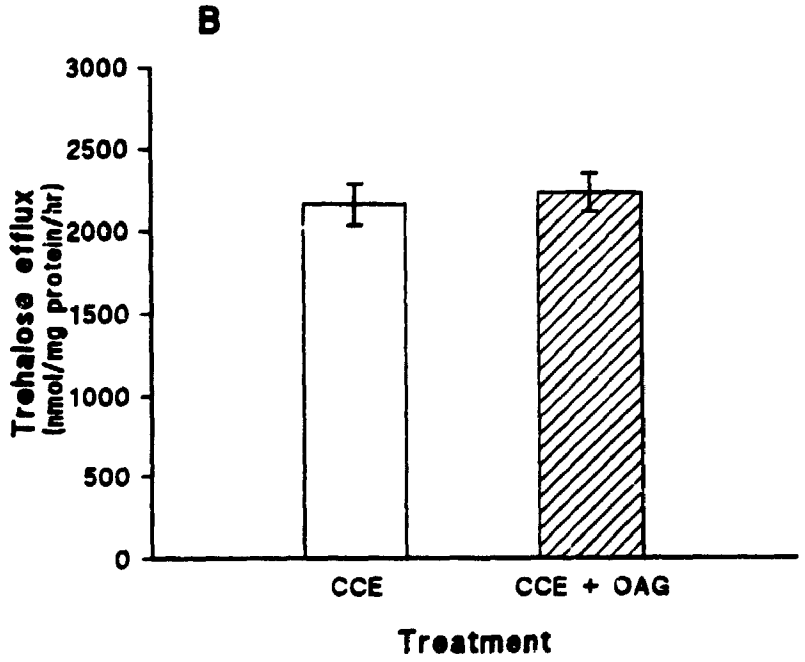

A
4000
3000
2000
1000
0
Trehalose efflux
(nmol/mg protein/hr)
CC I
CC I + H7
Treatment
B
2400
2000
1600
1200
800
400
0
Trehalose efflux
(nmol/mg protein/hr)
CCE
CCE + SPG
Treatment

Figure 6. The failure of a synthetic diacylglycerol analogue to alter trehalose efflux from fat body *in vitro*. A. Paired fat body lobes were incubated individually in 2 ml of physiological saline I at 30°C in a shaker water bath. One lobe of each pair was incubated with 50 μg/ml OAG (1-oleol-2-acetyl-glycerol). The remaining paired lobe was not treated. B. Paired fat body lobes were incubated individually in 2 ml of physiological saline I at 30°C in a shaker water bath. One lobe of each pair was incubated with 50 μg/ml OAG (1-oleol-2-acetyl-glycerol) and both lobes challenged with natural hormone (CCE, 0.2 GPE/ml). In both experiments 50 μl of medium was taken at zero time and again at 1h for trehalose determination with anthrone reagent. The protein content of the tissue was determined as described in the materials and methods. A paired t-test showed that OAG had no effect on trehalose efflux in either experiment (n = 8). Abbreviations used are CON, control; CCE, natural hypertrehalosemic hormones.


A
1500
1200
900
600
300
0
Trehalose efflux
(nmol/mg protein/hr)
CON
OAG
Treatment


B
3000
2500
2000
1500
1000
500
0
Trehalose efflux
(nmol/mg protein/hr)
CCE
CCE + OAG
Treatment

illustrates that OAG neither stimulates trehalose efflux from resting fat body (Fig. 6A) nor attenuates trehalose efflux stimulated by CC extract (Fig. 6B). These results suggest that PKC is not involved in hormonal stimulation of trehalose synthesis.

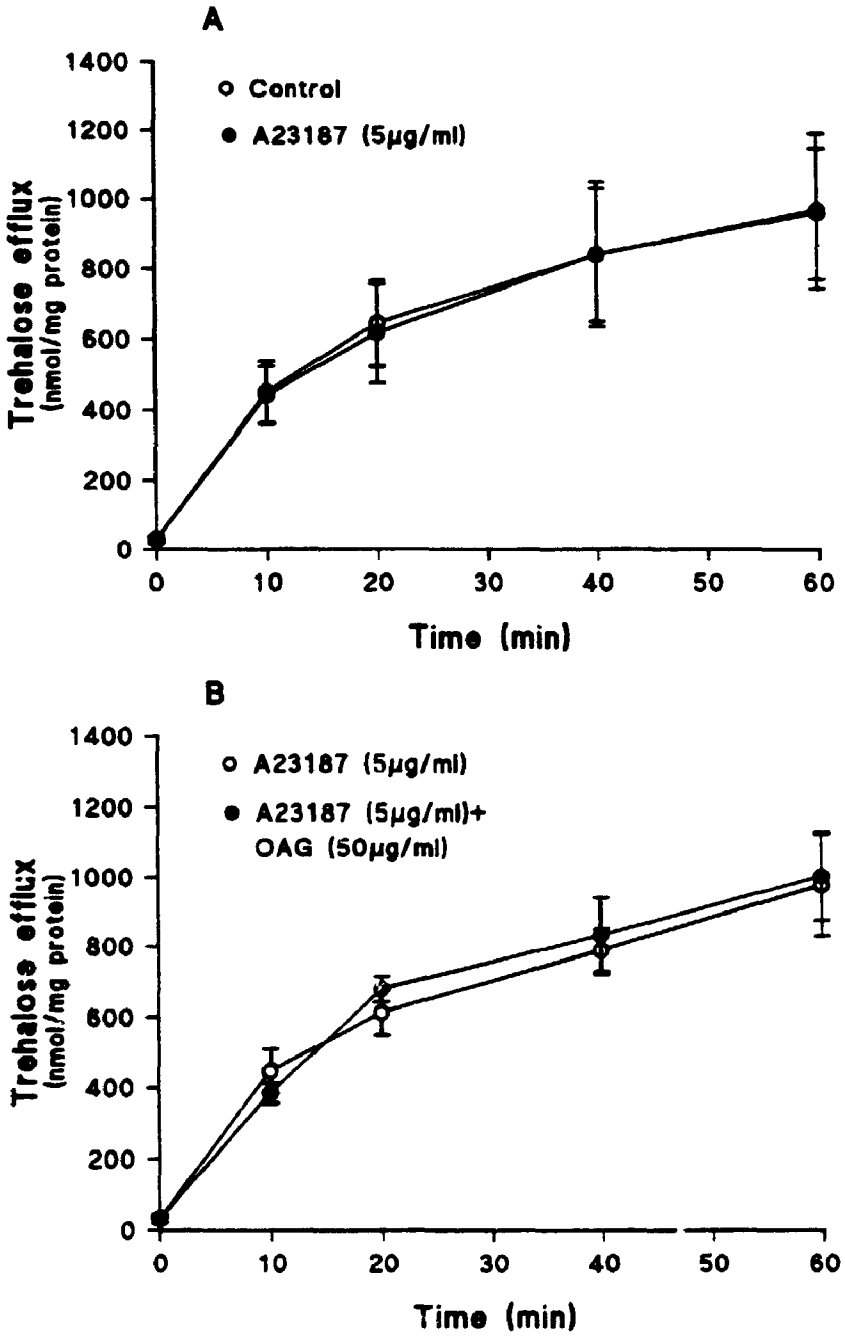
An increase in inositol 1,4,5-trisphosphate is usually followed by an increase in the intracellular concentration of calcium. This effect can be mimicked by treatment of the tissue with the calcium ionophore A23187 (Reed and Lardy, 1972). Fig. 7A indicates that addition of this agent to the saline bathing the fat body *in vitro* does not stimulate trehalose effflux from the tissue. Similarly, addition of OAG to the medium in combination with A23187 does not affect trehalose efflux (Fig. 7B). These results suggest that PKC is not involved in the stimulatory effect of the hypertrehalosemic hormones on trehalose efflux.

3.1.3.2 Evidence of a role for phospholipase A_2

Fatty acids are ubiquitous in cell membranes and because of the low concentration of free fatty acids in the cytosol are well suited to act as messenger molecules (Graber *et al.*, 1994). In the plasma membrane, they are covalently linked to other molecules to form phospholipids. These fatty acids can be liberated by cellular phospholipases such as phospholipase A_2.

Several inhibitors of this lipase have been developed

Figure 7. Failure of synthetic diacylglycerol to increase trehalose efflux from fat body *in vitro*. In panel A, paired fat body lobes were incubated individually in 2 ml of physiological saline I at 30°C in a shaker water bath. One lobe of each pair was incubated with the calcium ionophore A23187 (5 μg/ml). In panel B, paired fat body lobes were treated with A23187 (5 μg/ml) and one lobe of each pair also treated with OAG (1-oleol-2-acetyl-glycerol: 50 μg/ml). In both experiments a sample of medium was taken at zero time for analysis and incubation was continued for 1h during which 50 μl samples were taken for trehalose determination with anthrone reagent at the indicated times. The protein content of the tissue was determined as described in the materials and methods. Student's t-test performed on the data for both experiments revealed no significant differences (n = 8).

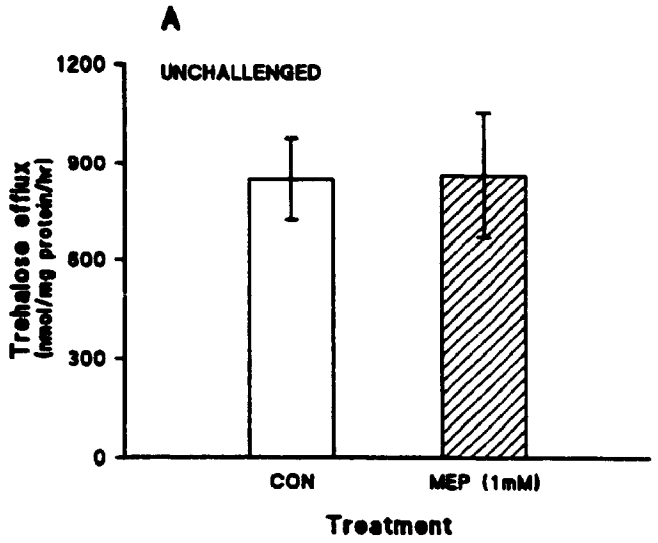

A
Control
A23187 (5µg/ml)
Trehalose efflux
(nmol/mg protein)
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
Time (min)
B
A23187 (5µg/ml)
A23187 (5µg/ml)+
OAG (50µg/ml)
Trehalose efflux
(nmol/mg protein)
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
Time (min)

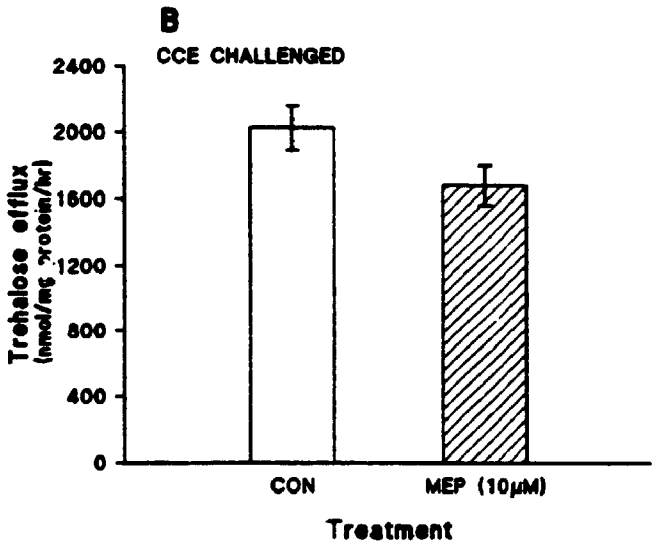
(Chang *et al.*, 1987). Mepacrine is an antimalarial agent which acts by interfering with the substrate-enzyme interface (Schaad *et al.*,1987; Chang *et al.*, 1987; Jancinova *et al.*, 1994; Fan, 1994). Fig. 8A illustrates that incubation of the fat body lobes with mepacrine does not affect trehalose efflux in the unstimulated tissue. However, trehalose efflux which has been stimulated by CC extract or CC I is significantly inhibited by mepacrine (Fig. 8B and C). These results suggest that PLA_2 may be associated with the stimulation of trehalose efflux by hypertrehalosemic hormone.

ρ-Bromophenacylbromide (BPB) has been used to indicate potential PLA_2 involvement in several physiological processes (Chang *et al.*, 1987). Incubation of fat body lobes with 1 mM BPB does not affect the release of trehalose from resting tissue (Fig. 9A). Trehalose efflux which has been stimulated by CC extract however, is significantly inhibited by 0.1 mM BPB but not by 0.01 mM (Fig. 9B and C). These results support the hypothesis that PLA_2 may be part of the hormonal mechanism.

Phospholipase A_2 can be activated by the amphiphillic peptide melittin (Mollay and Kriel, 1974). The effect of this peptide on trehalose efflux from the fat body was determined. Paired fat body lobes were incubated individually in 2.0 ml of saline I. To the treated sample, melittin (25 μg/ml) was added and allowed to incubate for 1h

Figure 8. Inhibition of hormone stimulated trehalose efflux from fat body *in vitro* by the phospholipase A_2 inhibitor mepacrine. A) Paired fat body lobes were preincubated for 1h at 30°C. The medium (physiological saline II) bathing one lobe contained 1 mM mepacrine. The tissue was then transferred to fresh medium (physiological saline I) again with mepacrine in the appropriate medium. After 1h samples (50 µl) of medium were taken for determination of the trehalose content (n=8). B) Paired fat body lobes were preincubated in 2 ml physiological saline II at 30°C. One lobe of each pair was treated with 10 µM mepacrine. After 1h the tissue was transferred to fresh medium (physiological saline I) in which the tissue was treated with mepacrine as before. Corpus cardiacum extract (0.2 GPE/ml) was added to the medium bathing both lobes and after an additional period of incubation 50 µl of medium were taken for trehalose analysis. The differences shown are significant, $p < 0.05$; n=15. C) Paired fat body lobes were preincubated in 2 ml physiological saline II at 30°C. The medium bathing one lobe of each pair contained 1 mM mepacrine. After 1h the tissues were transferred to fresh medium (physiological saline I) again with mepacrine in the appropriate vessel as before. The medium bathing both lobes contained 10pmol CC I per ml of medium. After further incubation for 1h 50 µl samples of medium were removed for trehalose determination with anthrone reagent. The difference shown is significant, $p < 0.025$; n=8. The protein content of the tissue was determined as described in the materials and methods. Abbreviations used are CON, control; CC I, synthetic hypertrehalosemic hormone I, CCE, Crude natural hypertrehlosemic hormone; MEP, mepacrine.


A
UNCHALLENGED
1200
900
600
300
0
Trehalose efflux
(nmol/mg protein/hr)
CON
MEP (1mM)
Treatment


B
CCE CHALLENGED
2400
2000
1600
1200
800
400
0
Trehalose efflux
(nmol/mg protein/hr)
CON
MEP (10μM)
Treatment

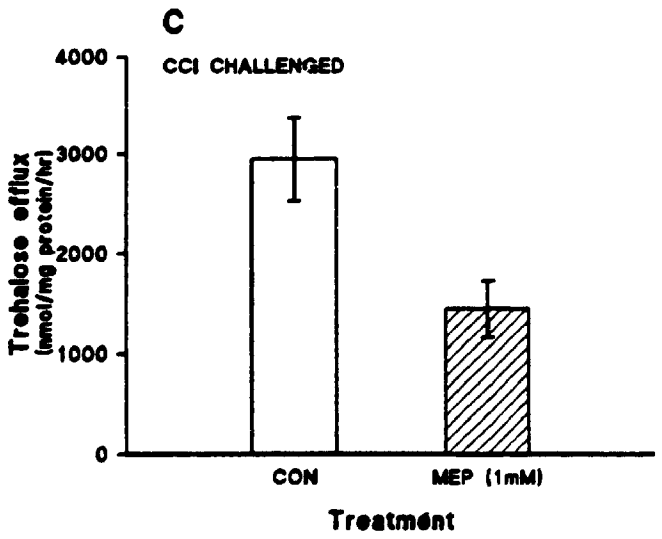
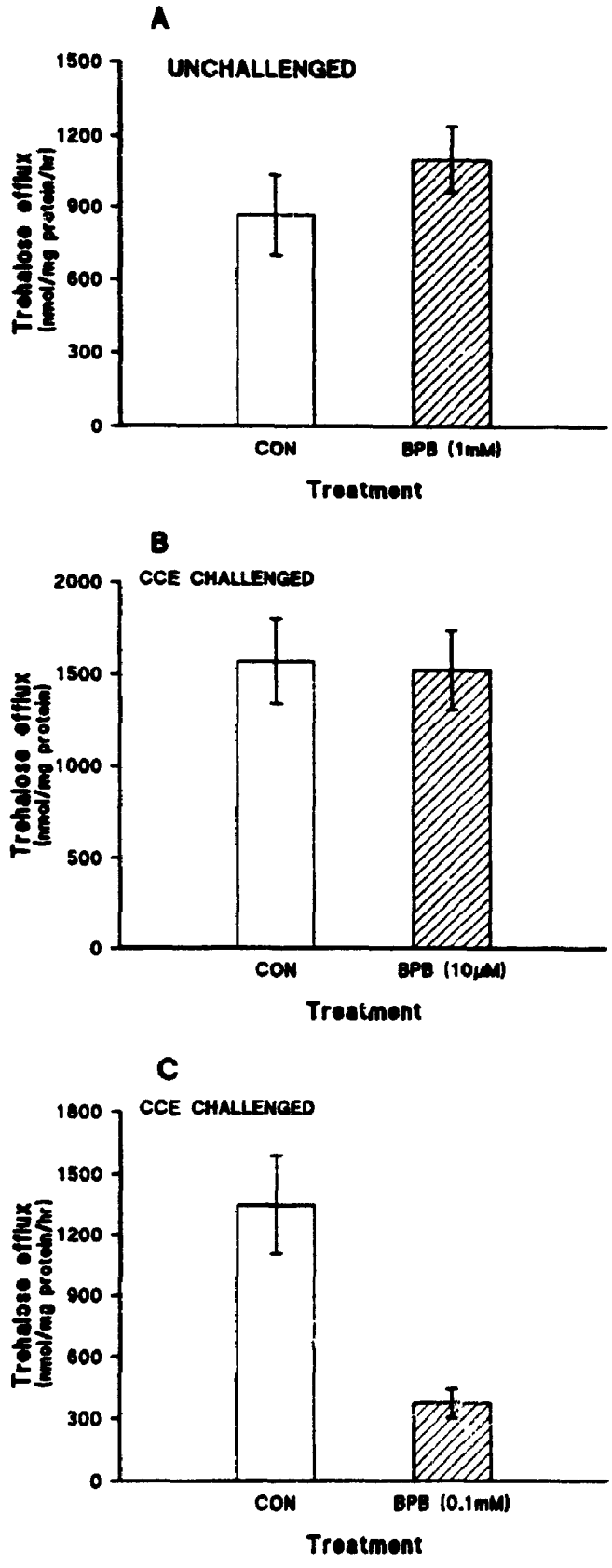

C
CCI CHALLENGED
4000
3000
2000
1000
0
Trehalose efflux
(nmol/mg protein/hr)
CON
MEP (1mM)
Treatment

Figure 9. Inhibition of hormone stimulated trehalose efflux from fat body *in vitro* by the phospholipase A_2 inhibitor ρ-bromophenacyl bromide . A) Paired fat body lobes were pre-incubated for 1h at 30°C. The medium (physiological saline II) bathing one lobe contained 1 mM BPB. Each tissue was then transferred to fresh medium (physiological saline I), again with BPB in the appropriate medium. At 1h 50 μl of medium was taken for determination of the trehalose content (n=8). B) Paired fat body lobes were pre-incubated in 2 ml physiological saline II at 30°C. One lobe of each pair was treated with 10 μM BPB. At 1h the tissues were transferred to fresh medium (physiological saline I) in which one of the tissues was treated with BPB as before. Corpus cardiacum extract (0.2 GPE/ml) was added to both lobes and after an additional period of incubation for 1h 50 μl of medium was taken for trehalose analysis (n=8). C) Paired fat body lobes were pre-incubated in 2 ml physiological saline II at 30°C. The medium bathing one lobe of each pair contained 1 mM BPB. At 1h both tissues were transferred to fresh medium (physiological saline I), one containing BPB and the other without BPB as before. The medium bathing both lobes contained CC extract (0.2 GPE/ml). After further incubation for 1h 50 μl of medium was removed for trehalose determination with anthrone reagent. The difference shown is significant, $p < 0.005$; n=8. The protein content of the tissue was determined as described in the materials and methods. Abbreviations used are, CON, control; CCE, Crude natural hypertrehlosemic hormone; BPB, ρ-bromophenacyl bromide.


A
UNCHALLENGED
Trehalose efflux
(nmol/mg protein/hr)
1500
1200
900
600
300
0
CON
BPB (1mM)
Treatment
B
CCE CHALLENGED
Trehalose efflux
(nmol/mg protein)
2000
1500
1000
500
0
CON
BPB (10μM)
Treatment
C
CCE CHALLENGED
Trehalose efflux
(nmol/mg protein/hr)
1800
1500
1200
900
600
300
0
CON
BPB (0.1mM)
Treatment

at 30°C in a shaking water bath after which 50 µl of medium was taken for trehalose determination. The sugar efflux in the control sample was 1667 ± 182 nmol trehalose/mg protein, whereas that in the melittin treated sample was 2194 ± 194 nmol trehalose/mg protein. A comparison of the means reveals that these differences are significant ($p < 0.05$, $n=6$).

Because glycogen phosphorylase is generally considered to be the rate determining reaction in trehalose synthesis it was of interest to know whether the PLA_2 inhibitors and activators which affected trehalose efflux had corresponding effects on glycogen phosphorylase. The action of BPB on phosphorylase in dispersed trophocytes is shown in Table 2. The data show that the percentage of enzyme in the *a* form is not changed significantly by the action of the inhibitor. The site of action of BPB must therefore be at some site downstream from phosphorylase. Not surprisingly, melittin, which increases trehalose efflux from the cells also activates phosphorylase (Table 3). However, the addition of melittin to trophocytes activated by either crude or synthetic hypertrehalosemic hormone blocked the normal activation of phosphorylase caused by hormone alone (Table 3).

Table 2. The failure of ρ-bromophenacyl bromide to inhibit hypertrehalosemic hormone activation of glycogen phosphorylase in trophocytes.

	Phosphorylase activity (nmol NADPH.min^{-1}.mg^{-1} protein)		Percent *a*
	a	Total	
Control	28.8±1.9	102.5±7.0	28.5±1.5
BPB	37.9±5.1	118.6±18.7	33.3±2.3
CCE	65.6±8.6	93.3±7.6	69.2±2.3
CCE+BPB	82.8±11.8	118.5±10.4	69.1±4.4
CCI	59.6±8.8	88.9±13.2	67.2±2.3
CCI+BPB	68.8±6.5	91.4±6.6	75.2±3.7
CCII	58.0±12.4	89.3±19.1	64.9±1.0
CCII BPB	57.0±6.6	97.7±15.6	59.1±2.6

Dispersed trophocytes (~30,000/sample) in 250 µl saline III were allowed to equilibrate for 30 min in a water bath at 30°C with gentle shaking. To one half the samples BPB (0.1 mM final concentration) was added and the incubation continued for 15 min. Some of the samples were then treated with hypertrehalosemic hormone, either CCE (0.2 gland pair equivalents/sample), CC I or CC II (10 pmol/sample). The incubation was continued for 5 min before the reaction was stopped by the addition of 750 µl ice-cold NaF/EDTA solution followed by sonification. The samples were then processed and phosphorylase activity determined as described in the materials and methods.
The data are expressed as means ± s.e.m.
ANOVA performed on the data reveals the differences between Control(n=13) and BPB(n=6). CCE(n=17) and CCE+BPB(n=3), CC I(n=5) and CC I+BPB(n=3) and CC II(n=4) and CC II+BPB(n=3) to be not significantly different.
Abbreviations used are CCE, CC extract containing both natural hypertrehalosemic hormones; CC I, synthetic hypertrehalosemic hormone I; CC II, synthetic hypertrehalosemic hormone II; BPB, ρ-bromophenacyl bromide.

Table 3. The activation of glycogen phosphorylase by the phospholipase A_2 activator, melittin and its inhibition of hypertrehalosemic hormone mediated stimulation of phosphorylase activity in trophocytes.

	Phosphorylase activity (nmol NADPH.min $^{-1}$.mg^{-1} protein)		Percent *a*
	a	Total	
Control	24.3±2.5	76.9±6.8	31.3±1.0
Melittin	57.3±10.4	118.5±18.0	47.8±3.9
CCE	65.6±8.6	93.3±7.6	69.2±2.3
CCE+Mel	54.2±15.9	132.4±12.9	39.5±7.7
CCI	59.6±8.8	88.9±13.2	67.2±2.3
CCI+Mel	31.2±7.5	98.2±11.2	30.8±4.6
CCII	58.0±12.4	89.3±19.1	64.9±1.0
CCII+Mel	56.6±9.4	143.5±11.8	39.1±4.6

Dispersed trophocytes (~30,000/sample) in 250 µl saline III in 15 ml round-bottomed glass tubes were allowed to equilibrate for 30 min in a water bath at 30°C with gentle shaking. To one-half the samples melittin (12.50 µg/ml final concentration) was added and the incubation continued for 15 min. Some of the samples were then treated with hypertrehalosemic hormone, either CC extract (0.2 gland pair equivalents/sample), CC I or CC II (10 pmol/sample). The incubation was allowed to continue for 5 min before the reaction was stopped by the addition of 750 µl ice-cold NaF/EDTA solution followed by sonification. The samples were then processed and phosphorylase activity determined as described in the materials and methods.
The data are expressed as means ± s.e.m.
ANOVA performed on the data reveals that the differences between Control (n=13) vs. Mel.(n=4) ($p < 0.0001$), CCE(n=17) vs. CCE+Mel.(n=3) ($p < 0.0005$), CC I(n=5) vs. CC I+Mel.(n=3) ($p < 0.005$) and CC II(n=4) vs. CC II+Mel.(n=3) ($p < 0.005$) to be statistically significant.
Abbreviations used are CCE, CC extract which contains natural hypertrehalosemic hormones; CC I, synthetic hypertrehalosemic hormone I; CC II, synthetic hypertrehalosemic hormone II; Mel, melittin.

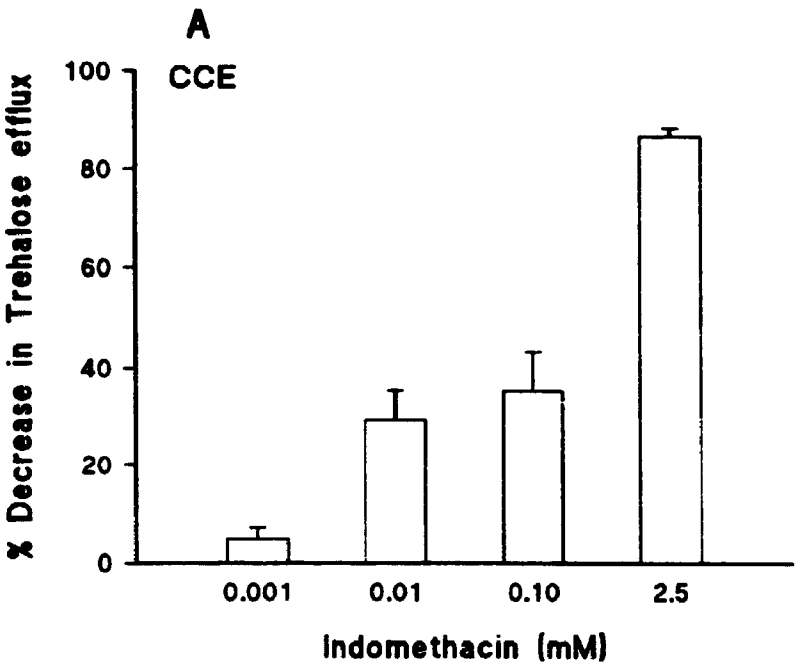
3.1.3.3 Evidence for a relationship between arachidonic acid metabolism and trehalose synthesis

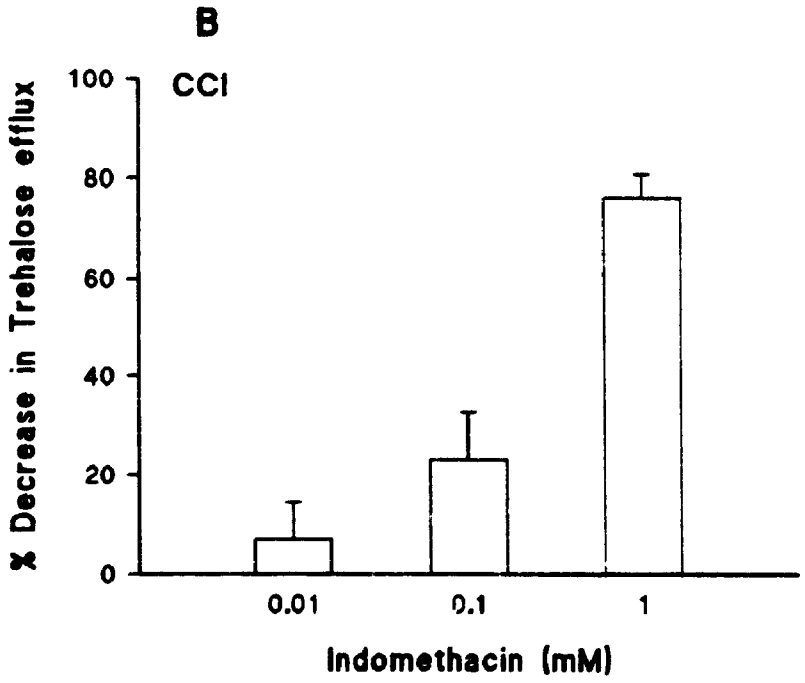
To determine whether trehalose efflux from resting tissue was affected by indomethacin, paired fat body lobes were pre-incubated in saline II containing 40 mM trehalose. Indomethacin was included in the saline bathing one lobe of each pair. At 1h the tissues were transferred to saline I without trehalose but with indomethacin present at the same concentration as before. After incubation for 1h at 30°C in a shaker water bath, 50 μl of medium were taken for determination of trehalose. The efflux of trehalose from the tissue with 1 mM indomethacin present was decreased by 38 percent. In contrast, the lower concentration of indomethacin (0.1 mM) increased trehalose efflux by 157 percent. A comparison of the means reveals that both the decrease ($p < 0.05$, n=8) and the increase ($p < 0.005$, n=8) are significant.

The effect of indomethacin on trehalose efflux in hormone treated tissue is different from that in resting tissue. The results obtained for both crude hormone and CC I (Fig. 10) show that indomethacin was inhibitory at all concentrations tested and did not display the stimulatory effect found with unchallenged tissue.

The effect of the cyclooxygenase inhibitor diclofenac, was similar to that of indomethacin. With unchallenged fat body 0.1 mM diclofenac increased the release of trehalose

Figure 10. The inhibitory effect of indomethacin on trehalose efflux from fat body stimulated by hypertrehalosemic hormone *in vitro*. A. Paired fat body lobes were pre-incubated in 2 ml of physiological saline II at 30°C. Indomethacin was included in the medium bathing one lobe of each pair. At 1h the tissues were transferred individually to fresh medium (saline I), with and without indomethacin as during the preincubation. At this time natural hypertrehalosemic hormone (0.2 GPE/ml) was added to the medium containing left and right lobes. B. Paired fat body lobes were pre-incubated in 2 ml physiological saline II at 30°C. Indomethacin was included in the medium bathing one lobe of each pair. After 1h the tissues were transferred to fresh medium (saline I), again with indomethacin present in the medium bathing those tissues previously treated with inhibitor. At this time synthetic hypertrehalosemic hormone CC I (10 pmol/ml) was added to the medium containing left and right lobes. Following incubation for 1h 50 μl of medium was taken for trehalose determination. Protein was determined as described in the material and methods. The results show the mean change in trehalose efflux due to indomethacin as a percentage of that for the paired control tissue. Indomethacin significantly decreases trehalose efflux induced by natural and synthetic (CC I) hypertrehalosemic hormone.


A
CCE
% Decrease in Trehalose efflux
100
80
60
40
20
0
0.001
0.01
0.10
2.5
Indomethacin (mM)

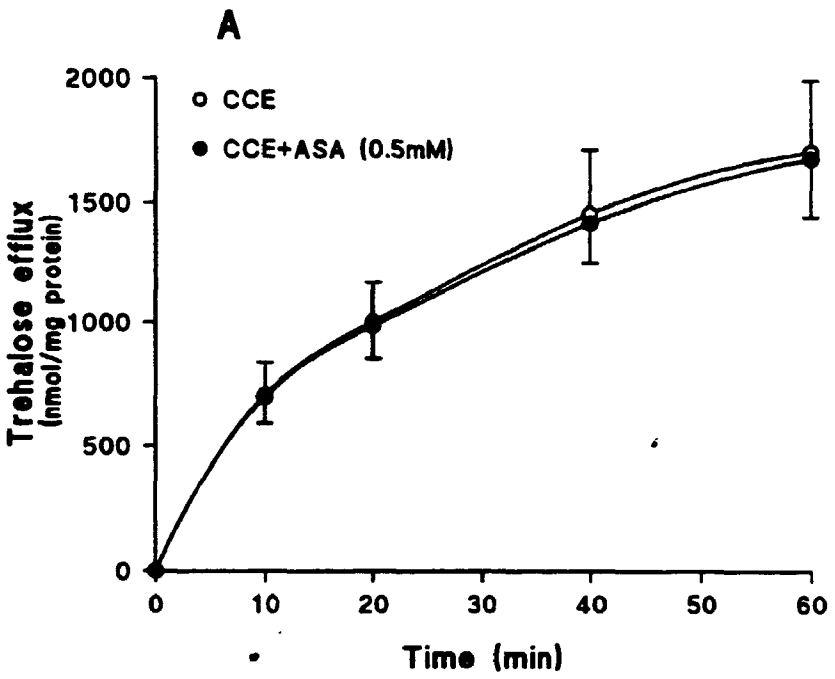

B
CCl
% Decrease in Trehalose efflux
100
80
60
40
20
0
0.01
0.1
1
Indomethacin (mM)

from 549 ± 40 nmol/mg protein to 1448 ± 141 nmol/mg protein, an increase of 164 percent ($p < 0.005$, n=8). The effect of the same concentration of inhibitor on fat body treated with crude hormone was to decrease trehalose efflux by 213 percent ($p < 0.005$, n=8). This result is comparable to that obtained with indomethacin.

Because acetylsalicylic acid is an effective inhibitor of cyclooxygenase in mammalian tissues (Needleman, 1986) an effect on the trophocytes was to be expected. Fig 11A and B show that at both concentrations tested there is no significant effect on trehalose efflux. Nevertheless, it should be noted that the values obtained for the higher concentration of ASA were consistently lower than the control values for all time intervals.

Because both indomethacin and diclofenac decreased trehalose efflux in hormone stimulated fat body it was of interest to know whether this effect was due to inhibition of glycogen phosphorylase. Table 4 shows that 0.1 and 1 mM indomethacin activate phosphorylase whereas 0.01 mM was ineffective. Diclofenac has a similar pattern of activity (Table 5). The data show that hormonal activation of phosphorylase is not prevented by the inhibitors. Thus inhibition of hormone stimulated trehalose efflux cannot be explained by inhibition of glycogen phosphorylase. The fact that both cyclooxygenase inhibitors blocked trehalose efflux but failed to block activation of phosphorylase by the

Figure 11. Failure of acetylsalicylic acid (ASA) to block hypertrehalosemic hormone stimulated trehalose efflux from fat body *in vitro*. In panel A, paired fat body lobes were preincubated in 2 ml of saline II at 30°C for 1h. ASA (0.5 mM) was included in the medium bathing one lobe of each pair. The tissues were then transferred to saline (I) with ASA added to the medium containing the tissues previously treated with ASA. At this time crude hypertrehalosemic hormone, CCE (0.2 GPE/ml), was added to the medium containing left and right lobes. Following incubation for 1h the samples were processed as indicated in the legend to Fig. 5. Panel B, shows a similar experiment in which 0.1 mM ASA was used. Protein content of the tissue was determined as described in materials and methods. A comparison of the data in each experiment shows that the differences due to the treatment are not significant (n = 8).


A
2000
1500
1000
500
0
o CCE
● CCE+ASA (0.5mM)
Trehalose efflux
(nmol/mg protein)
0
10
20
30
40
50
60
Time (min)

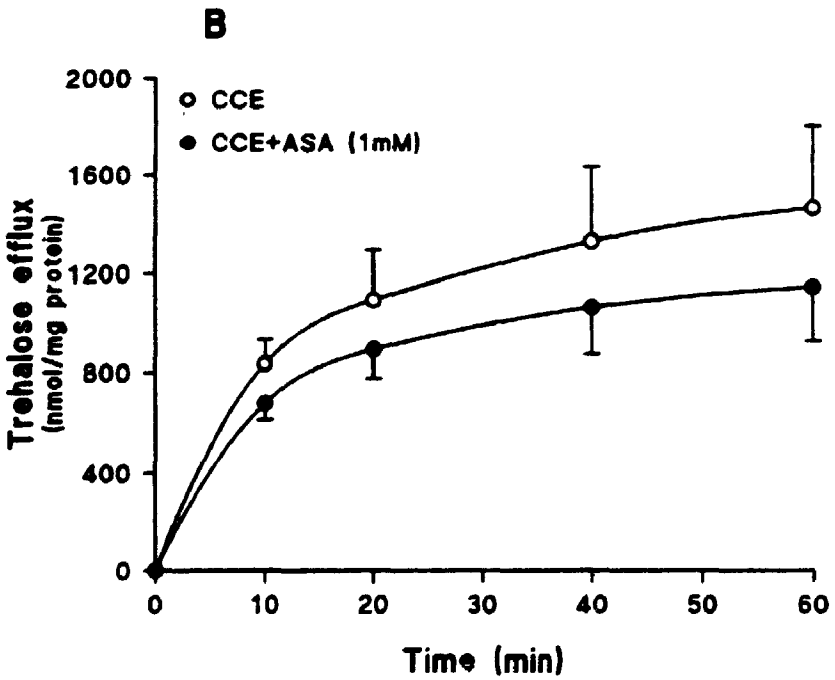

B
2000
1600
1200
800
400
0
o CCE
● CCE+ASA (1mM)
Trehalose efflux
(nmol/mg protein)
0
10
20
30
40
50
60
Time (min)

Table 4. The activation of glycogen phosphorylase by the cyclooxygenase inhibitor, indomethacin.

	Phosphorylase activity (nmol NADPH.min^{-1}.mg^{-1} protein)		Percent *a*
	a	Total	
Control	28.8±1.9	102.5±7.0	28.5±1.5
$INDO_1$	66.3±20.5	77.8±21.1	77.6±6.1
$INDO_2$	64.7±2.7	123.2±5.3	52.9±4.5
$INDO_3$	28.5±3.9	101.1±6.7	27.7±2.2
CCE	65.6±8.6	93.3±7.6	69.2±2.3
CCE+$INDO_1$	81.8±14.5	100.7±6.0	80.2±9.4
CCE+$INDO_2$	68.3±2.2	92.8±3.3	71.4±2.1
CCE+$INDO_3$	74.4±3.3	104.2±7.9	71.9±3.1
CCI	59.6±8.8	88.9±13.2	67.2±2.3
CCI+$INDO_2$	91.5±9.6	120.9±12.0	75.6±1.8
CCII	58.0±12.4	89.3±19.1	64.9±1.0
CCII+$INDO_2$	59.2±4.6	98.1±10.9	64.2±4.1

Dispersed trophocytes (~30,000/sample) in 250 µl saline III in 15 ml round-bottomed glass tubes were allowed to equilibrate for 30 min in a water bath at 30°C with gentle shaking. Indomethacin (1.0-0.01 mM final concentration) was added to the samples where indicated and the incubation continued for 15 min. Some of the samples were then treated with one of the hypertrehalosemic hormones, either CC I or CC II (10 pmol/sample) or the CC extract (0.2 gland pair equivalents/sample) or left untreated to serve as control samples. The incubation was to continued for an additional 5 min before the reaction was stopped by the addition of 750 µl ice-cold NaF/EDTA solution followed by sonification. The samples were then processed and phosphorylase activity determined as described in the materials and methods.
The data are expressed as means ± s.e.m.
Analysis of the data (ANOVA) shows that only Control(n=13) vs. $INDO_1$(n=3) ($p < 0.001$), Control(n=13) vs. $INDO_2$(n=3) ($p < 0.001$), and CC I(n=5) vs. CC I+$INDO_2$(n=3) ($p < 0.05$) are significantly different, whereas, Control(n=13) vs. $INDO_3$(n=3), CCE(n=17) vs. $INDO_1$(n=3), $INDO_2$(n=3) and $INDO_3$(n=6), and CC II(n=4) vs. CC II+$INDO_2$(n=6) are not.
Abbreviations used are CCE, CC extract which contains natural hypertrehalosemic hormones; CC I, synthetic hypertrehalosemic hormone I; CC II, synthetic hypertrehalosemic hormone II; $INDO_1$, 1.0 mM indomethacin; $INDO_2$, 0.1 mM indomethacin; $INDO_3$, 0.01 mM indomethacin.

Table 5. The activation of glycogen phosphorylase by the cyclooxygenase inhibitor diclofenac.

	Phosphorylase activity (nmol NADPH.min^{-1}.mg^{-1} protein)		Percent *a*
	a	Total	
Control	28.8±1.9	102.5±7.0	28.5±1.5
DICLO	59.5±2.5	110.1±3.0	54.0±1.2
CCE	65.6±8.6	93.3±7.6	69.2±2.3
CCE+DICLO	84.2±9.5	114.6±13.0	73.5±0.6
CCI	59.6±8.8	88.9±13.2	67.2±2.3
CCI+DICLO	88.3±5.3	99.9±2.3	88.3±3.3

Dispersed trophocytes (~30,000/sample) in of 250 µl saline III in 15 ml round-bottomed glass tubes were allowed to equilibrate for 30 min in a water bath at 30°C with gentle shaking. Diclofenac (0.1 mM final concentration) was added where indicated and the incubation continued for 15 min. Some of the samples were then treated with hypertrehalosemic hormones (CCE, 0.2 gland pair equivalent/sample or CC I, 10 pmol/sample) or left untreated to serve as controls. The incubation was continued for an additional 5 min before the reaction was stopped with the addition ice-cold 750 µl NaF/EDTA solution followed by sonification. The samples were then processed and phosphorylase activity determined as described in the materials and methods.
The data are expressed as the means ± s.e.m.
Anova performed on the data reveals that Control (n=13) vs. Diclo.(n=3) ($p < 0.001$), CCE(n=17) vs. CCE+Diclo.(n=3) ($p < 0.01$), CC I(n=5) vs. CC I+Diclo.(n=3) ($p < 0.005$) are significantly different.
Abbreviations used are CCE, CC extract which contains natural hypertrehalosemic hormones; DICLO, diclofenac.

hormones suggested that an accumulation of trehalose may have occurred within the tissue. To test the possibility that the hormone increases intracellular trehalose and that this is augmented by the inhibitor, the following series of experiments were undertaken.

Fig. 12 illustrates the time dependent stimulatory effect of CC extract on intracellular trehalose in the trophocytes. The data show that trehalose is elevated within 5 min in cells treated with hormone, reaches a maximum within 10 min, and remains elevated for 30 min after which the concentration of trehalose decreases to control levels. It was anticipated that treatment of the trophocytes with indomethacin would decrease the intracellular concentration of free trehalose because the inhibitor markedly decreases the efflux of trehalose from CC stimulated cells. However, the results do not support this view (Fig. 13) but instead show that elevated levels of free trehalose in CC stimulated trophocytes are not significantly affected by the inhibitor. It is also of interest to note that trophocytes treated with indomethacin alone also show an elevated level of intracellular trehalose.

Because indomethacin inhibited trehalose synthesis it was of interest to determine whether inhibitors of the lipoxygenase pathway also decreased production of trehalose. In resting tissue NDGA had no effect on trehalose efflux at the concentration tested. The rate of trehalose efflux in

Figure 12. The time dependent effect of crude hypertrehalosemc hormone on intracellular trehalose in dispersed trophocytes. Trehalose formation in untreated and hormone (CCE) challenged trophocytes (~30,000/sample) is illustrated using cells drawn from the same population. The trophocytes were incubated in 10 ml round-bottomed polyethylene tubes in 350 ul physiological saline IV at 30°C in a shaker water bath with and without CCE (0.2 GPE/sample) for the indicated samples. One-quarter ml samples were removed and processed as described in the materials and methods. Analysis (ANOVA) of the data indicates a significant difference at 5, 10, 15 and 30 min, $p < 0.05$, n=3. The results demonstrate that the hormone increased intracellular trehalose levels as early as 5 min and that this difference was maintained for up to 30 min. Abbreviation used is CCE, natural hypertrehalosemic hormone.

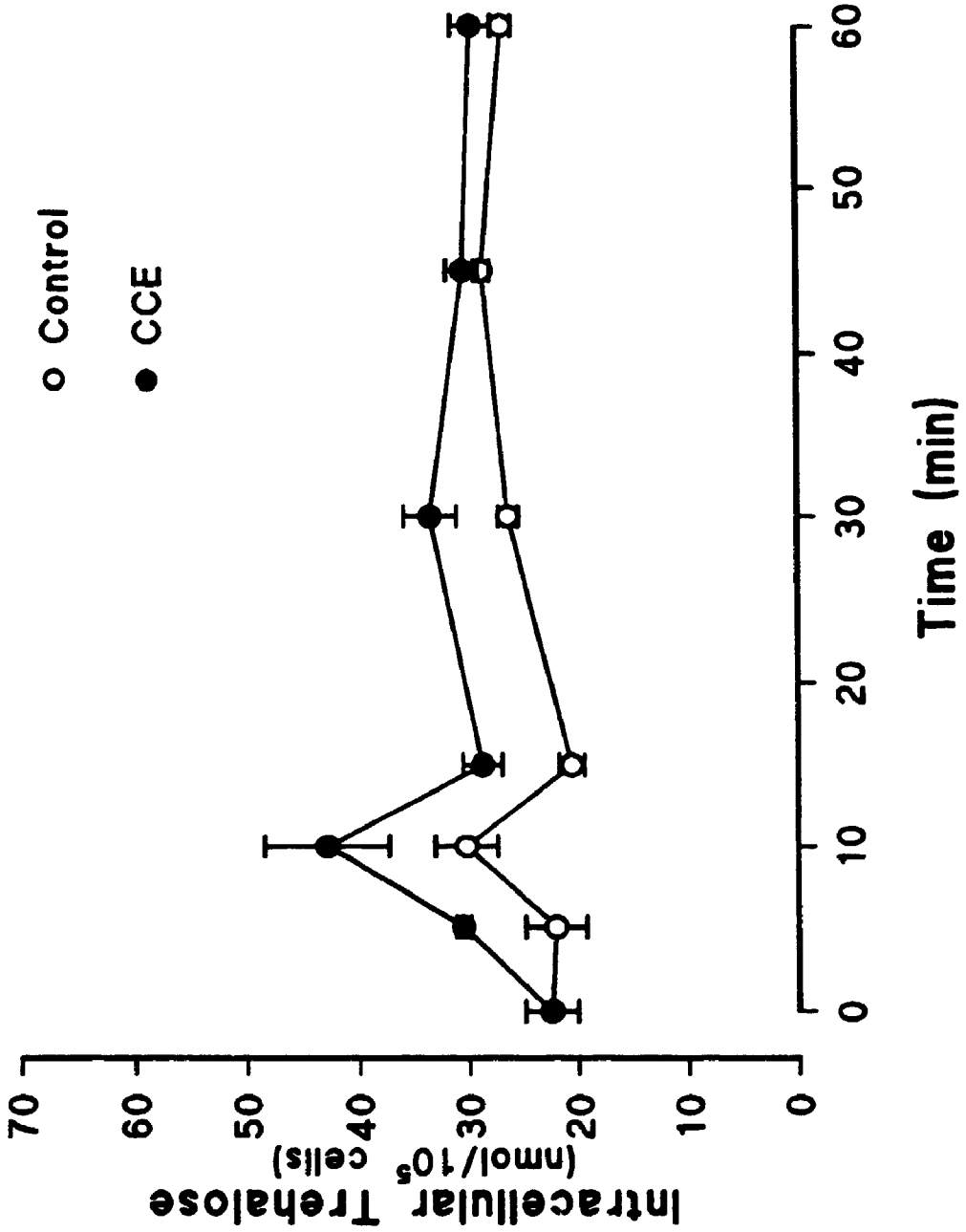
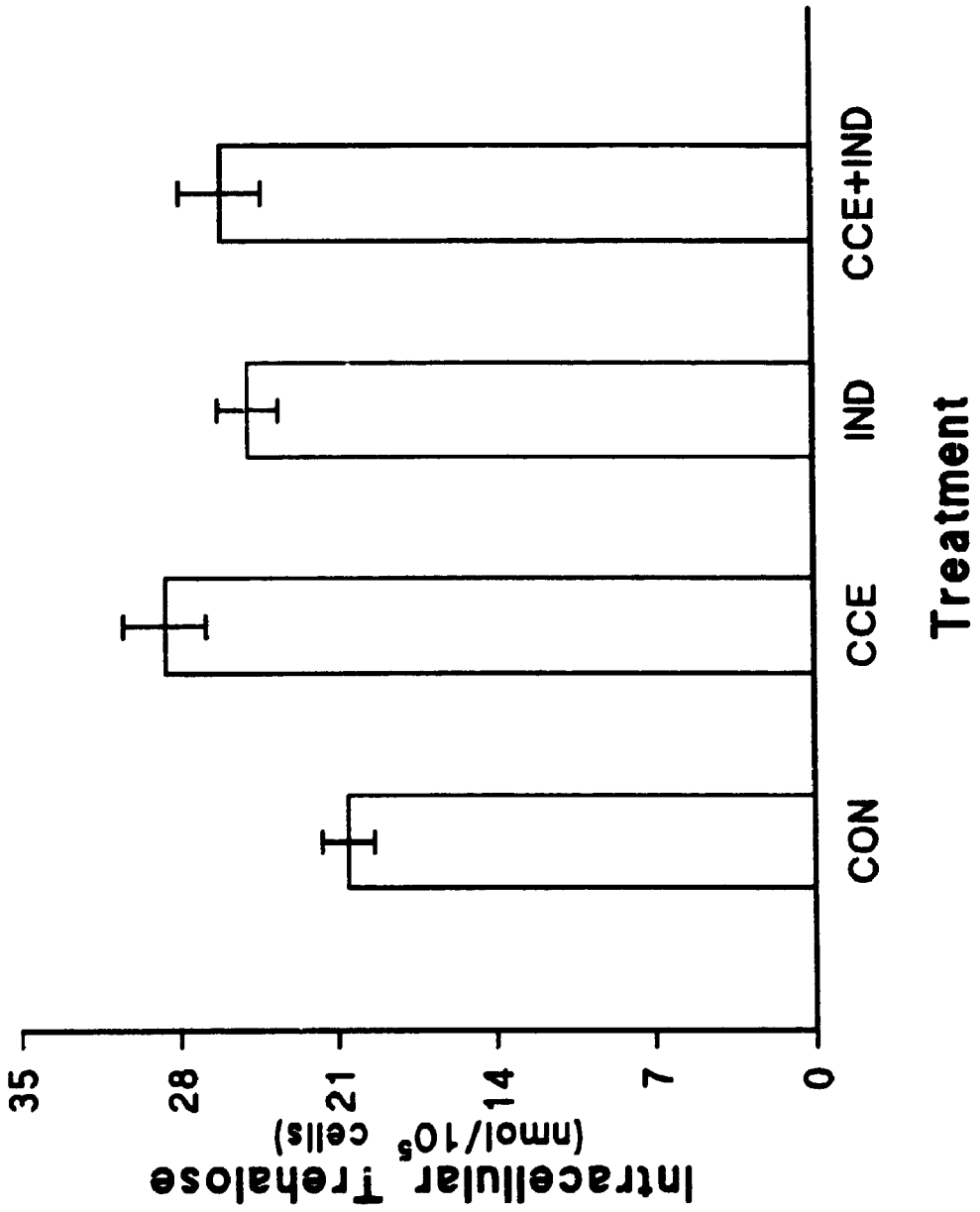

Control
CCE
Intracellular Trehalose
(nmol/10^5 cells)
Time (min)
0
10
20
30
40
50
60
70

Figure 13. The failure of indomethacin to inhibit the stimulatory effect of crude hypertrehalosemic hormone (CCE) on intracellular trehalose in dispersed trophocytes. Trehalose formation in indomethacin, and CCE challenged trophocytes (~45,000/sample) is illustrated using cells which have all been drawn from the same population. The dispersed cells were pre-incubated in 10 ml round-bottomed polyethylene tubes in 350 µl physiological saline IV at 30°C in a shaker water bath for 15 min. Indomethacin (0.1mM final concentration) was added where indicated. After the pre-incubation period, CCE (0.2 GPE/350µl) was added where shown. Following 15 min incubation, 250 ul of medium containing cells was removed and the intracellular trehalose determined as described in the materials and methods. Statistical analysis of the data reveals a significant difference only between control and CCE treated samples, $p < 0.01$, $n = 8$. The results show that intracellular trehalose levels elevated by CCE are not affected by indomethacin. Abbreviations used are CON, control; CCE, natural hypertrehalosemic hormone; INDO, indomethacin.


Intracellular Trehalose
(nmol/10^5 cells)
0
7
14
21
28
35
CON
CCE
IND
CCE+IND
Treatment

control tissues was 509 ± 68 nmol/mg protein compared to 389 ± 80 nmol/mg protein in fat body treated with 0.1 mM NDGA. This difference is not significant. However, when trehalose efflux was stimulated by CC extract it was inhibited by 0.1 mM NDGA. The production of trehalose decreased from 1851 ± 289 n mol/mg protein to 906 ± 159 nmol/mg protein. The difference is significant ($p < 0.01$. n=8). Decreasing the concentration of NDGA to 10 μM caused the effect of the inhibitor on hormone stimulated trehalose efflux to disappear. Since the inhibitory effect of indomethacin on trehalose efflux was not reflected in the activity of glycogen phosphorylase it was of interest to know whether NDGA was also inactive. Table 6 shows that treatment of the cells with NDGA results in minor activation of phosphorylase. The activation of phosphorylase by crude hypertrehalosemic hormone, CC I and CC II is not inhibited by NDGA. These results suggest that inhibition of trehalose efflux is not due to inhibition of glycogen phosphorylase.

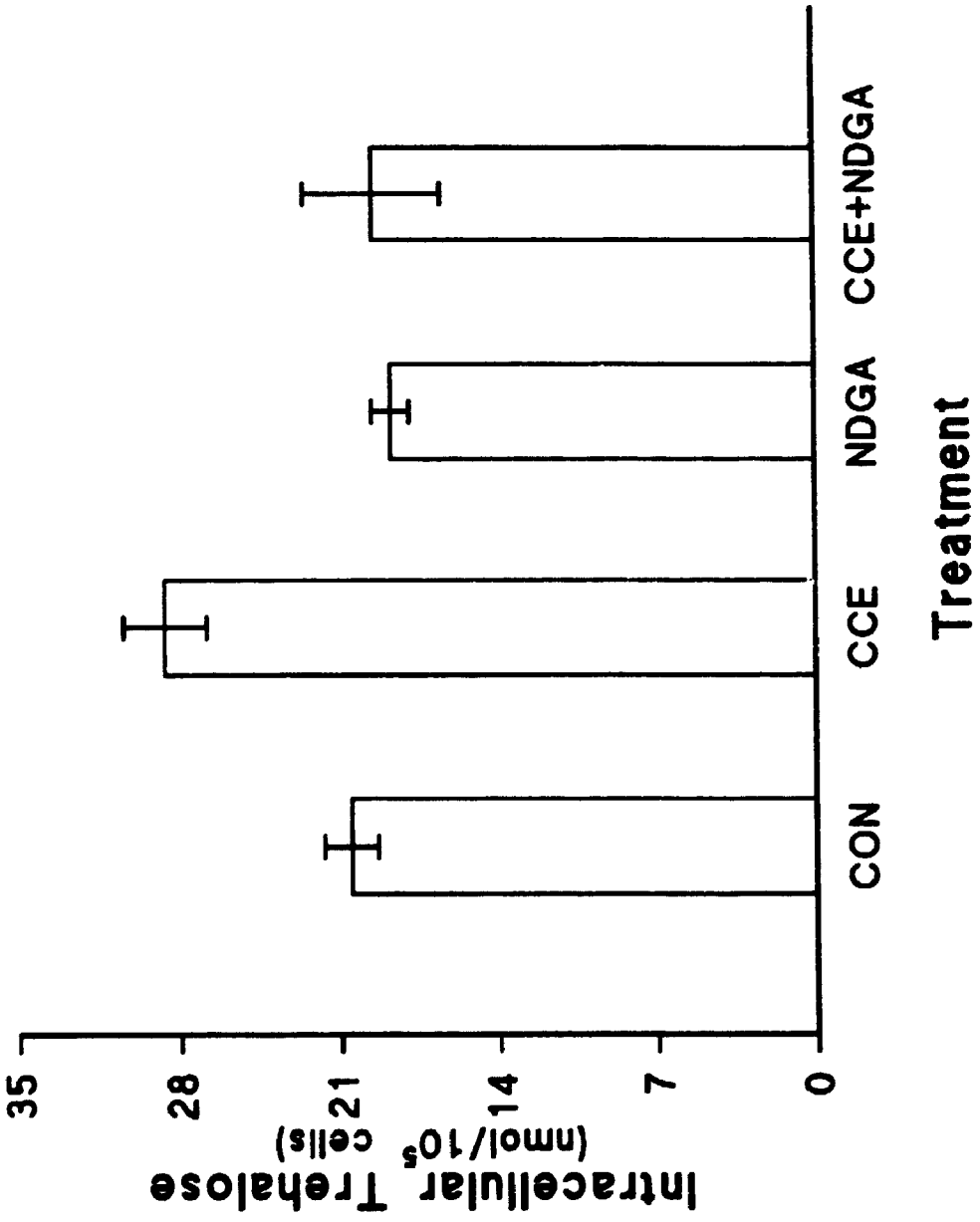
Since the lipoxygenase and cyclooxygenase pathways are present together in most tissues, it was of interest to know whether NDGA had an effect on intracellular trehalose in hormone treated fat body similar to that of indomethacin. The results shown in Fig. 14 suggest that NDGA has no effect on intracellular trehalose in unstimulated cells but decreases that in hormone treated trophocytes.

Table 6. The failure of lipoxygenase inhibitor, nordihydroguaiaretic (NDGA) to inhibit hormonal activation of phosphorylase activity in trophocytes.

	Phosphorylase activity (nmol NADPH.min $^{-1}$.mg^{-1} protein)		Percent *a*
	a	Total	
Control	24.3±2.5	76.9±6.8	31.3±1.0
NDGA	52.7±5.1	124.3±10.6	42.4±1.8
CCE	65.6±8.6	93.3±7.6	69.2±2.3
CCE+NDGA	96.1±23.7	129.7±15.2	71.8±10.1
CCI	59.6±8.8	88.9±13.2	67.2±2.3
CCI+NDGA	51.4±12.0	74.0±15.0	77.2±5.8
CCII	58.0±12.4	89.3±19.1	64.9±1.0
CCII+NDGA	74.6±7.4	91.6±6.4	81.1±2.4

Dispersed trophocytes (~30,000/sample) in 250 µl saline III in 15 ml round-bottomed glass tubes were allowed to equilibrate for 30 min in a water bath at 30°C with gentle shaking . NDGA (0.1 mM final concentration) was added where indicated and the incubation continued for 15 min. Hypertrehalosemic hormones, either CCE (0.2 gland pair equivalents/sample), CC I or CC II (10 pmol/sample), were then added as shown above. The incubation was allowed to continue for an additional 5 min before the reaction was stopped by the addition of 750 µl ice-cold NaF/EDTA solution followed by sonification. The samples were then processed and phosphorylase activity determined as described in the materials and methods.
Results are expressed as means ± s.e.m.
ANOVA performed on the data reveals that Control(n=13) vs. NDGA(n=6) ($p < 0.001$) and CC II(n=4) vs. CC II+NDGA(n=3) ($p < 0.005$) are significantly different, whereas CCE(n=17) vs. CCE+NDGA(n=3) and CC I(n=5) vs. CC I+NDGA(n=3) are not.
Abbreviations used are CCE, CC extract containing natural hypertrehalosemic hormones; CC I, synthetic hypertrehalosemic hormone I; CC II, synthetic hypertrehalosemic hormone II; NDGA, nordihydroguaiaretic acid.

Figure 14. Inhibition by NDGA of the increase in intracellular trehalose caused by crude hypertrehalosemic hormone (CCE) in dispersed trophocytes. Trehalose formation in NDGA, and CCE challenged trophocytes (~45,000/sample) is illustrated using cells which have all been drawn from the same population. The dispersed cells were pre-incubated in 10 ml round-bottomed polyethylene tubes in 350 µl physiological saline IV at 30°C in a shaker water bath for 15 min. NDGA (0.1mM final concentration) was added where indicated. After the preincubation period, CCE (0.2 GPE/350µl) was added where shown. After an additional 15 min incubation, 250 ul of medium containing cells was removed and the intracellular trehalose determined as described in the materials and methods. Statistical analysis of the data (n = 4) reveals significant differences between: Control and CCE ($p < 0.01$); CCE and NDGA ($p < 0.01$); CCE and CCE+NDGA ($p < 0.05$). The results show that the increase in intracellular trehalose due to CCE is inhibited by NDGA. Abbreviations used are CON, control; CCE, natural hypertrehalosemic hormone; NDGA, nordihydroguaiaretic acid.


Intracellular Trehalose
(nmol/10^5 cells)
35
28
21
14
7
0
CON
CCE
NDGA
CCE+NDGA
Treatment

3.2 Hormonal regulation of Free Fatty Acids

The demonstration in this study that putative blockade of arachidonic acid metabolism due to inhibition of the cyclooxygenase and lipoxygenase pathways causes a marked reduction in trehalose production by the trophocytes treated with hypertrehalosemic hormone argues strongly that arachidonic acid and possibly other fatty acids may be important elements in the hormonal mechanism. In the work described below evidence is presented to show that the action of the hypertrehalosemic hormones is accompanied by the production of free fatty acids.

3.2.1 Control of free fatty acid concentration in trophocytes

As illustrated in Fig. 15, intracellular free fatty acids are increased in trophocytes treated with the synthetic hypertrehalosemic hormone CC I. Four fatty acids are released in sufficient quantity to be detected. Showing quantitative change in response to the hormone are palmitic, stearic, oleic and linoleic acids. The same fatty acids are also released by the action of the synthetic hypertrehalosemic hormone, CC II (Fig. 16). The fatty acids do not appear to be released into the extracellular medium (Fig. 17). In contrast to trophocytes intracellular free fatty acids in urocytes and mycetocytes do not increase in response to the hormone (Fig. 18).

Figure 15. The stimulatory effect of synthetic hypertrehalosemic hormone (CC I) on free fatty acid concentration in dispersed trophocytes *in vitro*. Trophocytes (~50,000/sample) in 1 ml of saline III were pipetted into 40 ml plastic centrifuge tubes and allowed to equilibrate for 30 min at 30°C in a shaker water bath. At the end of the equilibration period CC I (100 pmol/ml) was added to one-half the samples which were then held at 30°C with gentle shaking for an additional 15 min. The reaction was stopped by pipetting the total incubation mixture into a 10 ml syringe fitted with a 20 gauge needle which was inserted through a rubber stopper supported in a 250 ml filter flask. The syringe contained a snug fitting disc of glass microfiber filter (GF/A) to retain the trophocytes. Mild vacuum was applied to the flask to draw the extracellular medium into the flask and away from the cells. The filter containing the cells was quickly withdrawn from the syringe with forceps and transferred to 1.5 ml of ice-cold methanol in a centrifuge tube and the tube sealed with a teflon-lined screw cap. The sample was mixed throughly by vortexing and allowed to stand for 20 min at 4°C. The samples were then centrifuged at 450g x 20 min. The supernatant was removed and the fatty acids extracted and derivatized with TMTFTH for GC analysis as described in the materials and methods. ANOVA performed on the data reveals that the intracellular levels of palmitic, stearic, oleic and linoleic acid were increased significantly by CC I ($p < 0.001$, $n = 14$).
Abbreviation used is CC I, synthetic hypertrehalosemic hormone I.

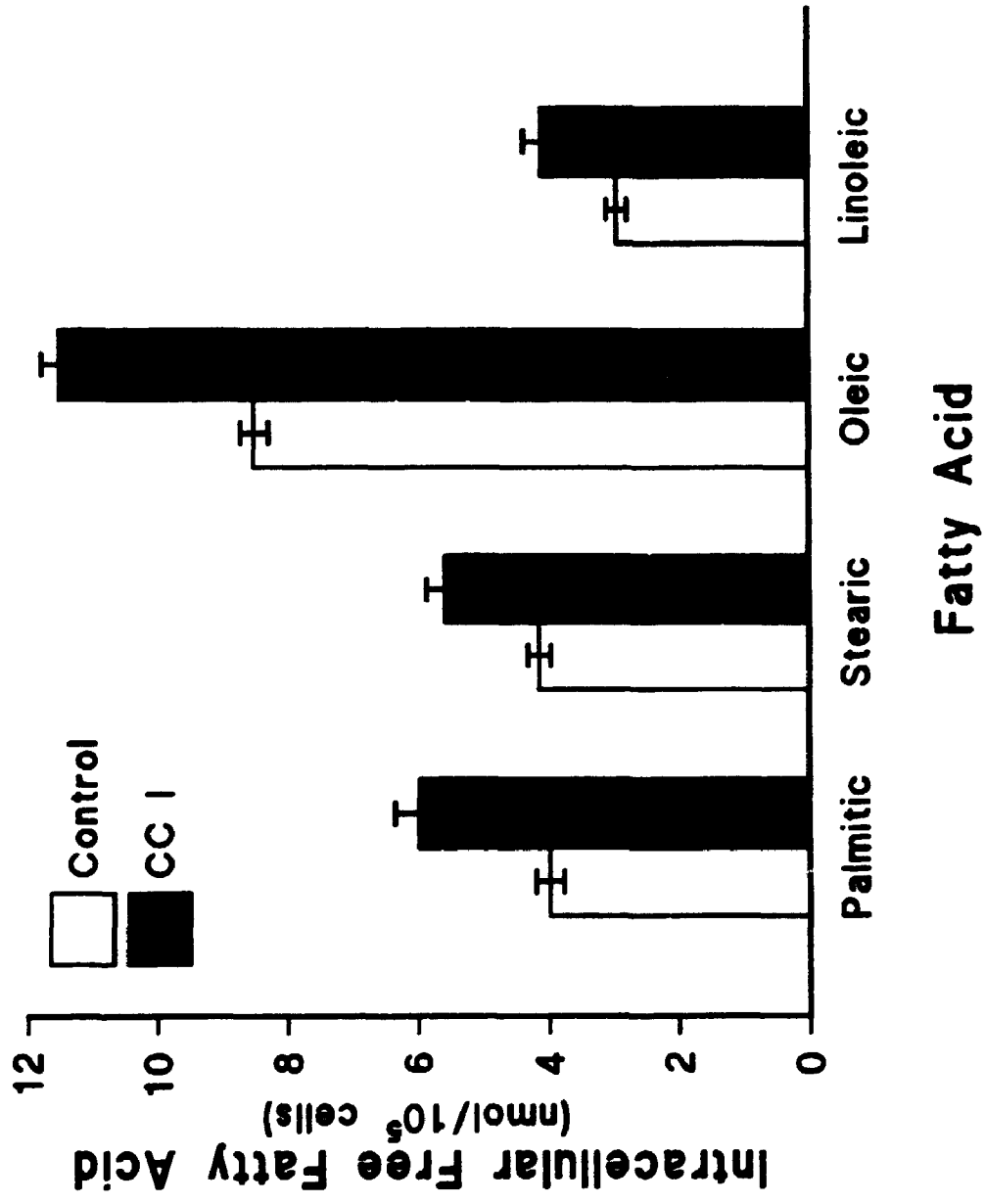

Intracellular Free Fatty Acid
(nmol/10^5 cells)
12
10
8
6
4
2
0
Control
CC I
Palmitic
Stearic
Oleic
Linoleic
Fatty Acid

Figure 16. The stimulatory effect of synthetic hypertrehalosemic hormone CC II on free fatty acid concentration in dispersed trophocytes *in vitro*. Trophocytes (~60,000/sample) in 1 ml of saline III were pipetted into 40 ml plastic centrifuge tubes and allowed to equilibrate for 30 min at 30°C in a shaker water bath. At the end of the equilibration period CC II (100 pmol/ml) was added to one-half the samples which were allowed to incubate at 30°C with gentle shaking for an additional 15 min. The reaction was stopped by pipetting the total incubation mixture into a 10 ml syringe and the sample processed as indicated in the legend of Fig. 15. Analysis of the data using ANOVA reveals that the intracellular levels of palmitic ($p < 0.025$, $n = 4$), stearic ($p < 0.005$, $n = 4$), oleic ($p < 0.005$, $n = 4$) and linoleic acid ($p < 0.005$, $n = 4$) were increased significantly by CC II. Abbreviation used is, CC II, synthetic hypertrehalosemic hormone II.

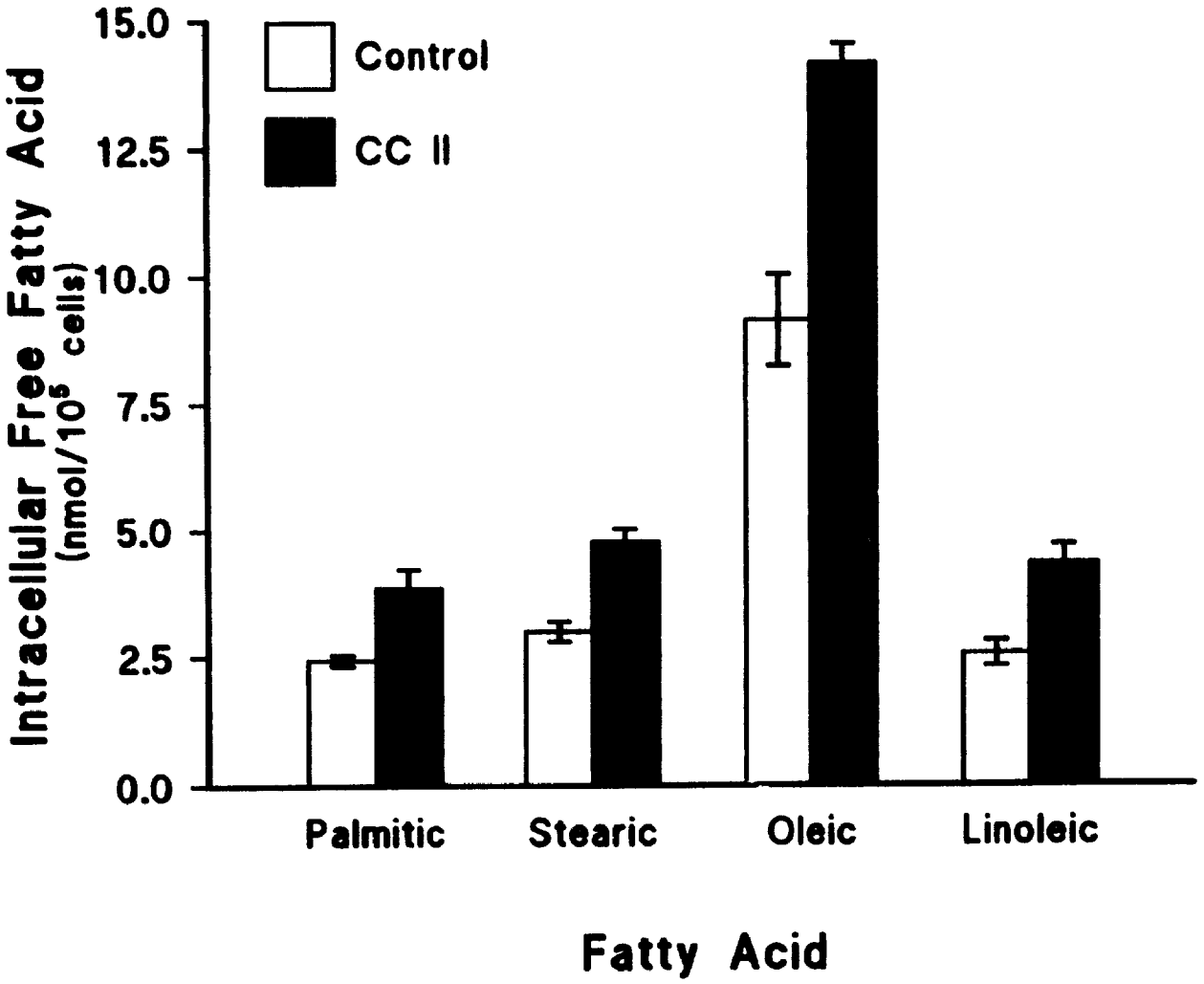

Intracellular Free Fatty Acid
(nmol/10^6 cells)
15.0
12.5
10.0
7.5
5.0
2.5
0.0
Control
CC II
Palmitic
Stearic
Oleic
Linoleic
Fatty Acid

Figure 17. Failure of the synthetic hormone CC I to stimulate release of free fatty acids from dispersed trophocytes into the incubation medium. Trophocytes (~50,000/sample) in 1 ml of saline III were pipetted into 40 ml plastic centrifuge tubes and allowed to equilibrate for 30 min at 30°C in a shaking water bath. At the end of the equilibration period CC I (100 pmol/ml) was added and the samples incubated at 30°C with gentle shaking for an additional 15 min. The reaction was stopped by pipetting the total incubation mixture into a 10 ml syringe with the needle (20G) inserted through a rubber stopper supported by a 250 ml filter flask. The flask had an ice bath at the bottom and a teflon-lined screw cap centrifuge tube containing 1.5 ml methanol was placed within the flask in such a way so that the needle end was within the centrifuge tube. The syringe contained a snug fitting disc of glass microfiber filter (GF/A) to retain the trophocytes. Mild vacuum was applied to the flask to draw the extracellular medium into the centrifuge tube within the flask and away from the cells. The centrifuge tube containing the incubation medium was removed from the flask and flushed with N_2, mixed thoroughly by vortexing, and allowed to stand for 20 min at 4°C. The samples were then centrifuged at 450g x 20 min to precipitate protein. After removal of the supernatant the fatty acids were extracted and derivatized with TMTFTH for GC analysis as described in the materials and methods. The levels of fatty acids were not significantly increased in the incubation medium by CC I (n = 4). Abbreviation used is, CC I, synthetic hypertrehalosemic hormone I.

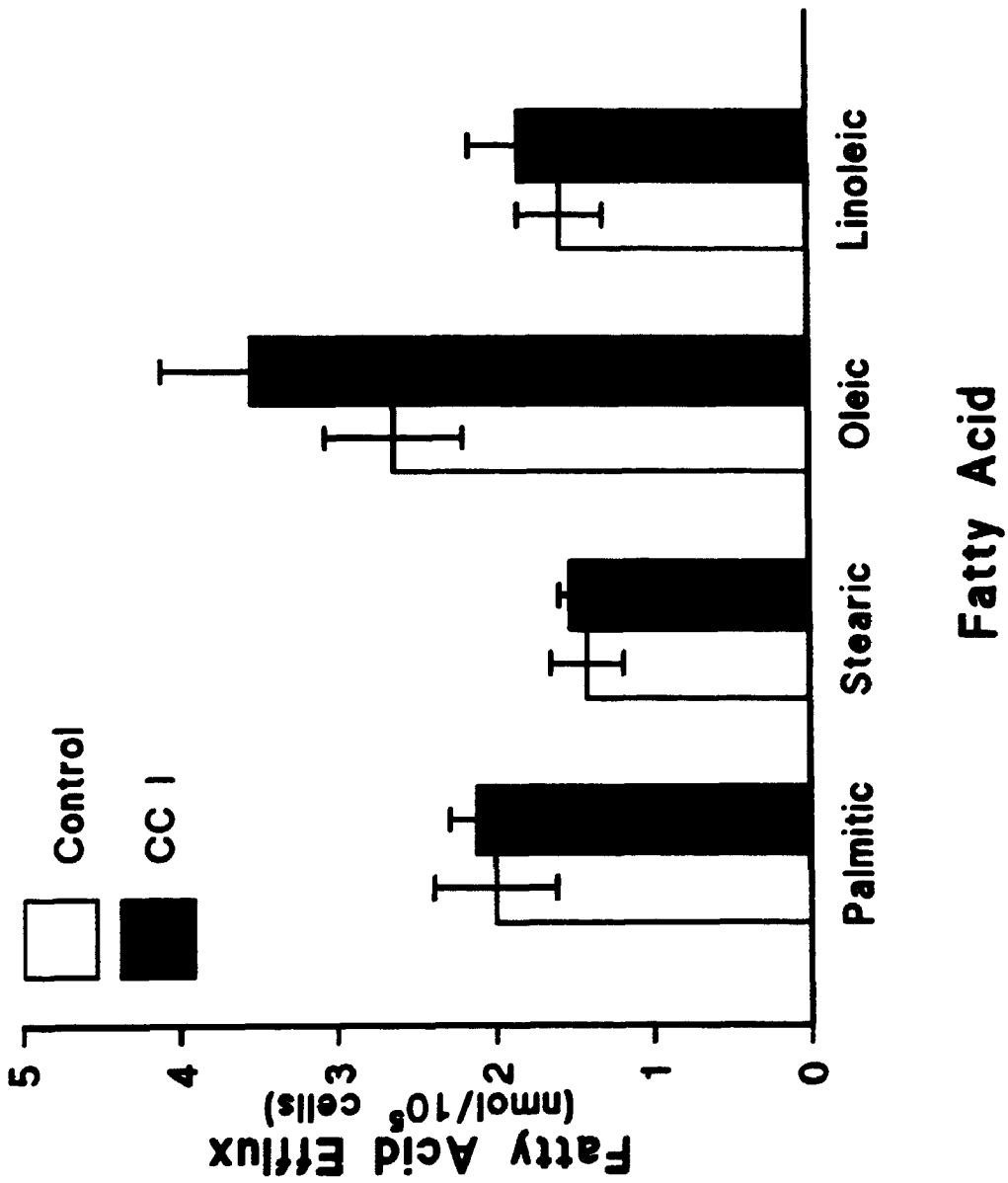
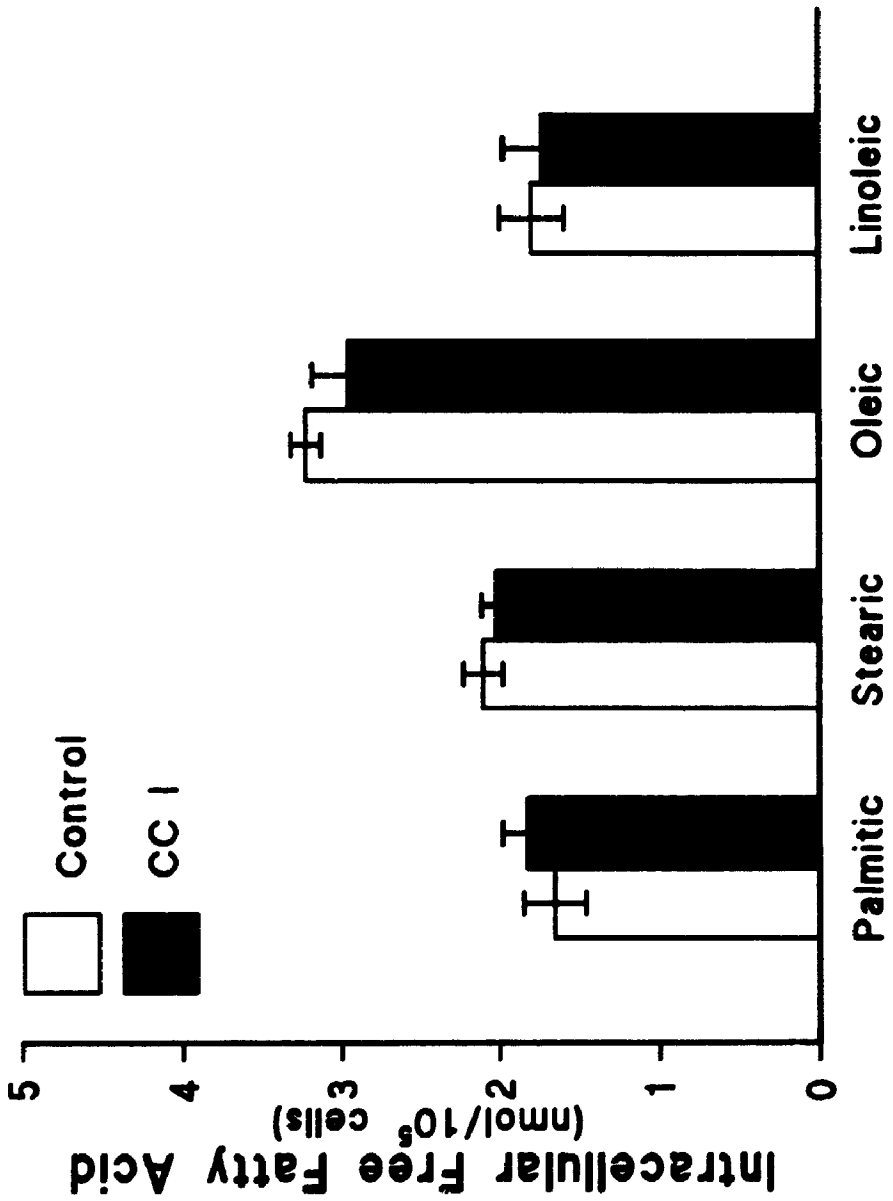

Control
CC I
Fatty Acid Efflux
(nmol/10^5 cells)
0
1
2
3
4
5
Palmitic
Stearic
Oleic
Linoleic
Fatty Acid

Figure 18. The failure of synthetic hypertrehalosemic hormone CC I to stimulate production of free fatty acids in urocytes and mycetocytes *in vitro*. Urates and mycetocytes (~120,000 cells/sample) in 1 ml of saline III were pipetted into 40 ml plastic centrifuge tubes and allowed to equilibrate for 30 min at 30°C in a shaker water bath. At the end of the equilibration period CC I (100 pmol/ml) was added to one-half the samples which were incubated at 30°C with gentle shaking for an additional 15 min. The reaction was stopped by pipettting the total incubation mixture into a 10 ml syringe with the needle (20 gauge) inserted through a rubber stopper supported in a 250 ml filter flask. The syringe contained a snug fitting disc of glass microfiber filter (GF/A) to retain the trophocytes. Mild vacuum was applied to the flask to draw the extracellular medium into the flask and away from the cells. The filter containing the cells was quickly withdrawn from the syringe with forceps and transferred to 1.5 ml of ice-cold methanol in a centrifuge tube and sealed with a teflon-lined screw cap. The sample was mixed thoroughly by vortexing and allowed to stand for 20 min at 4°C. The samples were then centrifuged at 450g x 20min to precipitate protein. The supernatant was removed and the fatty acids extracted and derivatized with TMTFTH for GC analysis as described in the materials and methods. Statistical analysis (ANOVA) of the data did not reveal any significant differences between the control and hormone treated samples, n = 5. Abbreviation used is, CC I, hypertrehalosemic hormone I.


Intracellular Free Fatty Acid
(nmol/10^6 cells)
0
1
2
3
4
5
Control
CC l
Palmitic
Stearic
Oleic
Linoleic
Fatty Acid

CC extract containing the natural hypertrehalosemic hormones has a dose dependent effect on the intracellular concentration of stearic, oleic and linoleic acids but no effect on palmitic acid (Fig. 19). The failure of palmitic acid to respond to the hormone is surprising since these measurements were derived from the same set of samples as those used for analysis of the remaining fatty acids. With the possible exception of stearic acid the major effect of the hormone appears to be realized with a low concentration of the active factor. Similar results were obtained with the synthetic hypertrehalosemic hormone CC II, with the exception that the increase in palmitic acid is comparable to that observed for the other fatty acids (Fig. 20). A maximal effect was obtained with 50 pmole of CC II per ml.

The action of the synthetic hypertrehalosemic hormone II is time dependent (Fig. 21). A maximal effect of the hormone on the intracellular free fatty acids appears to require approximately 30 min.

3.2.2 Hemolymph Free Fatty Acids

Very little is known about the nature, role, and concentration of free fatty acids in hemolymph. The most abundant lipid in hemolymph is diacylglycerol which is synthesized in the fat body and bound to the carrier protein, lipophorin, for transport in the hemolymph (Gilbert and Chino, 1974). Lipophorin carries esterified fatty acids

Figure 19. The concentration dependent stimulatory effect of crude natural hypertrehalosemic hormone (CCE) on the concentration of free fatty acids in dispersed trophocytes *in vitro*. Trophocytes (~60,000/sample) in 1ml of saline III were pipetted into 40 ml plastic centrifuge tubes and allowed to equilibrate for 30 min at 30°C in a shaker water bath. At the end of the equilibration period CCE (0.1-1.0 GPE/ml) was added as shown and the samples incubated at 30°C with gentle shaking for an additional 15 min. The reaction was stopped by pipetting the total incubation mixture into a 10 ml syringe to obtain the cells as described in the legend to Fig. 15. Analysis of the data using ANOVA reveals that the intracellular levels of stearic ($p < 0.01$, $n = 3$), oleic ($p < 0.05$, $n = 3$) and linoleic acid ($p < 0.05$, $n = 3$) are significantly increased by natural hypertrehalosemic hormone whereas palmitic acid was not. Abbreviation used is CCE, natural hypertrehalosemic hormone.

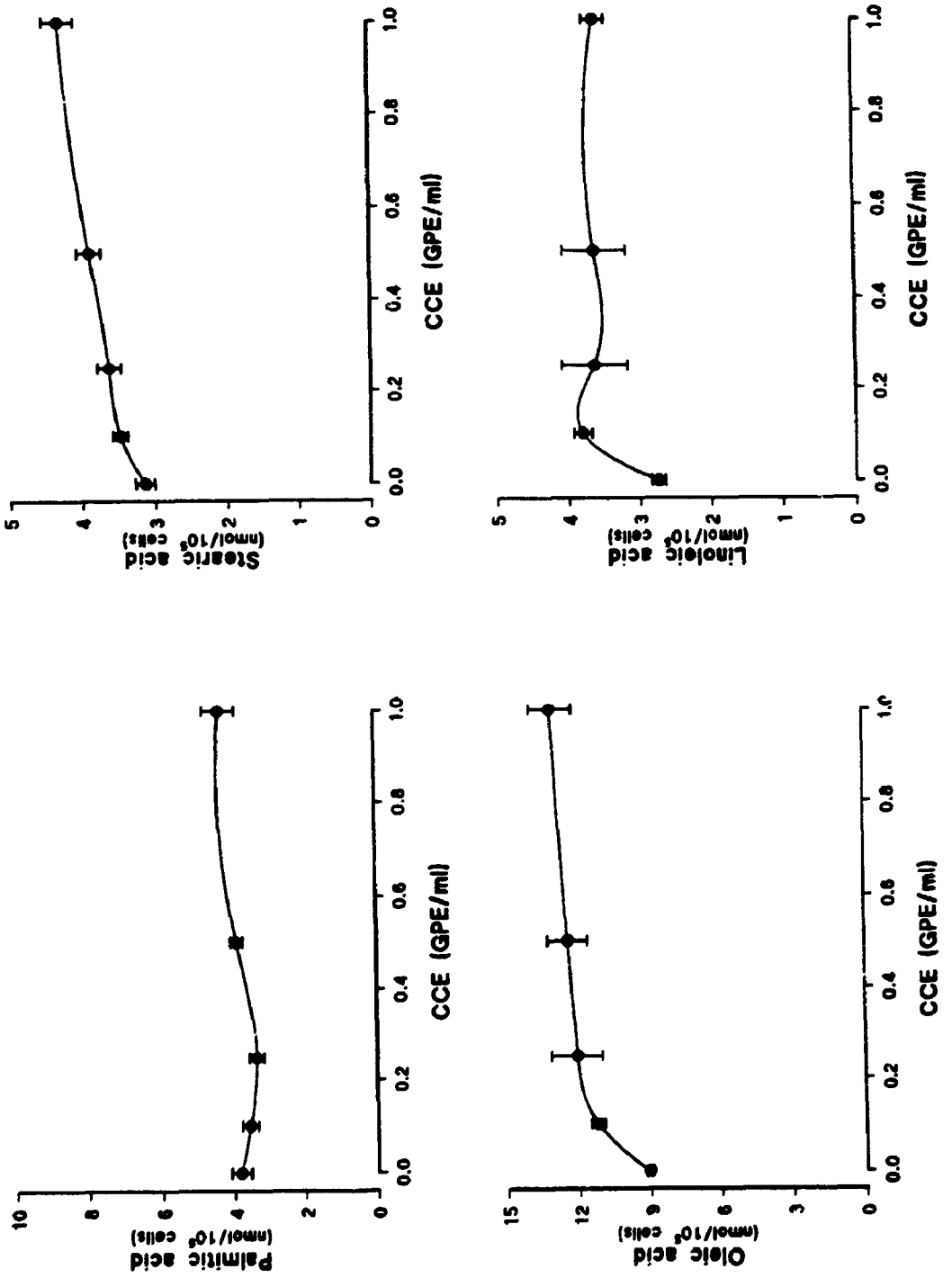
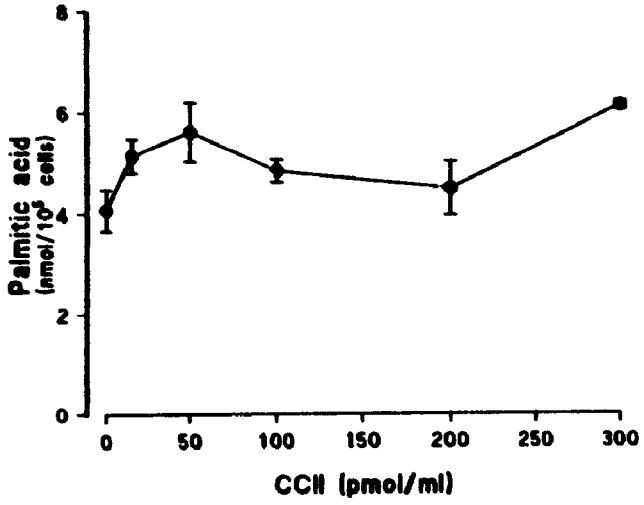
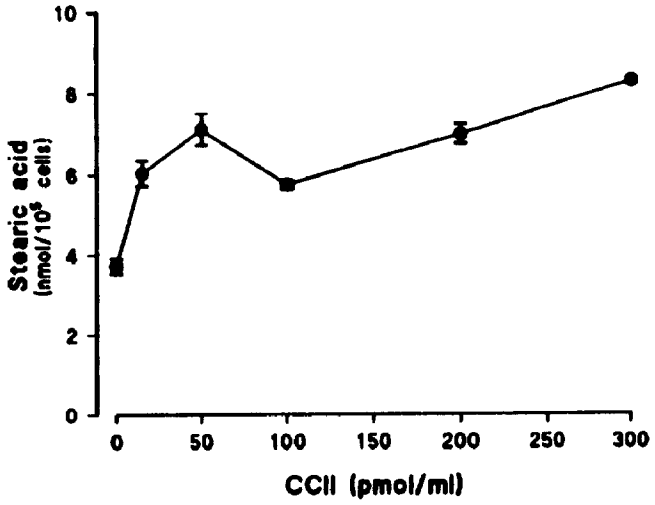
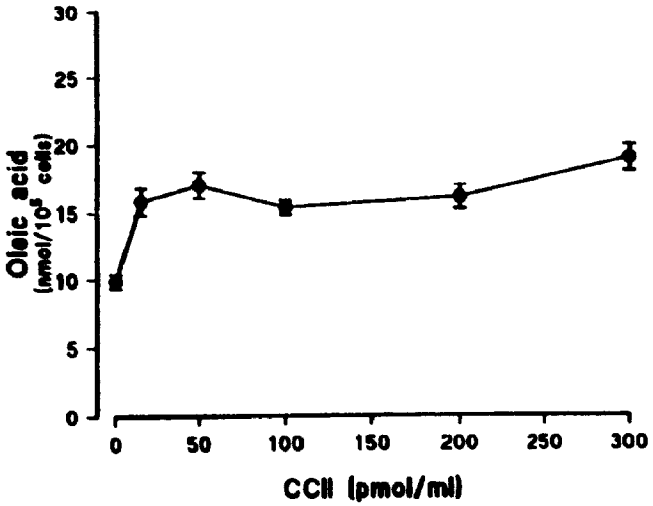

Stearic acid
(nmol/10^5 cells)
Linoleic acid
(nmol/10^5 cells)
Palmitic acid
(nmol/10^5 cells)
Oleic acid
(nmol/10^5 cells)
CCE (GPE/ml)
CCE (GPE/ml)
CCE (GPE/ml)
CCE (GPE/ml)

Figure 20. The stimulatory effect of the synthetic hypertrehalosemic hormone, CC II, on the concentration of free fatty acids in dispersed trophocytes *in vitro*. Trophocytes (~50,000/sample) in 1 ml of saline III were pipetted into 40 ml plastic centrifuge tubes and allowed to equilibrate for 30min at 30°C in a shaker water bath. At the end of the equilibration period CC II (15-300 pmol/ml) was added and incubation of the samples continued at 30°C with gentle shaking for an additional 15 min. The reaction was stopped and the samples processed as described in the legend to Fig. 15. Analysis of the data using ANOVA reveals that the intracellular levels of palmitic ($p < 0.01$, $n = 4$), stearic ($p < 0.001$, $n = 4$), oleic ($p < 0.0005$, $n = 4$) and linoleic acid ($p < 0.005$, $n = 4$) are significantly increased by CC II. Abbreviation used is CC II, synthetic hypertrehalosemic hormone I.


Palmitic acid
(nmol/10⁵ cells)
0
2
4
6
8
0
50
100
150
200
250
300
CCII (pmol/ml)


Stearic acid
(nmol/10⁵ cells)
0
2
4
6
8
10
0
50
100
150
200
250
300
CCII (pmol/ml)


Oleic acid
(nmol/10⁵ cells)
0
5
10
15
20
25
30
0
50
100
150
200
250
300
CCII (pmol/ml)

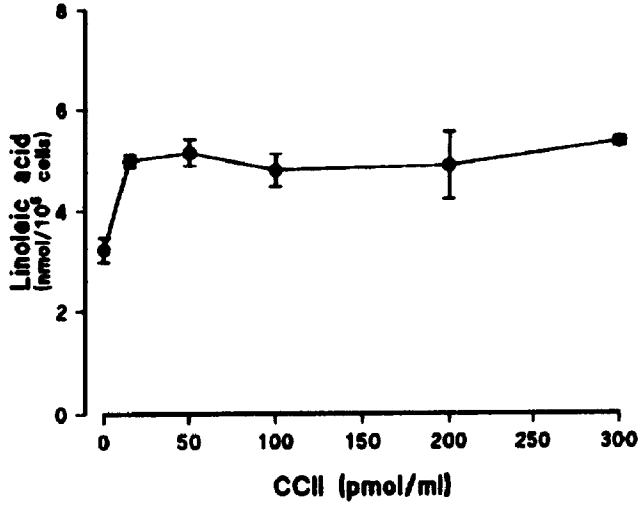
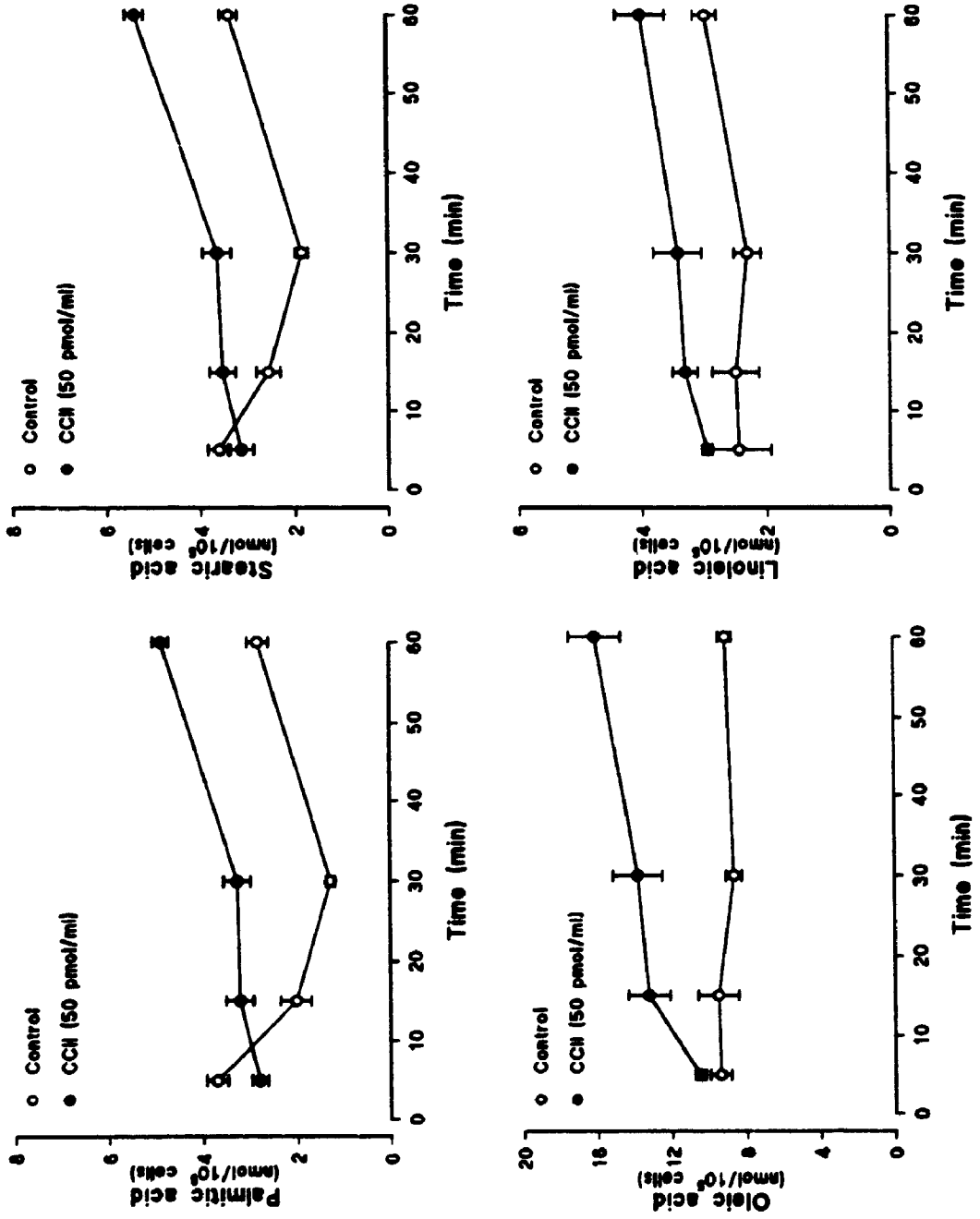

Linoleic acid
(nmol/10⁵ cells)
0
2
4
6
8
0
50
100
150
200
250
300
CCII (pmol/ml)

Figure 21. The time dependent effect of synthetic hypertrehalosemic hormone CC II on free fatty acid concentration in dispersed trophocytes *in vitro*. Trophocytes (~47,000/sample) in 1 ml of saline III were pipetted into 40 ml plastic centrifuge tubes and allowed to equilibrate for 30 min at 30°C in a shaker water bath. At the end of the equilibration period CC II (50 pmol/ml) was added and the samples incubated at 30°C with gentle shaking. AT 5, 15, 30 and 60 min intervals samples were taken by pipettting the total incubation mixture into a 10 ml syringe to obtain the cells which were processed as indicated in Fig. 15. Analysis of the data using ANOVA reveals that the intracellular levels of palmitic ($p < 0.005$, $n = 4$), stearic ($p < 0.005$, $n = 4$), oleic ($p < 0.05$, $n = 4$) and linoleic acid ($p < 0.05$, $n = 4$) are significantly increased by CC II. Abbreviation used is CC II, synthetic hypertrehalosemic hormone I.

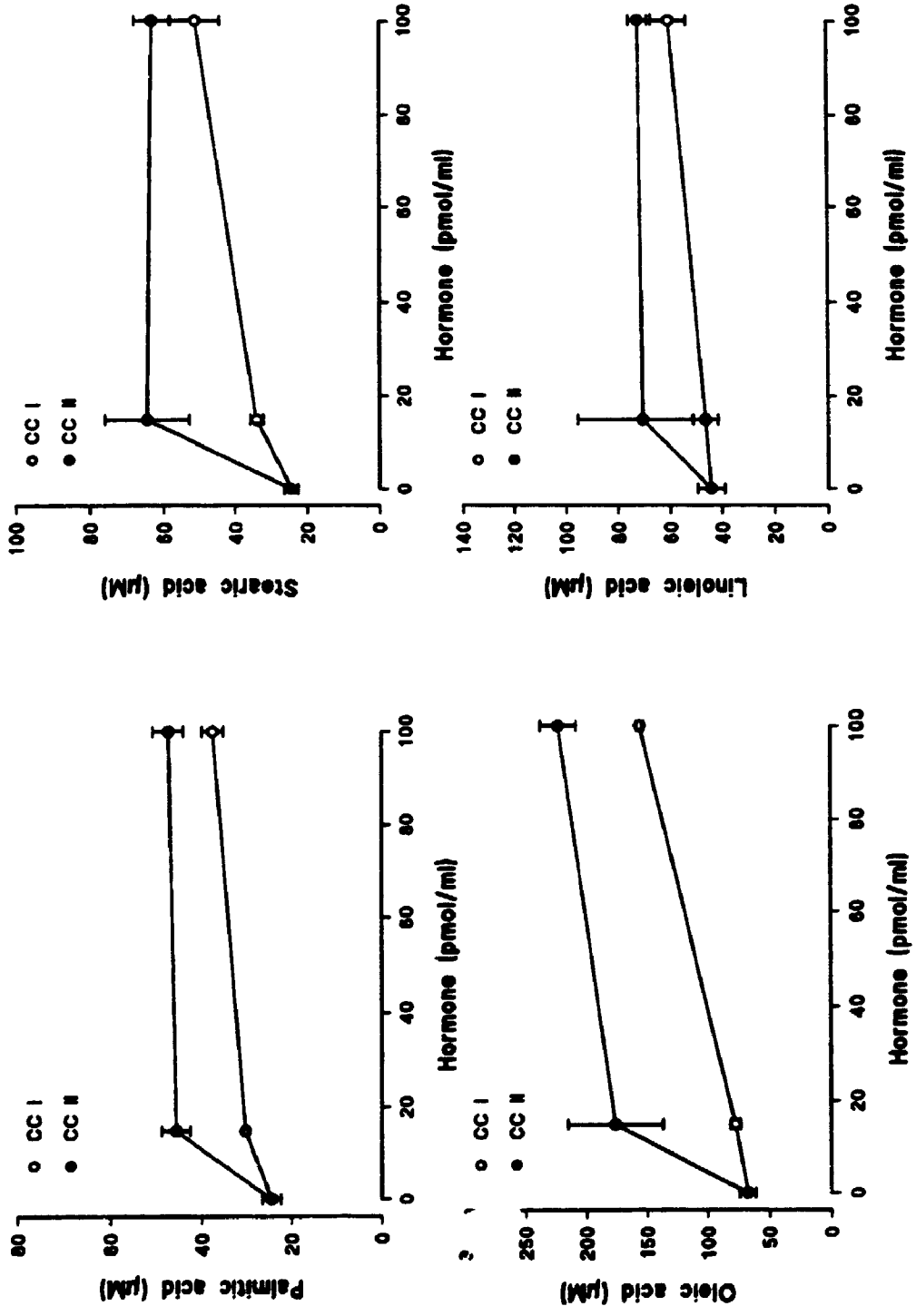

Palmitic acid
(nmol/10^6 cells)
Stearic acid
(nmol/10^6 cells)
Oleic acid
(nmol/10^6 cells)
Linoleic acid
(nmol/10^6 cells)
Time (min)
Control
CCH (50 pmol/ml)

to the site of utilization where they are released. It is possible that lipophorin may also bind free fatty acids for transport in the hemolymph. The fatty acids in the fat body which respond to the hormones are also present in the hemolymph and their concentration increases in response to the synthetic hypertrehalosemic hormones CC I and CC II (Fig. 22). The most notable feature of these results is that CC II appears to have a more potent effect on hemolymph free fatty acid concentration than does CC I.

3.2.3 Control of Free Fatty Acid Concentration in trophocytes

Because crude natural hormone as well as the synthetic hormone, CC II, increased intracellular levels of four fatty acids it was of interest to know the origin of these fatty acids. The data showing that the phospholipase A_2 inhibitors mepacrine and ρ-bromophenacyl bromide are potent inhibitors of trehalose efflux from trophocytes suggested that phospholipase A_2 might regulate free fatty acid levels in the cells. A series of experiments were therefore carried out using inhibitors and activators of phospholipase A_2 to determine whether a change in the activity of the enzyme would be reflected in the intracellular concentration of free fatty acids.

Figure 22. The stimulatory effect of the synthetic hypertrehalosemic hormones, CC I and CC II, on the appearance of free fatty acids in the hemolymph *in vivo*. Cockroaches were injected with the indicated amount of CC I and CC II in a final volume of 10 μl (ethanol 95%:saline I, 50:50: V/V). The controls were injected with 10 μl of the same solution without hormone. Following injection the insects were placed in glass jars for 1 h in the insectary. The cockroaches were anaesthetized by placing them in the freezer (-20^0C) for 10 minutes and the hemolymph then removed by centrifugation at 27g x 10 min. One hundred μl of hemolymph was pipetted directly into centrifuge tubes (fitted with teflon-lined screw caps) containing 1.5 ml ice-cold methanol. The tubes were flushed with N_2 and mixed thoroughly by vortexing and allowed to stand for 20 minutes at 4^cC after which they were centrifuged for 450g x 20min. The supernatant was transferred to a tube containing 5 ml hexane. The samples were then extracted and derivatized as described in the materials and methods. Analysis of the data using ANOVA reveals that levels of palmitic ($p < 0.005$, $n = 5$), stearic ($p < 0.005$, $n = 5$), oleic ($p < 0.001$, $n = 5$) and linoleic ($p < 0.025$, $n = 5$) acids in the hemolymph are significantly increased by CC I. The hemolymph levels of palmitic ($p < 0.001$, $n = 5$), stearic ($p < 0.005$, $n = 5$) and oleic acid ($p < 0.0025$, $n = 5$) are significantly increased by CC II whereas linoleic acid levels do not change. Abbreviations used are CC I, synthetic hypertrehalosemic hormone I; CC II, synthetic hypertrehalosemic hormone II.


Stearic acid (μM)
Linoleic acid (μM)
Palmitic acid (μM)
Oleic acid (μM)
Hormone (pmol/ml)
CC I
CC II

3.2.3.1 Role of Phospholipase A_2

Melittin, an activator of phospholipase A_2, increases intracellular free fatty acids in trophocytes (Fig. 23). With the exception of stearic acid, palmitic, oleic and linoleic acids show a strong concentration dependent effect of melittin. The increase in intracellular fatty acids due to melittin is significantly reduced by indomethacin (Fig. 24).

The stimulatory effect of melittin on intracellular free fatty acids does not appear to be similar to the action of the hypertrehalosemic hormone. The hypertrehalosemic hormone significantly increases the levels of all four fatty acids, whereas, melittin does not appear to increase stearic acid to the same degree as the other three fatty acids. The difference in the mode of action is also indicated by Fig. 25. The results show that CC I does not stimulate release of fatty acids from the membranes whereas melittin was highly effective in causing the release of palmitic, oleic and linoleic acids but not stearic acid. The data, therefore, support the view that melittin and the hormone do not have a common mode of action.

Using the same logic as that used for the previous experiments it would be expected that treatment of the trophocytes with BPB would decrease the concentration of intracellular free fatty acids. Figure 26 shows that both oleic and linoleic acids are decreased by treatment with BPB

Figure 23. The stimulatory effect of melittin on free fatty acid production in dispersed trophocytes *in vitro*. Trophocytes (~64,000/sample) in 1 ml of saline III were pipetted into 40 ml plastic centrifuge tubes and allowed to equilibrate for 30 min at 30°C in a shaker water bath. At the end of the equilibration period melittin (1-20 μg/ml) was added and the samples incubated at 30°C with gentle shaking for an additional 15 min. The reaction was stopped and the sample processed as indicated in the legend to Fig. 15. Analysis of the data using ANOVA shows that the intracellular levels of palmitic ($p < 0.0001$, $n = 4$), stearic ($p < 0.025$, $n = 4$), oleic ($p < 0.0001$, $n = 4$) and linoleic ($p < 0.0001$, $n = 4$) acids are increased by melittin. Abbreviation used is, CC I, synthetic hypertrehalosemic hormone I.

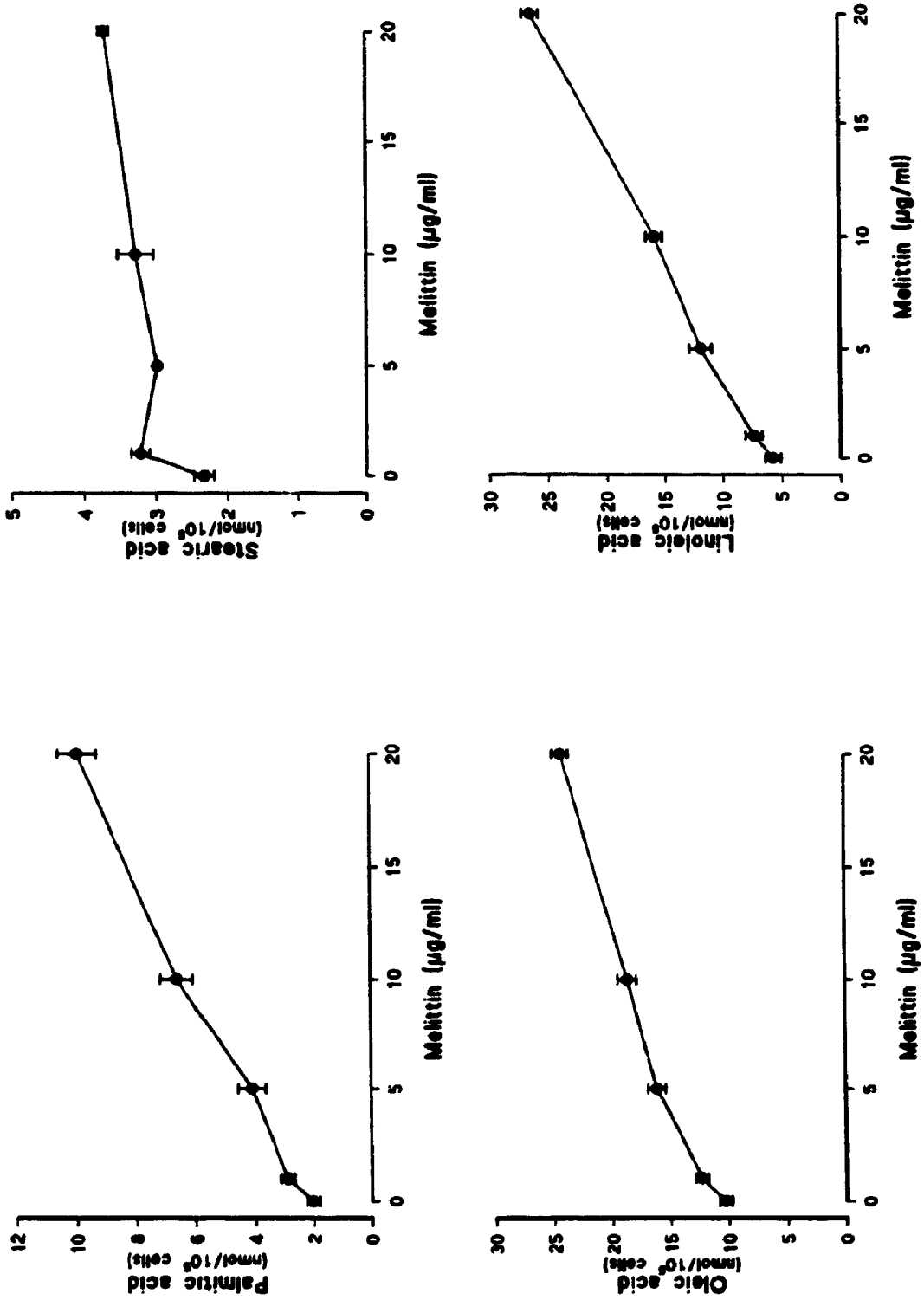

Stearic acid
(nmol/10^5 cells)
Melittin (μg/ml)
Linoleic acid
(nmol/10^5 cells)
Melittin (μg/ml)
Palmitic acid
(nmol/10^5 cells)
Melittin (μg/ml)
Oleic acid
(nmol/10^5 cells)
Melittin (μg/ml)

Figure 24. The inhibitory effect of indomethacin on melittin activated free fatty acid production in trophocytes *in vitro*. Trophocytes (~55,000/sample) in 1 ml of saline III were pipetted into 40 ml plastic centrifuge tubes and allowed to equilibrate for 30 min at 30°C in a shaker water bath. At the end of the equilibration period indomethacin (1 mM final concentration) was added to half of the samples and all were held at 30°C with gentle shaking for 15 min. At the end of this period melittin (10 μg/ml) was added to all the samples and the incubation continued for an additional 15 min. The reaction was stopped and the sample processed as indicated in the legend to Fig. 15. Analysis of the data using ANOVA reveals that the increase in intracellular fatty acids due to melittin was significantly inhibited by indomethacin as follows: palmitic acid ($p < 0.01$, $n = 5$), oleic acid ($p < 0.005$, $n = 5$), and stearic acid (N.S.). Abbreviations used are, Mel, melittin; IND, indomethacin.

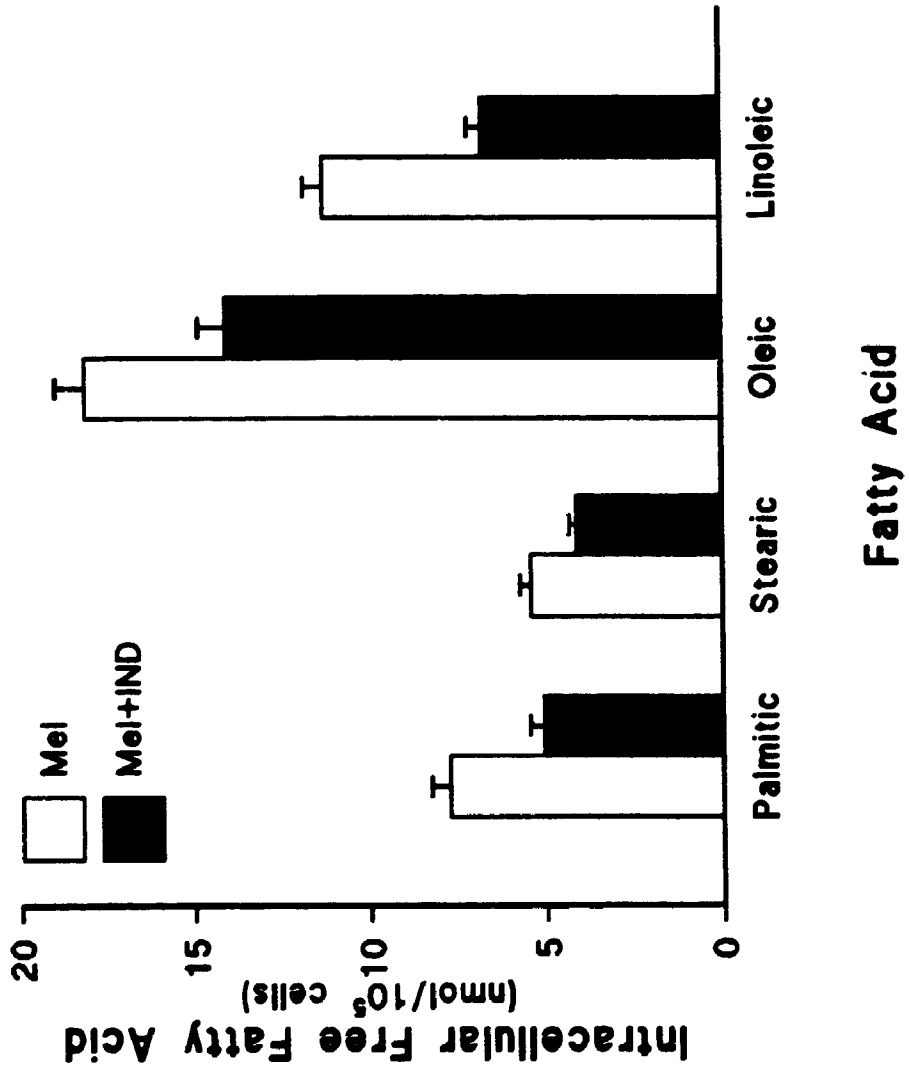

Intracellular Free Fatty Acid
(nmol/10^5 cells)
20
15
10
5
0
Mel
Mel+IND
Palmitic
Stearic
Oleic
Linoleic
Fatty Acid

Figure 25. A comparison of the effect of melittin and CC I on the release of free fatty acids from trophocyte membranes. Membranes were prepared according to the method of (Giudicelli, 1982). Trophocytes were homogenized with a hand-held homogenizer (OMNI 2000 fitted with a 5 mm generator) for 10s in a glycine-NaOH buffer (pH 9) immediately following dispersion of the cells. The homogenate was centrifuged at 20,000g x 15min at 4°C, the floating fat cake and infranatant discarded, and the pellet resuspended in 2.0 ml glycine-NaOH buffer followed by centrifugation at 20,000g x 20min. This was followed by an additional washing and centrifugation at the same speed for 10 min. The final pellet was resuspended in the glycine-NaOH buffer. The crude membrane preparation in 1.0 ml glycine-NaOH buffer was allowed to equilibrate at 30°C in a shaker water bath for 15 minutes after which melittin (10 µg/ml) and CC I (100 pmol/ml) were added. After 15 min the incubation was terminated by the addition of 1.5 ml ice-cold methanol. The mixture was transferred to a centrifuge tube which was then flushed with N_2 and capped with a teflon-lined screw cap. The sample was mixed thoroughly by vortexing and allowed to stand for 20 minutes at 4°C after which they were centrifuged at 450g x 20 min. The fatty acids were extracted and derivatized as described in the materials and methods. Protein was determined with the Folin phenol reagent (Lowry *et al.*, 1951). ANOVA performed on the data reveals that melittin stimulated release of palmitic ($p < 0.001$, $n = 4$), stearic ($p < 0.025$, $n = 4$), oleic ($p < 0.001$, $n = 4$) and linoleic ($p < 0.001$, $n = 4$) acids from membranes whereas that by CC I did not. Abbreviation used is, CC I, synthetic hypertrehalosemic hormone I.

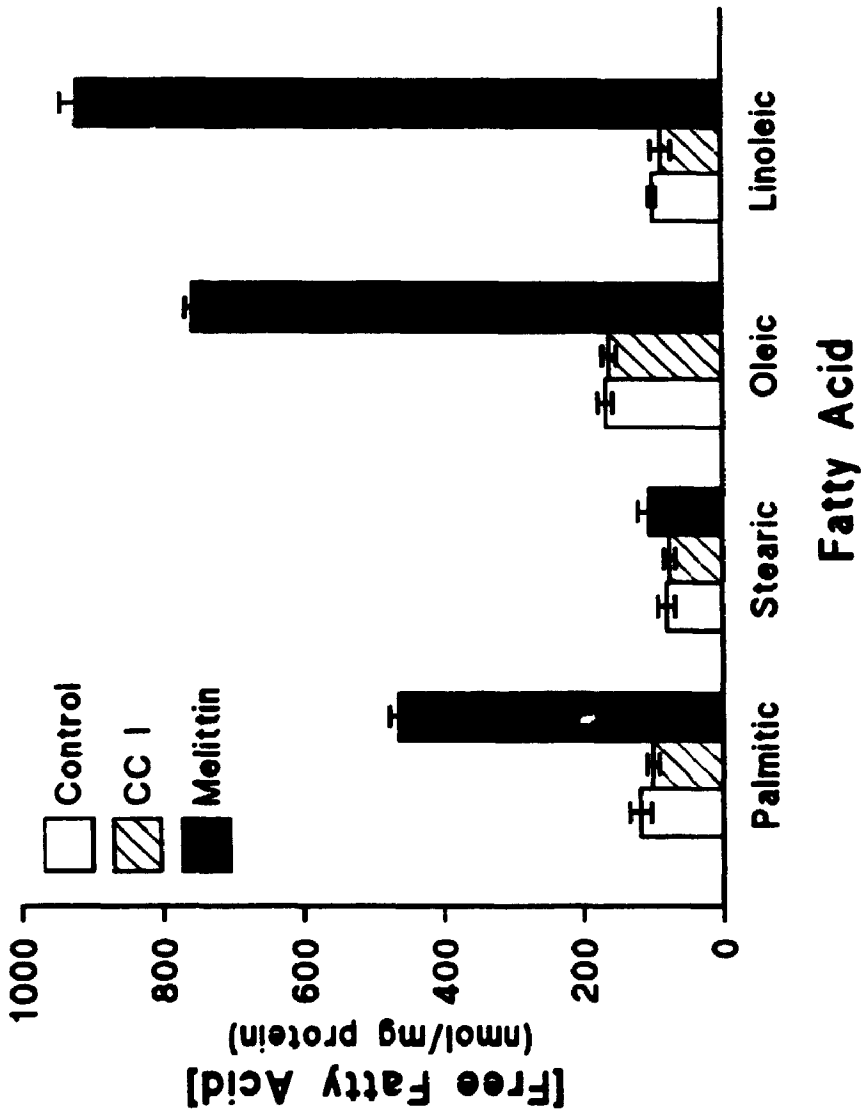
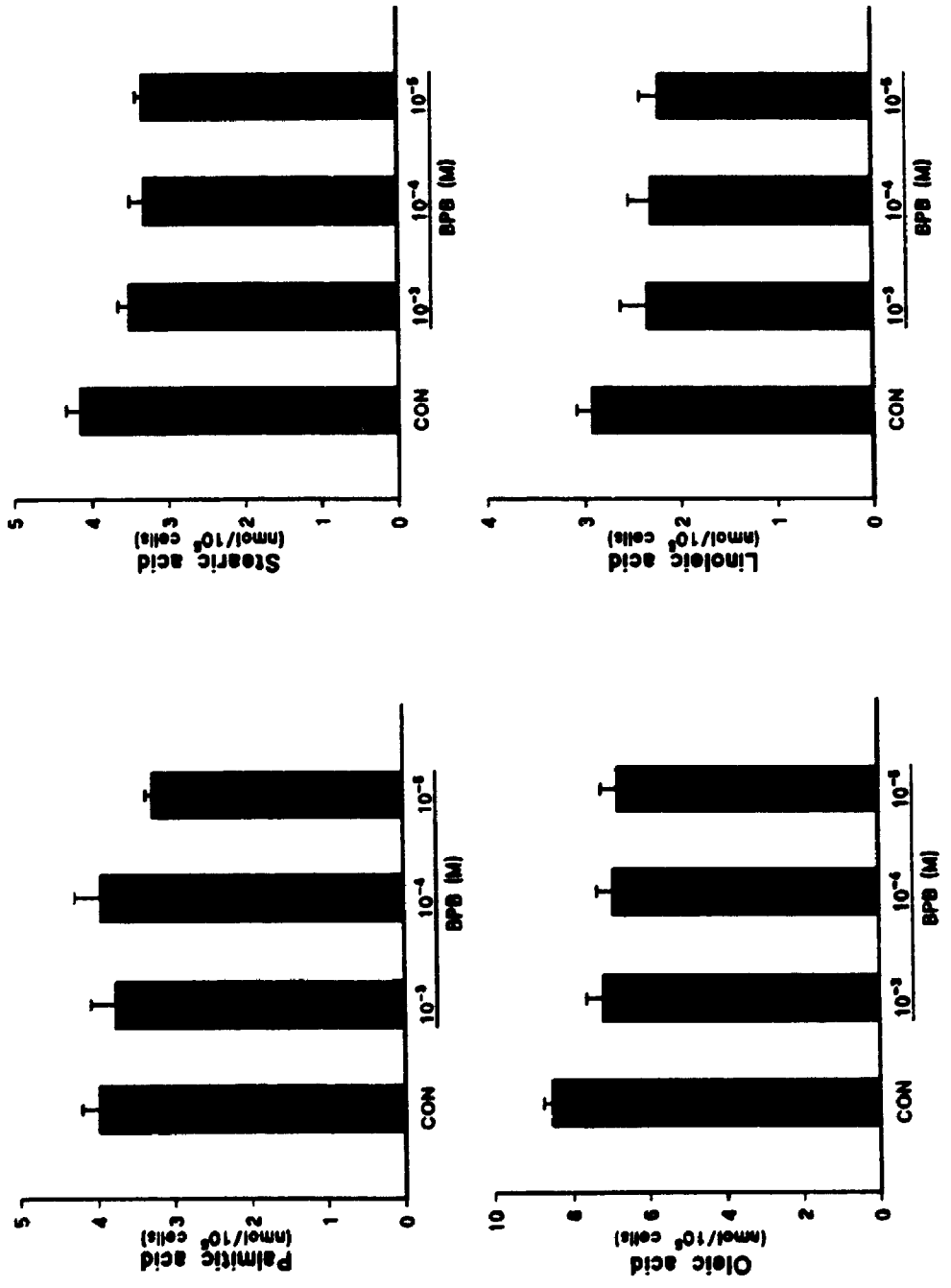

[Free Fatty Acid]
(nmol/mg protein)
1000
800
600
400
200
0
Control
CC 1
Melittin
Palmitic
Stearic
Oleic
Linoleic
Fatty Acid

Figure 26. The inhibitory effect of the phospholipase A_2 inhibitor ρ-bromophenacyl bromide (BPB) on free fatty acid concentration in dispersed trophocytes. Trophocytes (~50,000) in 1 ml of saline III were pipetted into 40 ml plastic centrifuge tubes and allowed to equilibrate for 30 min at 30°C in a shaker water bath. At the end of the equilibration period BPB was added to yield the concentration shown. The samples were allowed to incubate at 30°C with gentle shaking for an additional 30 min. The reaction was then stopped and the samples processed as indicated in the legend to Fig. 15. Duncan's test reveals that oleic acid levels in BPB treated samples are lower than control levels at all three concentrations tested ($p < 0.01$, $n = 4$). Linoleic acid in BPB (1 mM) treated cells is decreased compared to control ($p < 0.01$, $n = 4$). The other fatty acids were not affected by BPB. Abbreviations used are CON, control; BPB, ρ-bromophenacyl bromide.


Palmitic acid
(nmol/10^6 cells)
Stearic acid
(nmol/10^6 cells)
Oleic acid
(nmol/10^6 cells)
Linoleic acid
(nmol/10^6 cells)
CON
10^-3
10^-4
10^-5
BPB (M)

in unchallenged cells. More significantly, the increase in concentration of the four fatty acids due to CC I is significantly reduced by BPB (Fig. 27). These results are evidence that phospholipase A_2 may be an important site of regulation of intracellular fatty acid concentration.

3.2.3.2 Role of Cyclooxygenase

Numerous studies suggest that metabolites of fatty acids are potent mediators of cellular activity (Chang *et al.*, 1987; Needleman *et al.*, 1986; Irvine, 1982). Thus the rate of appearance of a fatty acid may be a rate-limiting step in the formation of active metabolites (Chang *et al.*, 1987). To determine whether inhibition of the cyclooxygenase pathway might cause an accumulation of fatty acids the trophocytes were incubated with indomethacin. As illustrated in Fig. 28, indomethacin increases the concentration of palmitic, stearic, oleic and linoleic acids in the trophocytes. The effect of indomethacin in the presence of CC I appears stimulatory but the effect is more pronounced at low concentrations of the inhibitor (Fig. 29). Similar results were obtained when CC II was used to stimulate fatty acid production but in these experiments the highest concentration of indomethacin used was inhibitory to the release of the fatty acids (Fig. 30).

Figure 27. Inhibition by ρ-bromophenacyl bromide of the stimulatory effect of the synthetic hypertrehalosemic hormone CC I on trophocytes. Trophocytes (~50,000) in 1 ml of physiological saline III were pipetted into 40 ml plastic centrifuge tubes and allowed to equilibrate for 30 min at 30°C with shaking. At the end of the equilibration period BPB was added to three of the five samples to give the concentration shown in the figure. Incubation of the samples was continued for an additional 15 min. CC I was then added to each of the BPB treated samples as well as one of the untreated samples to yield a final concentration of 100 pmol/ml. The remaining sample which did not receive BPB or CC I was used as the control. After incubation for a further 15 min the reaction was stopped and the free fatty acids determined as described in the legend to Figure 15. Duncan's test reveals that BPB, at all concentrations, inhibits the increase in intracellular palmitic ($p < 0.05$, $n = 4$), stearic ($p < 0.05$, $n = 4$), oleic ($p < 0.01$, $n = 4$) and linoleic ($p < 0.05$, $n = 4$) acids due to CC I. Abbreviations used are CON, control; CCI, synthetic hypertrehalosemic hormone I; BPB, ρ-bromophenacyl bromide.

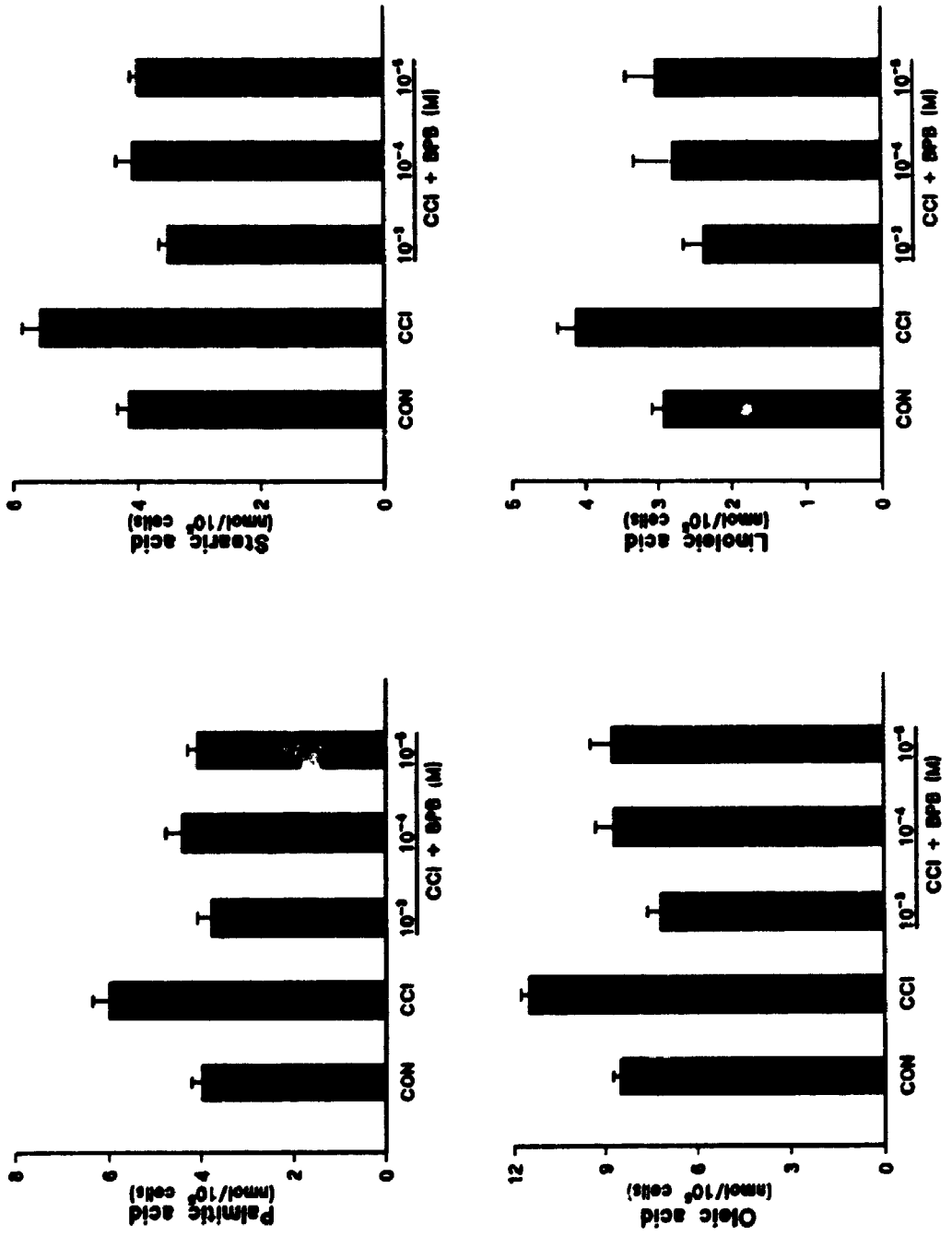

Palmitic acid
(nmol/10^6 cells)
Stearic acid
(nmol/10^6 cells)
Oleic acid
(nmol/10^6 cells)
Linoleic acid
(nmol/10^6 cells)
CON
CCl
CCl + BPB (M)

Figure 28. The stimulatory effect of indomethacin on the intracellular concentration of free fatty acids in trophocytes. Trophocytes (~32,000/sample) in 1 ml of saline III were pipetted into 40 ml plastic centrifuge tubes and allowed to equilibrate for 30 min at 30°C in a shaker water bath. At the end of the equilibration period indomethacin was added to yield the concentration shown. The samples were allowed to incubate at 30°C with gentle shaking for an additional 15 min. The reaction was stopped and the free fatty acids determined as described in the legend to Figure 15. Duncan's test reveals that indomethacin, at all concentrations, increased intracellular palmitic ($p < 0.01$, $n = 4$), stearic ($p < 0.05$, $n = 4$), oleic ($p < 0.01$, $n = 4$) and linoleic ($p < 0.05$, $n = 4$) acids with the exception of linoleic acid with 0.01 mM indomethacin. Abbreviations are CON, control; IND, indomethacin.

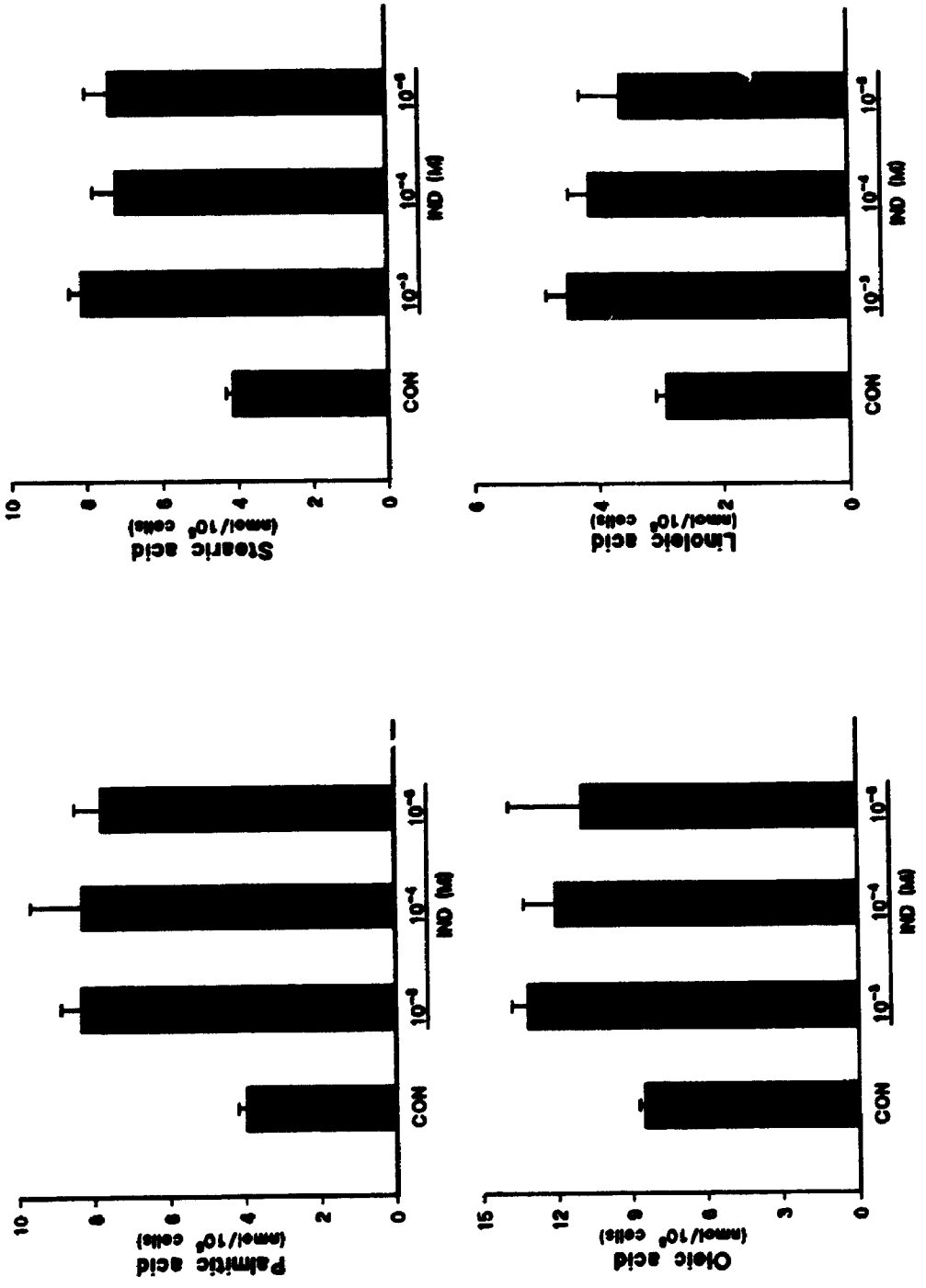
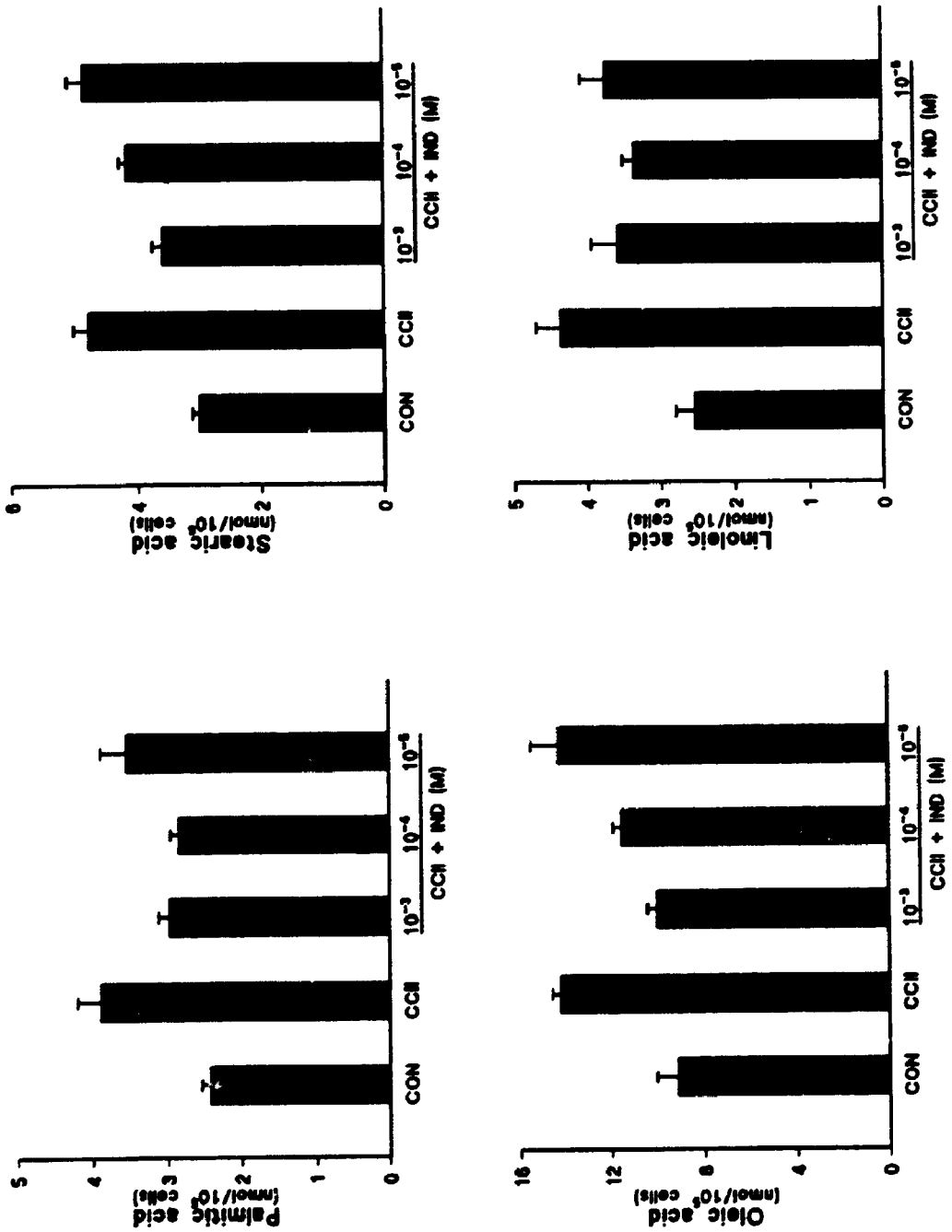

Stearic acid
(nmol/10⁶ cells)
Linoleic acid
(nmol/10⁶ cells)
Palmitic acid
(nmol/10⁶ cells)
Oleic acid
(nmol/10⁶ cells)
CON
IND (M)

Figure 29. Enhancement of the stimulatory effect of the synthetic hypertrehalosemic hormone CC I on intracellular free fatty acids in trophocytes by indomethacin (IND). Trophocytes (~32,000/sample) in 1 ml of saline III were pipetted into 40 ml plastic centrifuge tubes and allowed to equilibrate for 30 min at 30°C with shaking. At the end of the equilibration period indomethacin was added to three of the five samples to give the concentration shown in the figure. Incubation of the samples was continued for an additional 15 min. CC I was then added to each of the indomethacin treated samples as well as one of the untreated samples to yield a final concentration of 100 pmol/ml. The remaining sample which did not receive indomethacin or CC I was used as the control. After incubation for a further 15 min the reaction was stopped and the free fatty acids determined as described in the legend to Figure 15. Data analysis reveals that the stimulatory effect of CC I on fatty acid production is not inhibited by indomethacin (n = 4). Abbreviations used are CON, control; CCI, synthetic hypertrehalosemic hormone I; IND, indomethacin.

Stearic acid
(nmol/10⁶ cells)
10
8
6
4
2
0
CON
CCl
CCl + IND (M)
10⁻³
10⁻⁴
10⁻⁵
Linoleic acid
(nmol/10⁶ cells)
6
5
4
3
2
1
0
Palmitic acid
(nmol/10⁶ cells)
Oleic acid
(nmol/10⁶ cells)
15
12
9
6
3

Figure 30. The inhibitory effect of indomethacin (IND) on the release of intracellular fatty acids in CC II challenged trophocytes. Trophocytes (~60,000/sample) in 1 ml of physiological saline III were pipetted into 40 ml plastic centrifuge tubes and allowed to equilibrate for 30 min at 30°C with shaking. At the end of the equilibration period indomethacin was added to three of the five samples to give the concentration shown in the figure. Incubation of the samples was continued for an additional 15 min. CC II was then added to each of the indomethacin treated samples as well as one of the untreated samples to yield a final concentration of 100 pmol/ml. The remaining sample which did not receive indomethacin or CC II was used as the control. After incubation for a further 15 min the reaction was stopped and the free fatty acids determined as described in the legend to Figure 15. Duncan's test reveals that indomethacin blocks the increase in free fatty acids due to the action of CC II. Indomethacin (0.1 mM) inhibited the appearance of palmitic ($p < 0.05$, $n = 5$), oleic ($p < 0.05$, $n = 5$) and linoleic acid ($p < 0.05$, $n = 5$) due to the action of CC II. With 1 mM indomethacin the release of stearic and oleic acid due to CC II was also inhibited ($p < 0.01$, $n = 5$).


Stearic acid (nmol/10^6 cells)
Linoleic acid (nmol/10^6 cells)
Palmitic acid (nmol/10^6 cells)
Oleic acid (nmol/10^6 cells)
CON
CCl
CCl + IND (M)
10^-3
10^-4
10^-5

3.2.3.3 Role of Lipoxygenase

Of the four fatty acids examined the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) significantly increased the concentration only of oleic and linoleic acids in the trophocytes (Fig. 31). Interestingly, this effect was obtained with 0.1 mM NDGA: both 1 mM and 0.01 mM were ineffective in this regard. When trophocytes were challenged with CC I the concentration of each of palmitic, stearic, oleic and linoleic acids was significantly decreased by 1 mM NDGA (Fig. 32). This effect of the inhibitor was also apparent with lower concentration of the inhibitor.

3.3 Mechanism of Fatty Acid Action

3.3.1 Stimulatory effect of fatty acids on trehalose efflux

An important finding in this study has been the demonstration that the hypertrehalosemic hormones increase the concentration of intracellular fatty acids. Of equal interest is the finding that inhibitors of the cyclooxygenase and lipoxygenase pathways oppose this tendency of the hormones to increase intracellular free fatty acids. Because the action of these agents can be correlated with their effects on trehalose efflux it was of interest to determine whether the fatty acids alone could influence trehalose efflux from the tissue. Figure 33 shows that palmitic acid has no effect on trehalose efflux from

Figure 31. The stimulatory effect of nordihydroguaiaretic acid (NDGA) on the intracellular concentration of free fatty acids in trophocytes. Trophocytes (~60,000/sample) in 1 ml of physiological saline III were pipetted into 40 ml plastic centrifuge tubes and allowed to equilibrate for 30 min at 30°C in a shaker water bath. At the end of the equilibration period NDGA was added to yield the concentrations shown. The samples were incubated at 30°C with gentle shaking for an additional 15 min after which the reaction was stopped and the free fatty acids determined as described in the legend to Figure 15. Duncan's test reveals that the concentrations of oleic and linoleic acids were significantly increased in cells treated with 0.1 mM NDGA ($p < 0.01$, $n = 4$). Abbreviations used are CON, control; NDGA, nordihydroguaiaretic acid.

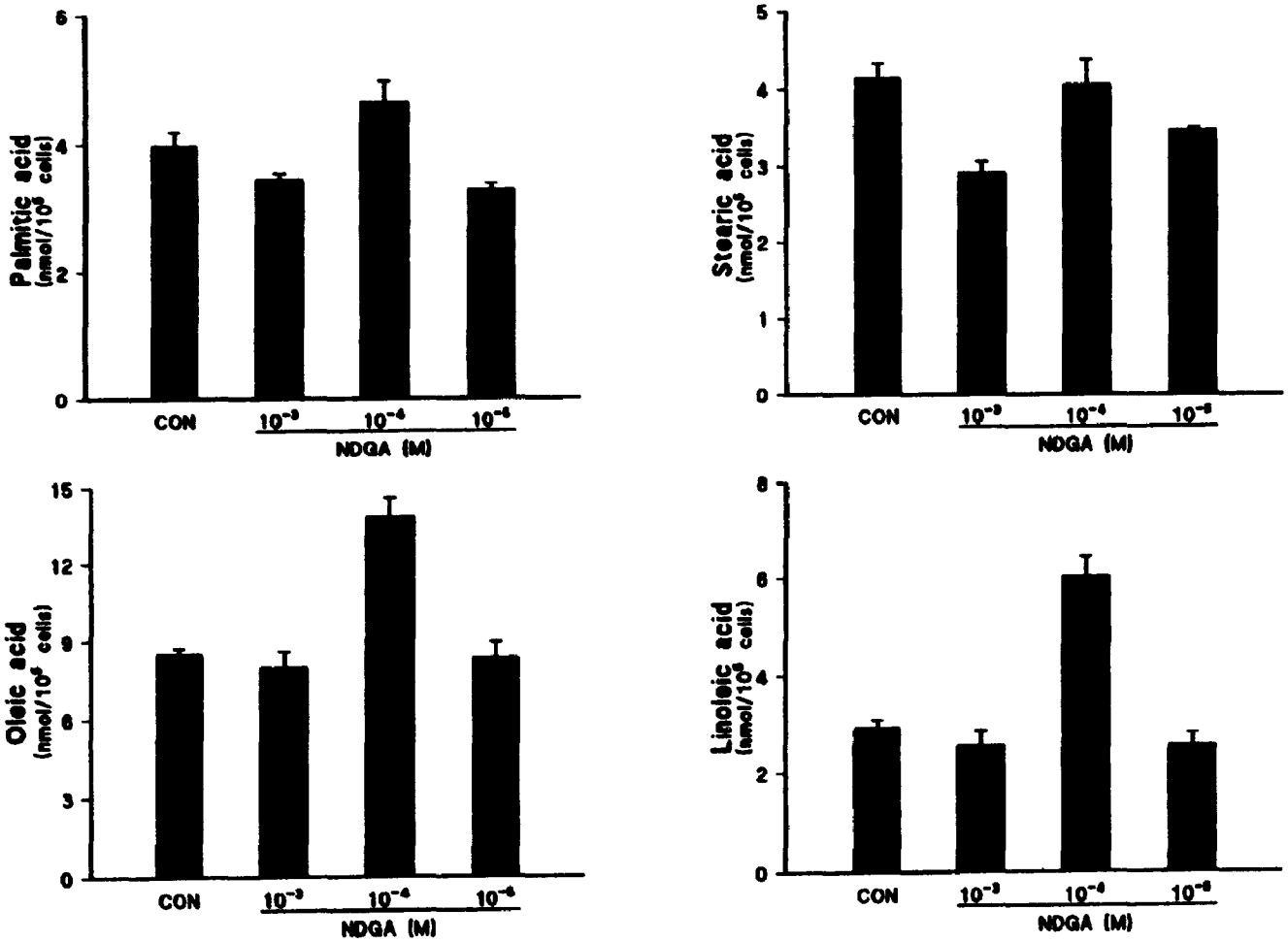
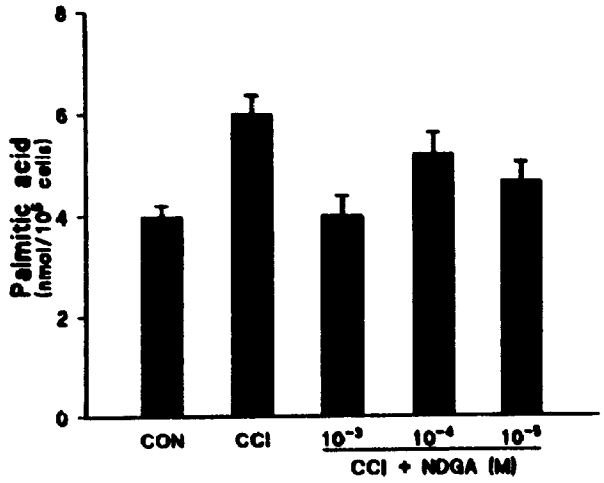
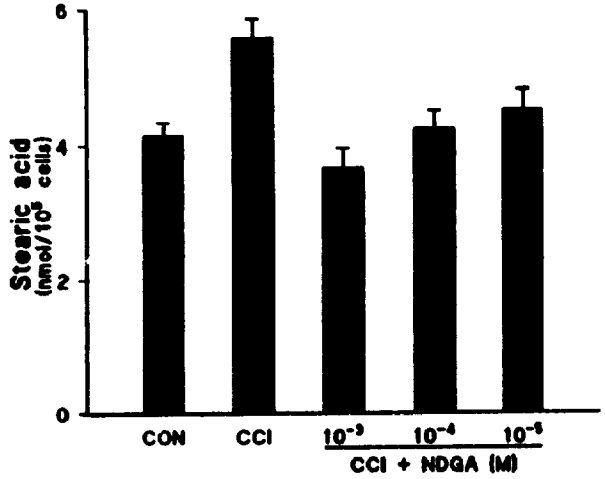
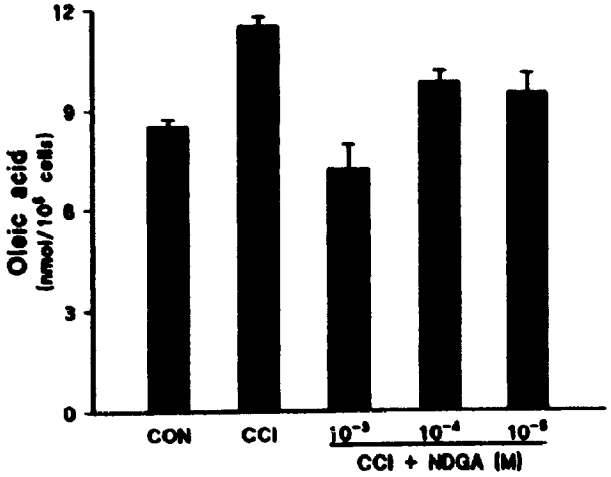

Palmitic acid (nmol/10^5 cells)
Stearic acid (nmol/10^5 cells)
Oleic acid (nmol/10^5 cells)
Linoleic acid (nmol/10^5 cells)
CON
10^-3
10^-4
10^-5
NDGA (M)

Figure 32. Inhibition by nordihydroguaiaretic acid (NDGA) of intracellular free fatty acid accumulation in trophocytes challenged with the synthetic hypertrehalosmic hormone CC I. Trophocytes (~37,000/sample) in 1 ml of saline III were pipetted into 40 ml plastic centrifuge tubes and allowed to equilibrate for 30 min at 30°C with shaking. At the end of the equilibration period NDGA was added to three of the five samples to yield the concentrations shown. Incubation of the samples was continued for an additional 15 min. CC I was then added to each of the NDGA treated samples as well as one of the untreated samples to yield a final concentration of 100 pmol/ml. The remaining sample which did not receive indomethacin or CC I was used as the control. After incubation for a further 15 min the reaction was stopped and the free fatty acids determined as described in the legend to Figure 15. Duncan's test reveals that the appearance of each of the free fatty acids due to the action of CC I is blocked by the highest concentration of NDGA tested ($p < 0.01$, $n = 4$). Abbreviations used are CON, control; CCI, synthetic hypertrehalosemic hormone I; NDGA, nordihydroguaiaretic acid.


Palmitic acid
(nmol/10⁶ cells)
8
6
4
2
0
CON
CCl
10⁻³
10⁻⁴
10⁻⁵
CCl + NDGA (M)


Stearic acid
(nmol/10⁶ cells)
6
4
2
0
CON
CCl
10⁻³
10⁻⁴
10⁻⁵
CCl + NDGA (M)


Oleic acid
(nmol/10⁶ cells)
12
9
6
3
0
CON
CCl
10⁻³
10⁻⁴
10⁻⁵
CCl + NDGA (M)

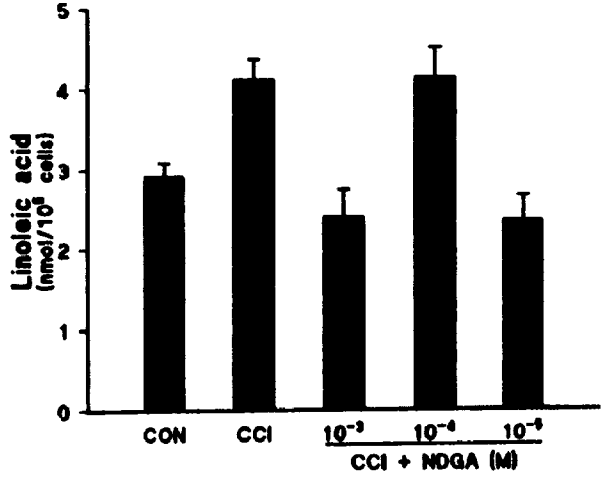
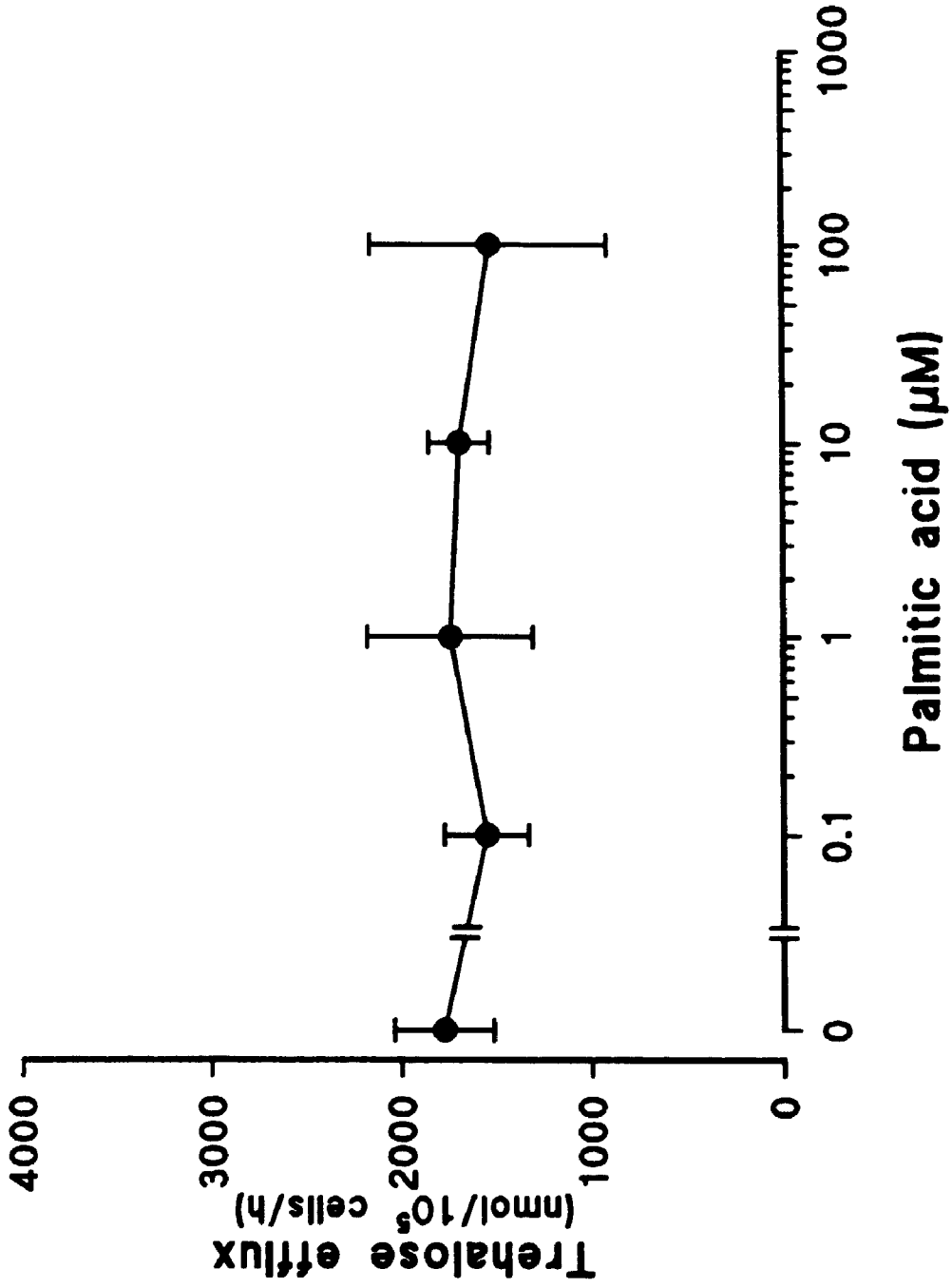

Linoleic acid
(nmol/10⁶ cells)
5
4
3
2
1
0
CON
CCl
10⁻³
10⁻⁴
10⁻⁵
CCl + NDGA (M)

Figure 33. The failure of palmitic acid to stimulate trehalose efflux from trophocytes *in vitro*. Dispersed trophocytes (~10,000/sample) in 0.5 ml of saline IV were pipetted into 10 ml round-bottomed polyethylene tubes. Palmitic acid (in 95% ethanol) was added to the cell sample to yield a final concentration of 0.1-100μM. The control sample containing no palmitic acid received an appropriate volume of ethanol, which never exceeded a final concentration of 0.5 %. The samples were incubated for 1h at 30°C in a shaker water bath. On completion of incubation 100μl of the medium containing the cells was removed from the sample and transferred to a stoppered centrifuge tube containing 2.0 ml chloroform/methanol (2:1, V/V). The extraction of trehalose from the samples and its measurement by gas chromatography is described in the materials and methods. The values shown are the means ± SEM (n=4). The results show that palmitic acid does not affect trehalose efflux from the trophocytes.


Trehalose efflux
(nmol/10^5 cells/h)
4000
3000
2000
1000
0
0
0.1
1
10
100
1000
Palmitic acid (μM)

trophocytes. Stearic acid, however, does stimulate trehalose efflux (Fig. 34), and a similar effect was obtained with oleic, linoleic and arachidonic acids (Fig. 35, 36, 37) each of which significantly increased trehalose efflux.

3.3.2 Synthesis of Arachidonic

Arachidonic acid was not detected in this study although it is present in low concentration in certain insect tissues. In mammals arachidonic acid may be released from membranes following stimulation by an agonist, thus the rate of release of this fatty acid may be limiting in the formation of eicosanoids (Chang *et al.*, 1987). Arachidonic acid is an essential fatty acid and is synthesized from linoleic acid via the pathway linoleic acid-----> γ-linolenic acid-----> di-homo-γ-linolenic acid-----> arachidonic acid (Gurr and James, 1975). Since the present study shows that intracellular linoleic acid increases in response to the hypertrehalasemic hormones it was of interest to determine whether it could serve as a substrate for arachidonic acid synthesis.

Fig. 38A shows that trophocytes are capable of converting linoleic acid to γ-linolenic acid and that this conversion is increased by the synthetic hypertrehalosemic hormone CC I. The synthesis of γ-linolenic acid in resting tissue was not affected by indomethacin although its

Figure 34. The stimulatory effect of stearic acid on trehalose efflux from trophocytes *in vitro*. Dispersed trophocytes (~10,000/sample) in 0.5 ml of physiological saline IV were pipetted into 10 ml round-bottomed polyethylene tubes. Stearic acid (in 95% ethanol) was added to the cell sample to yield a final concentration of 0.5-100 μM. The control sample containing no stearic acid received an appropriate volume of ethanol, which never exceeded a final concentration of 0.5 %. The samples were incubated for 1h at 30°C in a shaker water bath. On completion of incubation 100μl of the medium containing the cells was removed from the sample and transferred to a stoppered centrifuge tube containing 2.0 ml chloroform/methanol (2:1, V/V). The extraction of trehalose from the samples and its measurement by gas chromatography is described in the materials and methods. The values shown are the means ± SEM. Analysis of the data (ANOVA) shows that stearic acid stimulates trehalose efflux from the trophocytes ($p < 0.001$, $n=4$).

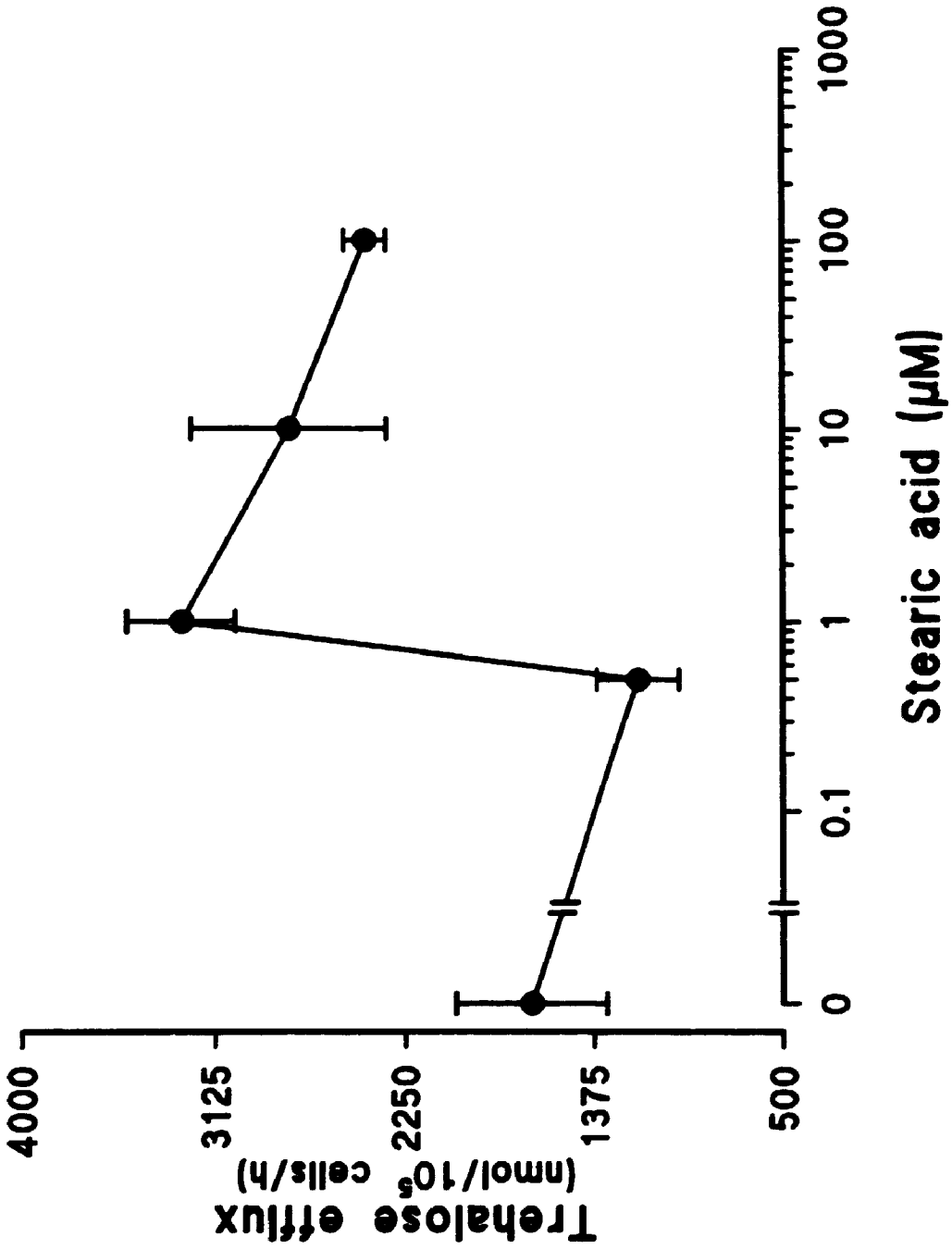

Trehalose efflux
(nmol/10^6 cells/h)
4000
3125
2250
1375
500
0
0.1
1
10
100
1000
Stearic acid (μM)

Figure 35. The stimulatory effect of oleic acid on trehalose efflux from trophocytes *in vitro*. Dispersed trophocytes (~10,000/sample) in 0.5 ml of physiological saline IV were pipetted into 10 ml round-bottomed polyethylene tubes. Oleic acid (in 95% ethanol) was added to the cell sample to yield a final concentration of 0.5-100 μM. The control sample containing no oleic acid received an appropriate volume of ethanol, which never exceeded a final concentration of 0.5 %. The samples were incubated for 1h at 30°C in a shaker water bath. On completion of incubation 100μl of the medium containing the cells was removed from the sample and transferred to a stoppered centrifuge tube containing 2.0 ml chloroform/methanol (2:1, V/V). The extraction of trehalose from the samples and its measurement by gas chromatography is described in the materials and methods. The values shown are the means ± SEM. Analysis of the data (ANOVA) shows that oleic acid stimulates trehalose efflux from the trophocytes ($p < 0.001$, $n=4$).

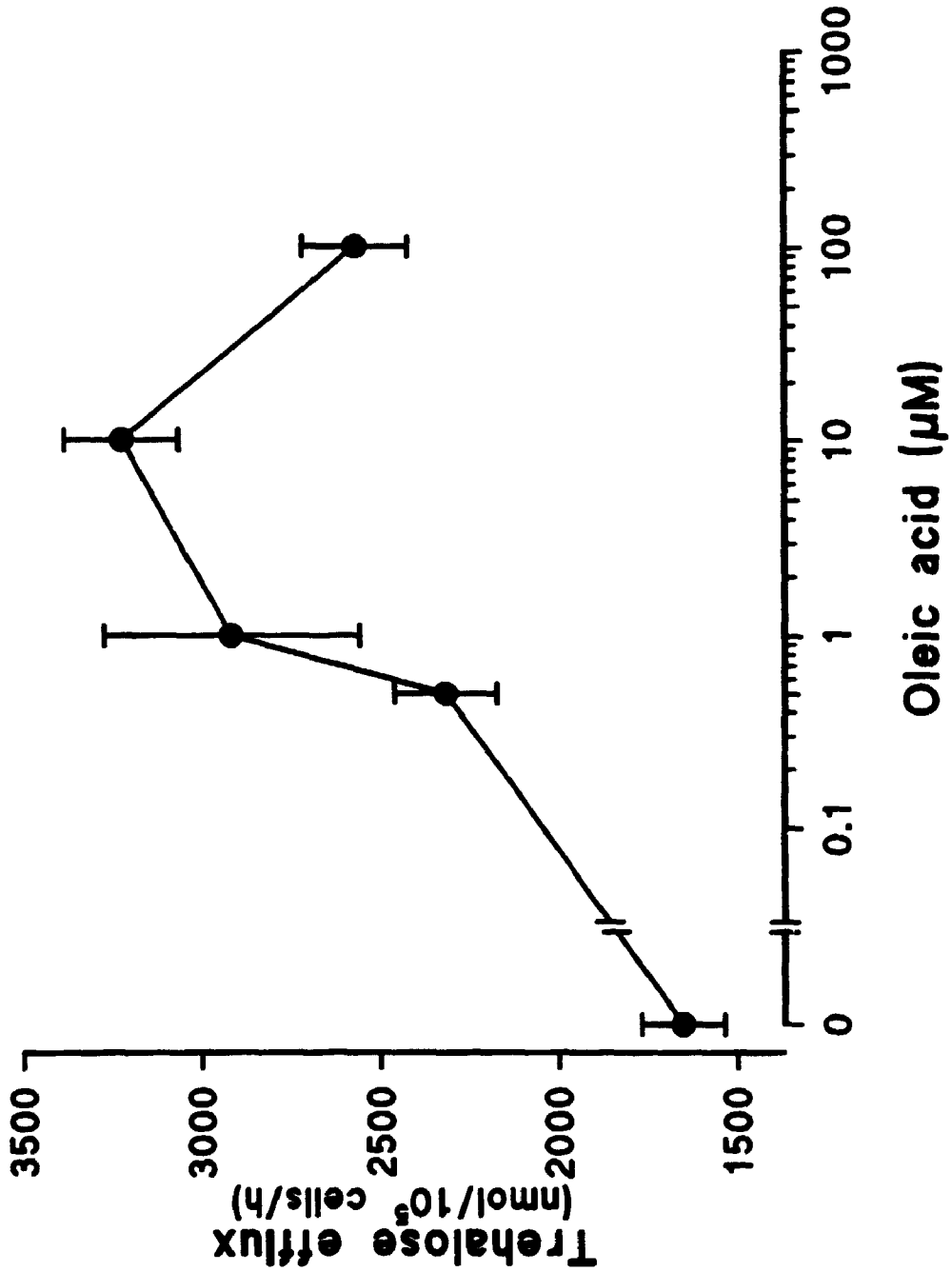

Trehalose efflux
(nmol/10⁵ cells/h)
3500
3000
2500
2000
1500
0
0.1
1
10
100
1000
Oleic acid (μM)

Figure 36. The stimulatory effect of linoleic acid on trehalose efflux from trophocytes *in vitro*. Dispersed trophocytes (~10,000/sample) in 0.5 ml of physiological saline IV were pipetted into 10 ml round-bottomed polyethylene tubes. Linoleic acid (in 95% ethanol) was added to the cell sample to yield a final concentration of 0.1-100 μM. The control sample containing no linoleic acid received an appropriate volume of ethanol, which never exceeded a final concentration of 0.5 %. The samples were incubated for 1h at 30°C in a shaker water bath. On completion of incubation 100μl of the medium containing the cells was removed from the sample and transferred to a stoppered centrifuge tube containing 2.0 ml chloroform/methanol (2:1, V/V). The extraction of trehalose from the samples and its measurement by gas chromatography is described in the materials and methods. The values shown are the means ± SEM. Analysis of the data (ANOVA) shows that linoleic acid stimulates trehalose efflux from the trophocytes ($p < 0.001$, $n=4$).

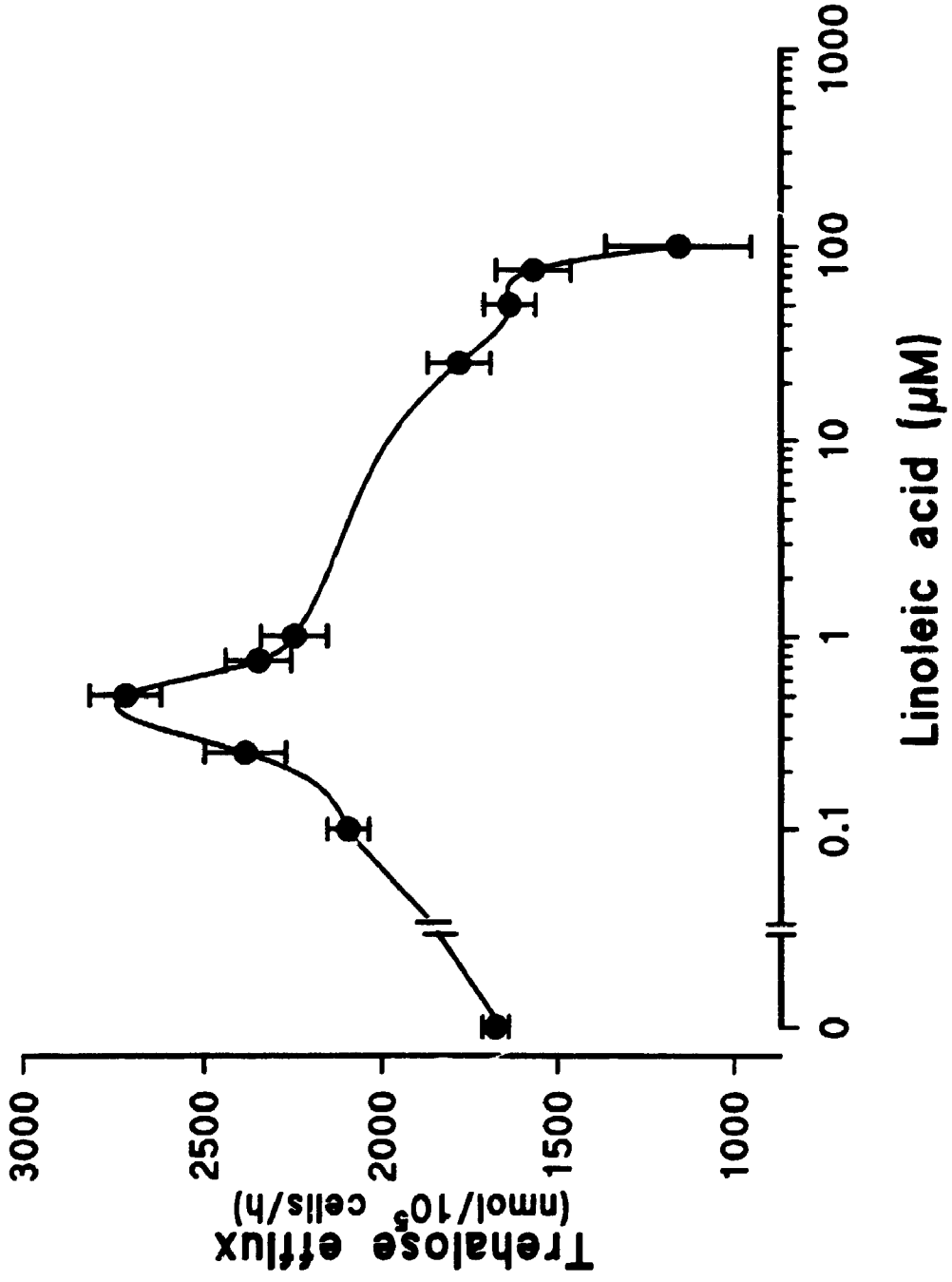

Trehalose efflux
(nmol/10⁵ cells/h)
3000
2500
2000
1500
1000
0
0.1
1
10
100
1000
Linoleic acid (μM)

Figure 37. The stimulatory effect of arachidonic acid on trehalose efflux from trophocytes *in vitro*. Dispersed trophocytes (~10,000/sample) in 0.5 ml of physiological saline IV were pipetted into 10 ml round-bottomed polyethylene tubes. Arachidonic acid (in 95% ethanol) was added to the cell sample to yield a final concentration of 0.1-100 μM. The control sample containing no arachidonic acid received an appropriate volume of ethanol, which never exceeded a final concentration of 0.5 %. The samples were incubated for 1h at 30°C in a shaker water bath. On completion of incubation 100μl of the medium containing the cells was removed from the sample and transferred to a stoppered centrifuge tube containing 2.0 ml chloroform/methanol (2:1, V/V). The extraction of trehalose from the samples and its measurement by gas chromatography is described in the materials and methods. The values shown are the means ± SEM. Analysis of the data (ANOVA) shows that arachidonic acid stimulates trehalose efflux from the trophocytes ($p < 0.001$, $n=3$).

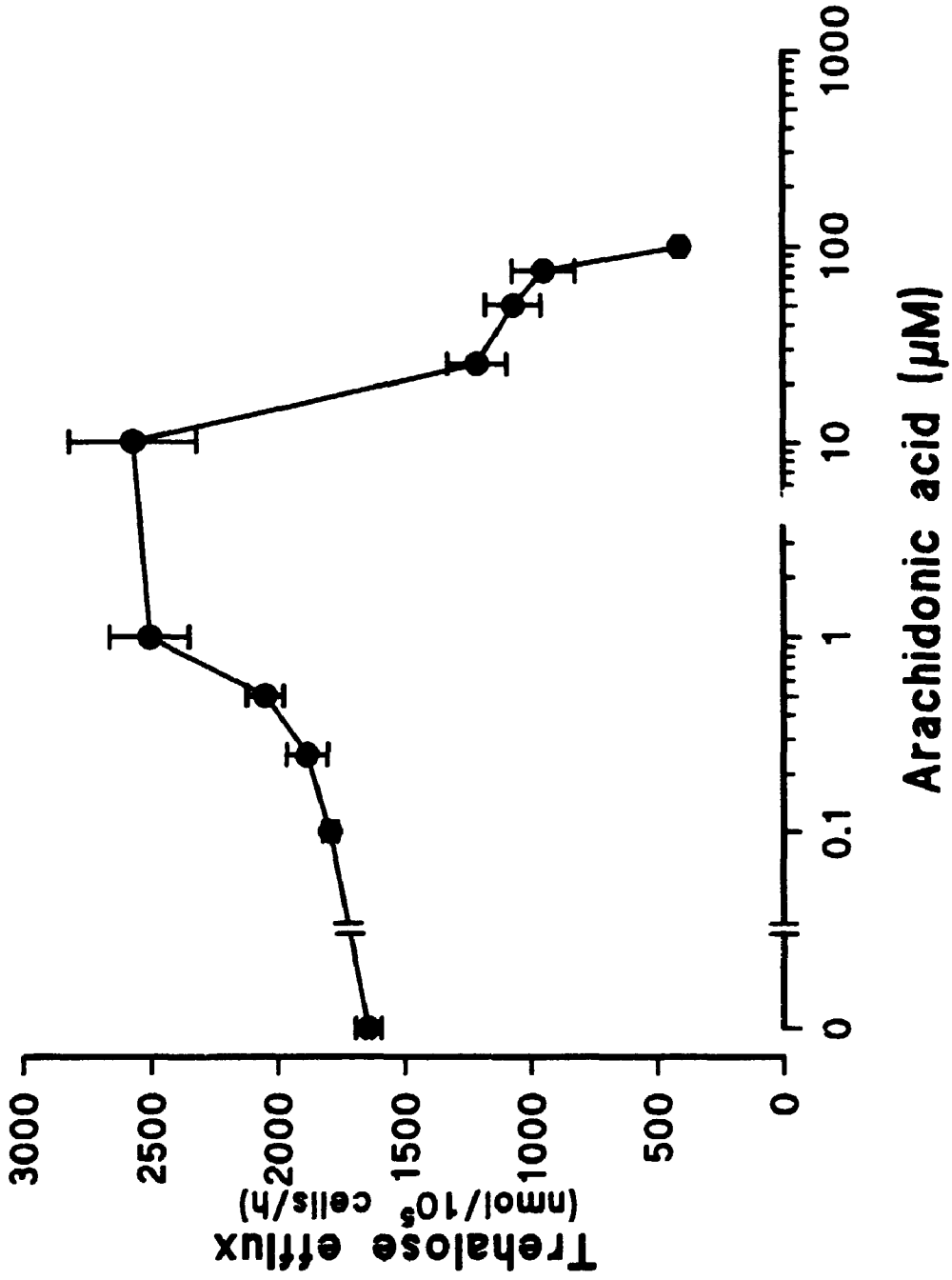
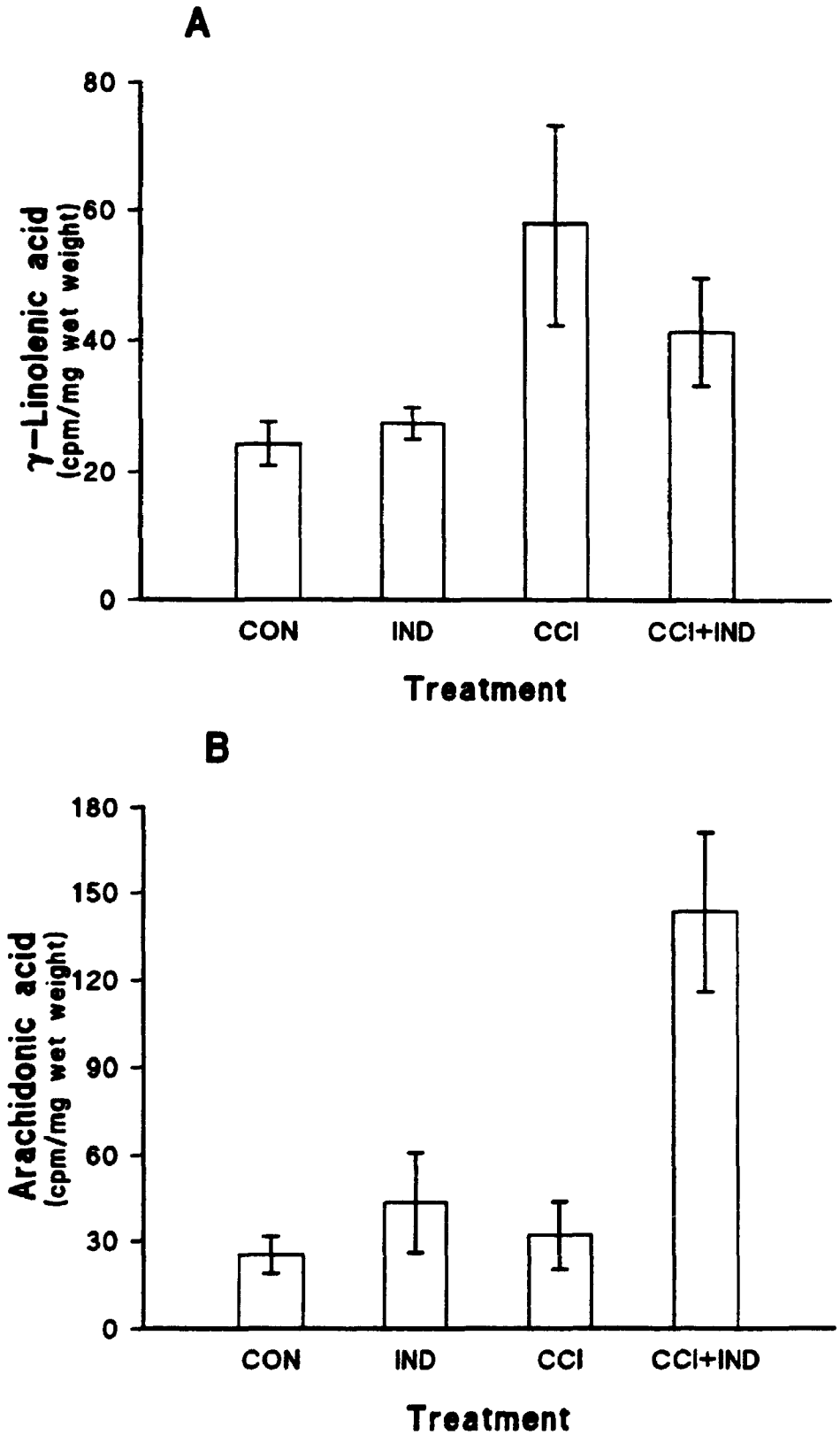

Trehalose efflux
(nmol/10^5 cells/h)
3000
2500
2000
1500
1000
500
0
0
0.1
1
10
100
1000
Arachidonic acid (μM)

Figure 38. The conversion of ^{14}C-linoleic acid into ^{14}C-γ-linolenic acid and ^{14}C-arachidonic acid in intact fat body. Four fat body (eight lobes) were used to prepare four samples comprising two lobes each, none of which contained two lobes from the same cockroach. The tissue in each group was labelled by incubation with 1.0 μCi of ^{14}C-linoleic acid in 2 ml of physiological saline II at 30°C in a shaker water bath for 1h. The tissue was then rinsed thoroughly with fresh physiological saline II. To half the samples indomethacin in fresh physiological saline II was added to give a final concentration of 1 mM. The incubation was continued for 1h after which CC I (100 pmol/ml) was added to one of the untreated and one of the indomethacin treated samples. After an additional 15 min the incubation was terminated by the addition of 1.5 ml of ice-cold methanol followed by homogenization. The samples were then transferred to centrifuge tubes fitted with teflon-lined screw caps, flushed with N_2 and allowed to stand at 4°C for 20 min. After centrifugation at 450g x 20 min the supernatant was transferred to a centrifuge tube containing 5 ml hexane. The fatty acids were extracted and processed as described in the materials and methods. Statistical analysis of the data reveals that the hypertrehalosemic hormone CC I significantly stimulates the conversion of linoleic acid into γ-linolenic acid ($p < 0.015$, $n = 4$) (A) and that there is a significant increase of arachidonic acid in the samples treated with indomethacin and challenged with CC I ($p < 0.0001$, $n = 4$) (B). Abbreviations used are CON, control; CC I, synthetic hypertrehalosemic hormone I; IND, indomethacin.


A
80
60
40
20
0
γ-Linolenic acid
(cpm/mg wet weight)
CON
IND
CCI
CCI+IND
Treatment
B
180
150
120
90
60
30
0
Arachidonic acid
(cpm/mg wet weight)
CON
IND
CCI
CCI+IND
Treatment

production in CC I treated trophocytes was slightly decreased by the inhibitor. Figure 38B illustrates the formation of arachidonic from linoleic acid in trophocytes. The fatty acid is elevated only in those cells challenged with CC I and treated with indomethacin. The apparent failure of arachidonic acid to increase in cells treated with CC I in the absence of indomethacin is likely due to the rapid utilization of this fatty acid following its formation.

3.3.3 Evidence for the production of active fatty acid metabolites

Fatty acids, especially arachidonic acid are metabolized to yield active metabolites, including prostaglandins. These metabolites are extractable from tissues with 30 % methanol and can be separated by reverse phase chromatography (Henke *et al.*, 1984).

The possibility that fatty acids arising as a result of the hormone action might have been converted into active products was tested by preparing a 30 percent methanol extract of trophocytes for analysis. Because the methanolic extracts would contain free fatty acids in addition to compounds such as eicosanoids the latter were obtained free of the former by first passing the methanolic extract through a reverse phase column and eluting any potential eicosanoids with 30 percent acetonitrile. Free fatty acids

are retained by the column.

The 30 percent acetonitrile fraction was dried and taken up in a small volume of 50 percent ethanol for assay using the trehalose efflux assay system. The results are presented in Fig. 39 and show that one or more substances in the eicosanoid fraction have a potent effect on the efflux of trehalose from the trophocytes.

The absorbance of material eluted from the reverse phase column with 30 percent acetonitrile is illustrated in Fig. 40. The data show that treatment of the trophocytes with crude hypertrehalosemic hormone produces a series of compounds having strong absorbance in the region of 192 nm. It is interesting that the amount of this material is greatly diminished if the cells treated with hormone are also treated with BPB or indomethacin. It is of further interest that prostaglandin $F_{2\alpha}$ and prostaglandin E_2 are also eluted from this column with 30 percent acetonitrile in the same region as the unidentified peaks (data not shown).

3.3.4 Stimulatory effect of Prostaglandins on trehalose efflux

To test the possibility that either prostaglandin $F_{2\alpha}$ or E_2 might have some activity in the trophocyte trehalose efflux assay system both of these agents were tested for activity. The results, presented in Fig. 41 show that 1μM prostaglandin $F_{2\alpha}$ has a potent effect on trehalose efflux

Figure 39. Stimulation of trehalose efflux from trophocytes treated with an acetonitrile soluble fraction from CCE challenged trophocytes. Trophocytes (1.0×10^6) were incubated in 40 ml plastic centrifuge tubes in 1 ml of physiological saline IV and allowed to equilibrate at 30°C in a shaker water bath for 30 min. CCE (0.2 GPE/ml) was added to the cells which were then incubated for an additional period of 1h. The incubation was terminated by the addition of 4.0 ml of ice-cold methanol. The solution was transferred to a centrifuge tube, flushed with N_2, and capped. After mixing thoroughly by vortexing and allowing to stand at 4°C for 20 min the sample was centrifuged at 450g x 20 min to remove precipitated proteins. The supernatant was then dried at 30°C using a rotary evaporator and the sample taken up in 2 x 250 µl of 30% methanol. The sample was transferred to an Eppendorf centrifuge tube and centrifuged at 5000rpm x 6 min. The entire sample was injected into the HPLC fitted with a C-18 (WATERS µBONDAPAK, reverse phase, 3.9 mm x 30 cm) column and a UV (WATERS LAMBDA MAX, Model 480, LC Spectrophotometer)detector set at 192 nm. The sample was eluted isocratically with 30% acetonitrile containing 0.008 percent trifluoroacetic acid. Solvent was delivered at the rate of 1 ml/min. The material eluted in the first 12 minutes were collected and dried at 30°Cusing a rotary evaporator. The dry sample was taken up in 2 x 250 µl of 50% ethanol (ethanol/physiological saline I). To determine the effect of the eluted material on trehalose efflux, trophocytes (~10,000/sample) were incubated in 10 ml round-bottomed polyethylene tubes in 0.5 ml physiological buffer IV. The eluted material was serially diluted and 1.0 µl was added to the cells. One equivalent of the metabolites represents 1.0 µl of the original 500 µl of 50% ethanol containing the total eluted material. After incubation for one hour 100 µl of the sample was removed and added to a centrifuge tube containing 2.0 ml chloroform/methanol (2/1; V/V). The samples were derivatized and processed as indicated in the materials and methods. Analysis of the data (ANOVA) reveals that trehalose efflux is significantly stimulated, $p < 0.001$, $n = 4$. The eluted material is extremely active in its ability to stimulate trehalose efflux from trophocytes.

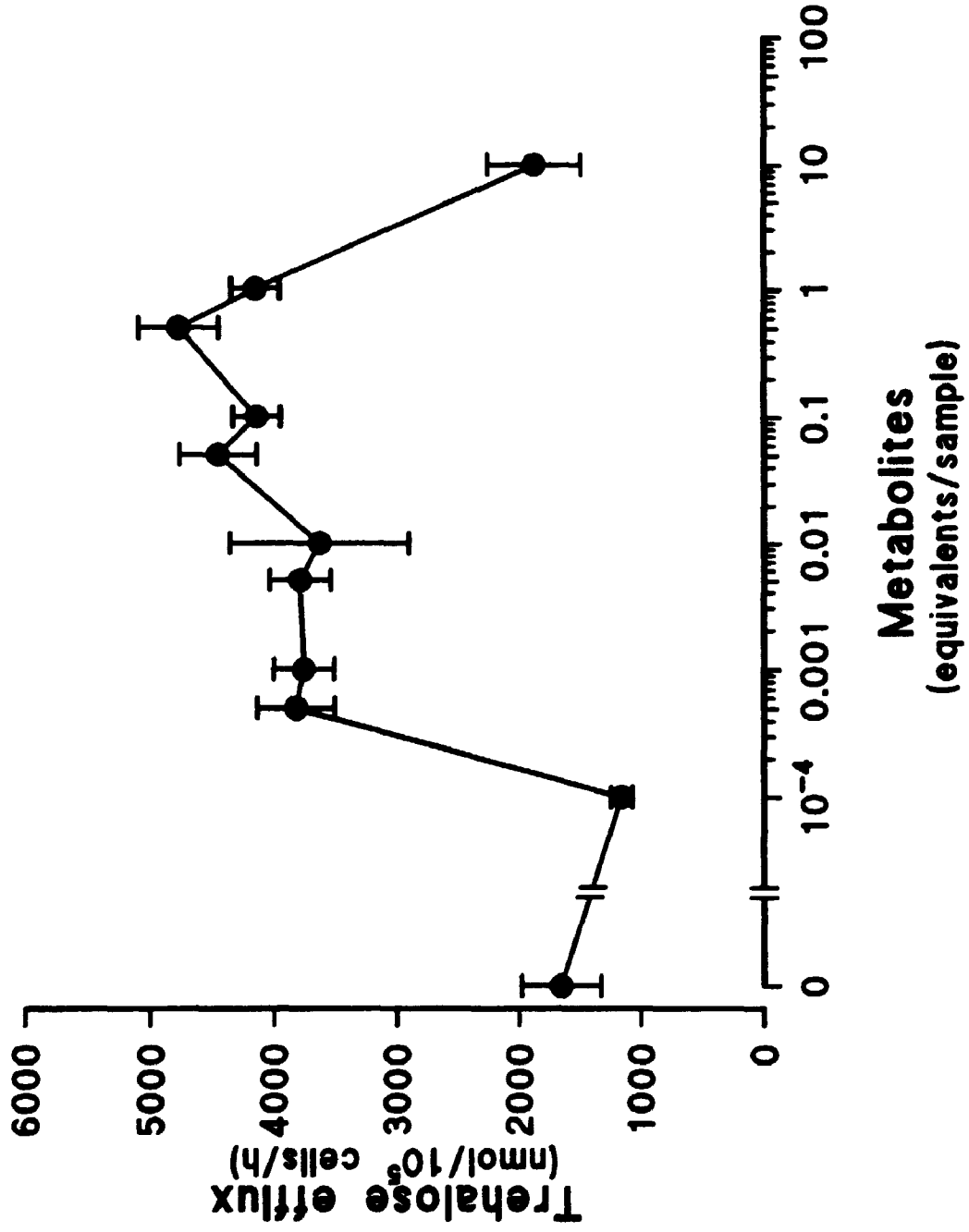

Trehalose efflux
(nmol/10^5 cells/h)
6000
5000
4000
3000
2000
1000
0
0
10^-4
0.001
0.01
0.1
1
10
100
Metabolites
(equivalents/sample)

Figure 40. Analysis, by reverse phase HPLC, of the methanol extractable fraction of fat body trophocytes challenged with CC extract. Four samples containing ~100,000 trophocytes each were incubated in 40 ml plastic centrifuge tubes for 30 min in 1.0 ml physiological saline III at 30°C in a shaker water bath. At this time either indomethacin or BPB (final concentration 0.1 mM) was added to two of the samples and all four samples were allowed to incubate for an additional 15 min. CCE (0.2 GPE/ml) was added to each of the samples containing inhibitor and one untreated sample. The other untreated sample was used as the control. All the samples were then incubated for an additional 15 min. The reaction was terminated by the addition of 4.0 ml of ice-cold methanol. The mixture was transferred to centrifuge tubes fitted with teflon-lined screw caps, flushed with N_2, mixed thoroughly by vortexing and allowed to stand at 4°C for 20 min. The sample was then centrifuged at 450g x 20 min to remove the precipitated protein. The supernatant was evaporated to dryness on a rotary evaporator at 30°C and the residue taken up in 2 x 250 μl of 30% methanol in H_2O. The sample was then transferred to Eppendorf centrifuge tubes and centrifuged at 5000rpm x 6 min to remove particulate matter. One hundred μl of the sample was injected into the HPLC fitted with a C-18 reverse phase column (WATERS μBONDAPAK, 3.9mm x 30cm) and a UV detector (WATERS LAMBDA MAX, Model 480, LC Spectrometer) set at 192 nm. The sample was eluted isocraticly with 30% acetonitrile buffered with 0.008 % trifluoroacetic acid solvent delivered at the rate of 1 ml/min. The results indicate that the hormone increased the concentration of substances that absorb at 192 nm. This increase is inhibited by indomethacin and BPB. Abbreviations used are CON, control; CCE, natural hypertrehalosemic hormone (I and II); INDO, indomethacin; BPB, ρ-bromophenacyl bromide.

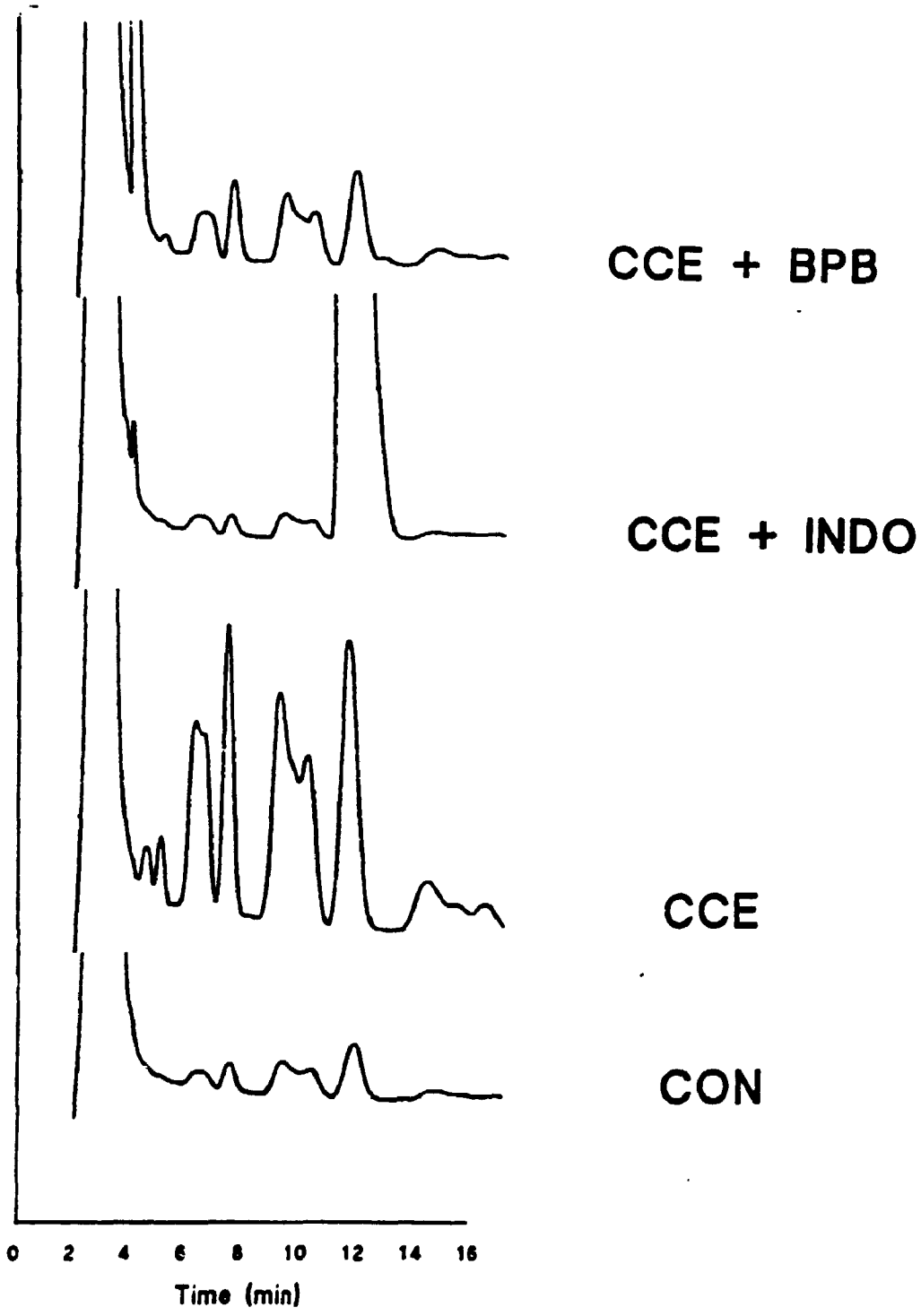
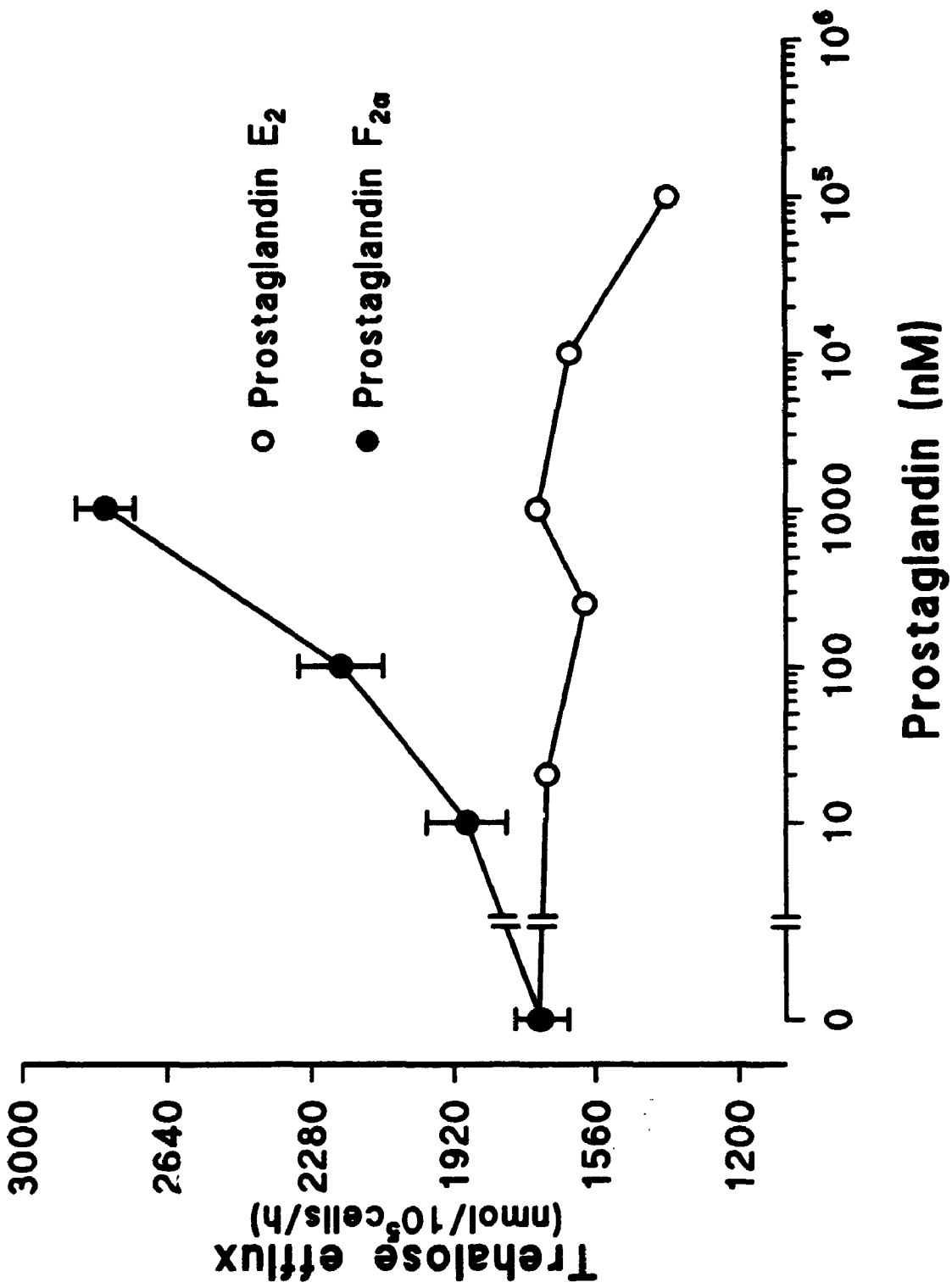

CCE + BPB
CCE + INDO
CCE
CON
0
2
4
6
8
10
12
14
16
Time (min)

Figure 41. The stimulatory effect of prostaglandin $F_{2\alpha}$ on trehalose efflux from trophocytes *in vitro*. Dispersed trophocytes (~10,000/sample) in 0.5 ml of physiological saline IV were pipetted into 10 ml round-bottomed polyethylene tubes. Prostaglandin $F_{2\alpha}$ (in 95% ethanol) was added to the cell sample to yield a final concentration of 0.01-1 μM. To the other cell samples prostaglandin E_2 was added to yield a final concentration of 20nM-100 μM. The control sample containing no prostaglandins received an appropriate volume of ethanol, which never exceeded a final concentration of 0.5 %. The samples were incubated for 1h at 30°C in a shaker water bath. On completion of incubation 100 μl of the medium containing the cells was removed from the sample and transferred to a stoppered centrifuge tube containing 2.0 ml chloroform/methanol (2:1, V/V). The extraction of trehalose from the samples and its measurement by gas chromatography is described in the materials and methods. Analysis of the data (ANOVA) for prostaglandin $F_{2\alpha}$ reveals the means to be significantly different from control, $p < 0.0025$, $n=4$. The data for prostaglandin E_2 represents single points. The figure illustrates that prostaglandin $F_{2\alpha}$ stimulates trehalose efflux from the trophocytes whereas prostaglandin E_2 does not.


Trehalose efflux
(nmol/10⁵cells/h)
3000
2640
2280
1920
1560
1200
Prostaglandin (nM)
0
10
100
1000
10⁴
10⁵
10⁶
○ Prostaglandin E₂
● Prostaglandin F₂α

from the dispersed trophocytes whereas prostaglandin E_2 was inactive. A sample of prostaglandin A_2 was also assayed for activity. The concentration tested was 100 μM. This is probably unphysiological but it is of interest that it had a stimulatory effect on trehalose efflux (Fig. 42).

3.4 Control of trehalose-6-phosphate phosphatase

Trehalose-6-phosphatase is a key enzyme in the synthesis of trehalose because of its role in the removal of phosphate from trehalose-6-phosphate thereby allowing the sugar to leave the cell. This suggests that any change in the activity of the enzyme would significantly change the rate of trehalose efflux from the cell. It was of interest to know the affinity of this enzyme for its substrate. This was done using trophocytes as the starting material. As illustrated in Fig. 43 the Km of this enzyme was found to be 2.70 mM which is very similar to the value obtained by Friedman and Hsueh (1979). An enzyme preparation prepared from trophocytes which had been incubated with and without CC I revealed no difference in enzyme activity due to the hormone treatment indicating that the enzyme does not undergo covalent modification due to the action of the hormone.

Because the change in concentration of free fatty occurs in trophocytes exposed to hypertrehalosemic hormones, and the apparent relationship between fatty acid

Figure 42. The stimulatory effect of prostaglandin A_2 on trehalose efflux from the fat body *in vitro*. Paired fat body lobes were incubated individually in 2ml of physiological saline I at 30°C in a shaking water bath. Prostaglandin A_2 (in 95% ethanol) was added to the physiological saline I containing one lobe of each pair of fat body lobes to yield a concentration of 100 μM. The control tissue was untreated but received an appropriate volume of ethanol, which never exceeded a final concentration of 0.5 %. At the indicated times 50 μl of medium was withdrawn for measurement of trehalose with anthrone reagent. The fat body lobes were placed in centrifuge tubes containing 1ml 0.9% saline for homogenization and the protein determined as described in the materials and methods. Analysis of the data shows that the effect of prostaglandin A_2 is significant ($p < 0.025$, $n = 6$).

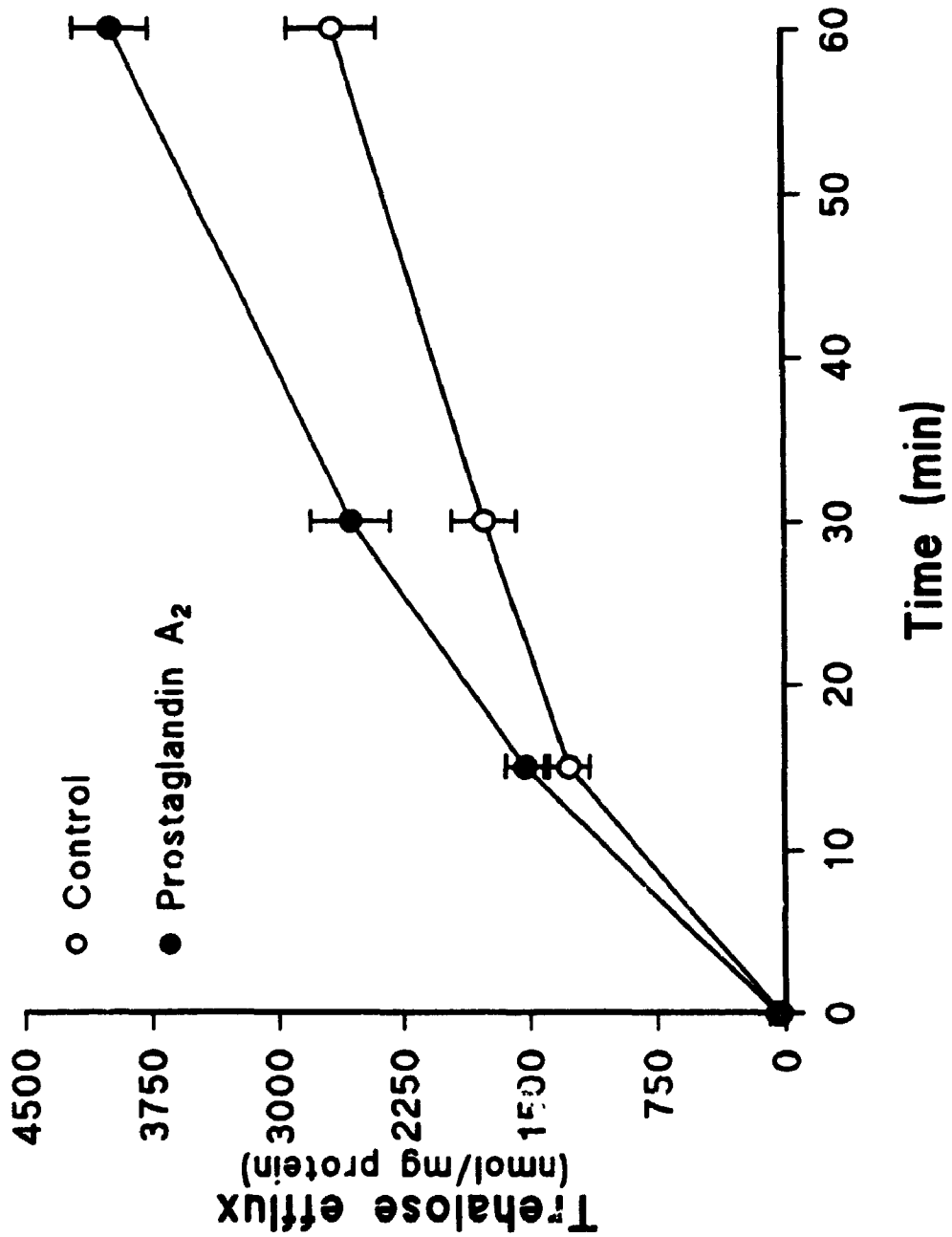
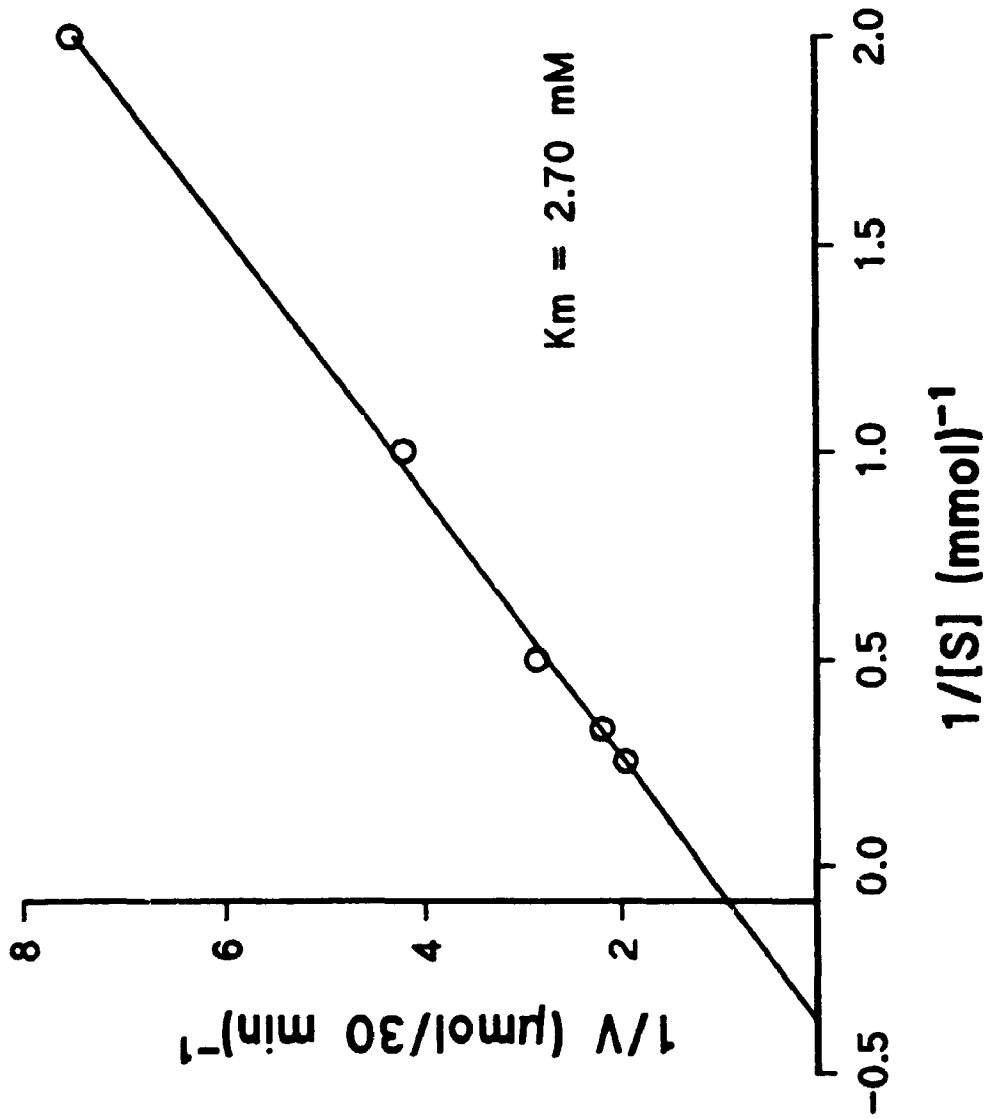

Trehalose efflux
(nmol/mg protein)
4500
3750
3000
2250
1500
750
0
0
10
20
30
40
50
60
Time (min)
o Control
● Prostaglandin A_2

Figure 43. Determination of the Km for trophocyte trehalose-6-phosphatase using a double reciprocal plot. Dispersed trophocytes were homogenized in a buffer composed of 0.5 M TES (N-Tris(Hydroxymethyl) methyl-2-aminoethanesulfonic acid) and 1.0 mM EDTA (ethylenediamine tetraacetic acid) adjusted to pH 8.0. The cells were homogenized in a round-bottomed centrifuged tube and centrifuged at 10,000g x 5 min. This was followed by recentrifugation of the supernatant recentrifuged at 33,000g x 15 min. The supernatant from the second centrifugation was used as the source of enzyme. The reaction was carried out in a buffer composed of 50 mM TES, 1.0 mM $MgCl_2$, 3.3 mM T-6-P (titrated to pH 7.0 with 1.0 N NaOH) and enzyme preparation in total volume of 0.5 ml. The mixture was incubated for 30 minutes at 32°C and the reaction halted by the addition of 0.5 ml 10% trichloroacetic acid (W/V). The samples were centrifuged and 0.5 ml of the supernatant used to determine inorganic phosphate. The data show that the enzyme has a Km = 2.70 mM.


Km = 2.70 mM
1/V (μmol/30 min)⁻¹
1/[S] (mmol)⁻¹
8
6
4
2
0.0
0.5
1.0
1.5
2.0
-0.5

concentration and trehalose efflux it was of interest to determine whether fatty acids affected the activity of trehalose-6-phosphatase. The results of this experiment show that whereas stearic and palmitic acids have no effect on the activity of the enzyme the remaining unsaturated fatty acids do (Fig. 44). One hundred μM oleic acid decreased the activity of the enzyme by half while 40 μM linoleic and arachidonic acid decreased the activity of the enzyme by 50 percent. Fig. 45 shows that the Ki for linoleic acid is estimated to be 13.27 μM. This would appear to be within the concentration range that is physiologically significant.

Figure 44. The inhibitory effect of free fatty acids on the activity of trophocyte trehalose-6-phosphatase. Dispersed trophocytes were homogenized in a buffer comprising 0.5 M TES (N-Tris(Hydroxymethyl) methyl-2-aminoethanesulfonic acid) and 1.0 mM EDTA (ethylenediamine tetraacetic acid) adjusted to pH 8.0. The trophocytes were homogenized and the homogenate centrifuged at 10,000g x 5 min. The supernatant was decanted and recentrifuged at 33,000g x 15 min and the supernatant from this second centrifugation was used as a source of enzyme. The reaction was carried out in a buffer composed of 50 mM TES, 1.0 mM $MgCl_2$, 3.3 mM T-6-P (titrated to pH 7.0 with 1.0 N NaOH) and enzyme preparation in a total volume of 0.5 ml. The fatty acids were added to the enzyme assay medium in 1.0 µl 95% ethanol. The mixture was incubated for 30 minutes at 32°C and the reaction halted by the addition of 0.5 ml of 10% trichloroacetic acid (W/V). The samples were centrifuged and 0.5 ml used to determine inorganic phosphate. The data show that palmitic and stearic acid do not affect the activity of the enzyme. Oleic acid inhibits 50% of the activity at a concentration of 100 µM whereas linoleic and arachidonic acid inhibit 50% of the activity at a lower concentration of 40 µM.

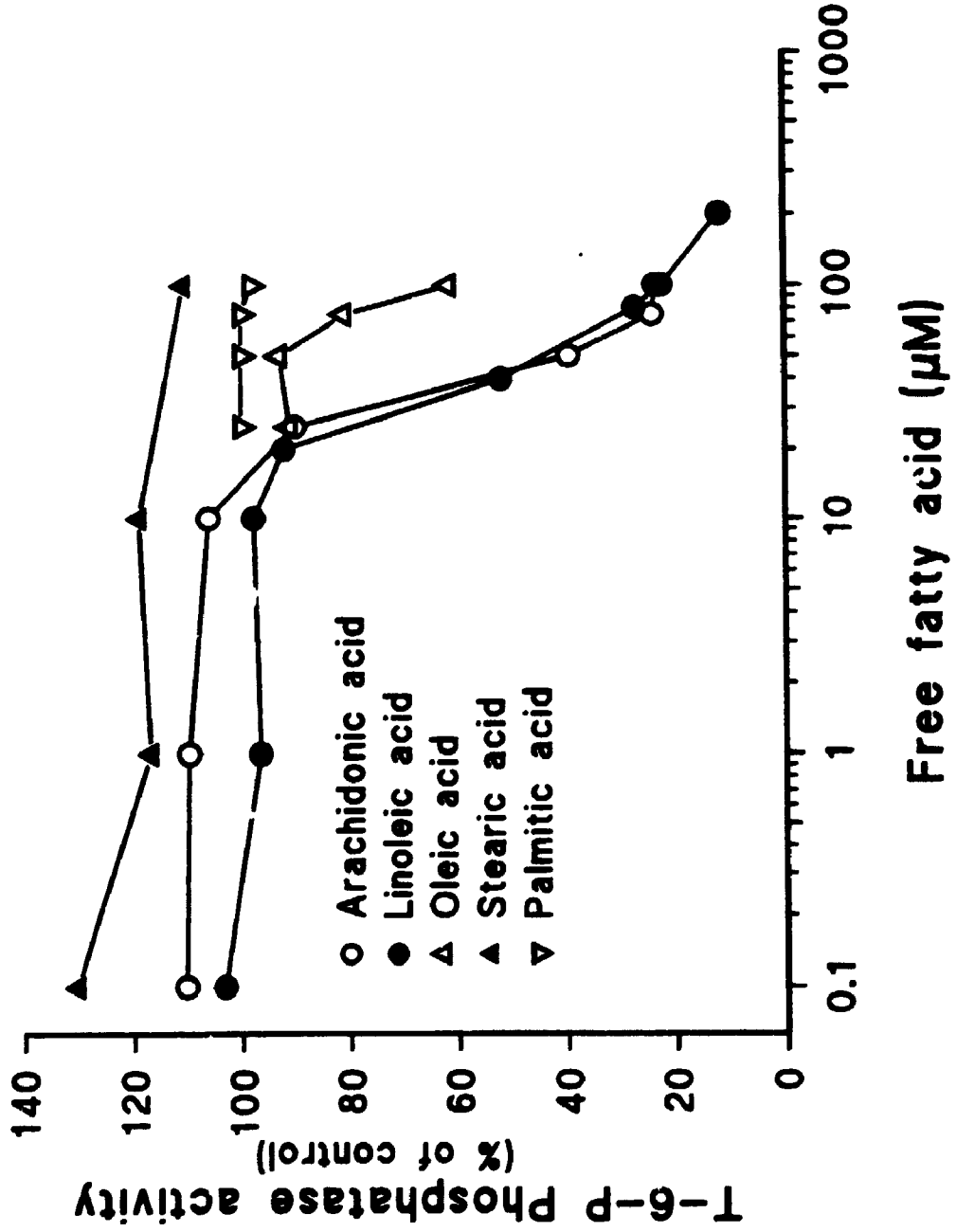
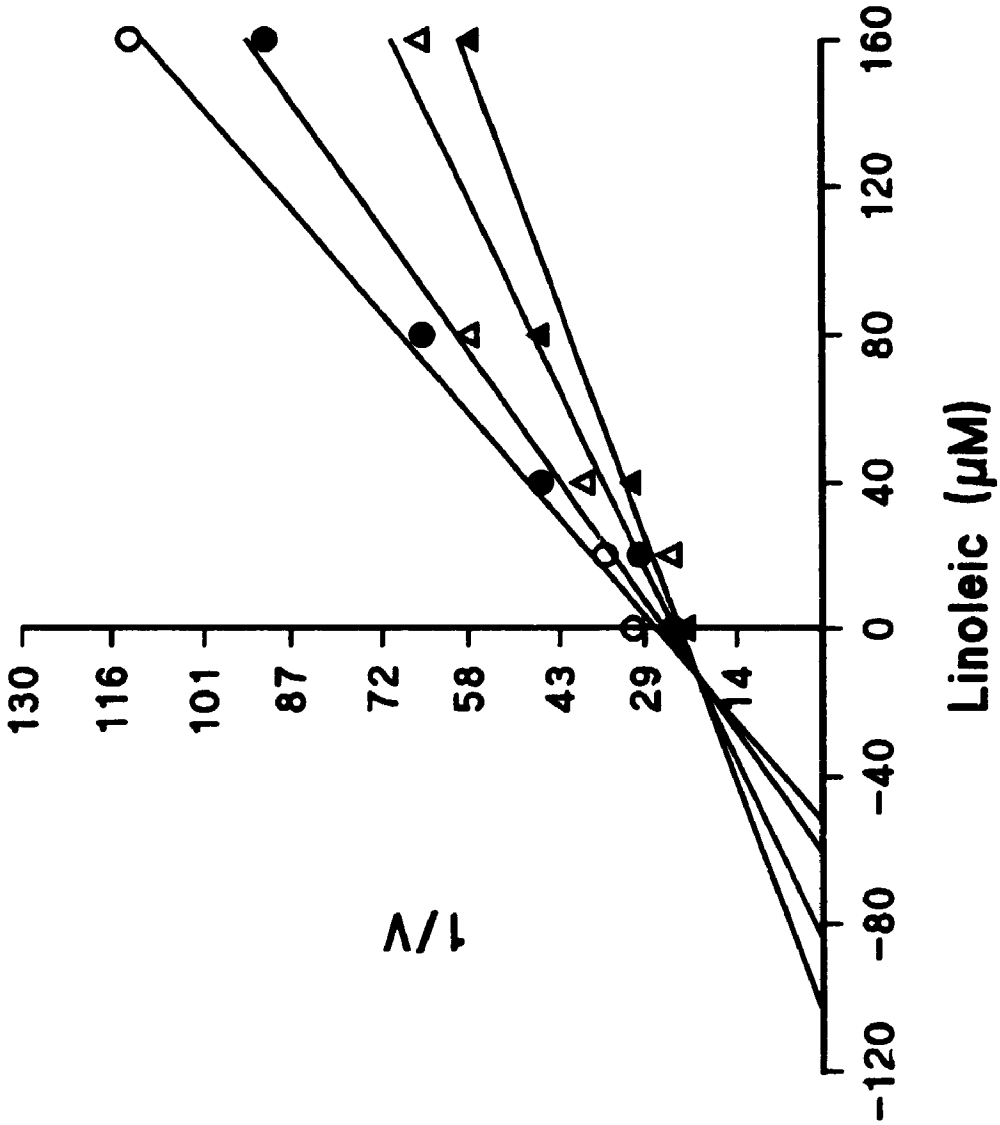

T-6-P Phosphatase activity
(% of control)
140
120
100
80
60
40
20
0
0.1
1
10
100
1000
Free fatty acid (μM)
Arachidonic acid
Linoleic acid
Oleic acid
Stearic acid
Palmitic acid

Figure 45. The determination of the Ki for linoleic acid as an inhibitor of trophocyte trehalose-6-phosphatase. Dispersed trophocytes were homogenized in a buffer comprising 0.5 M TES (N-tris(hydroxymethyl) methyl-2-amonoethanesulfonic acid), 1.0 mM EDTA (ethylenediamine tetraacetic acid) adjusted to pH 8.0. The cells were homogenized and centrifuged at 10,000g x 5 min following which the supernatant was decanted and recentrifuged at 33,000g x 15 min. The supernatant from the second centrifugation was used as the source of enzyme. Assay of trehalose-6- phosphatase was carried out in a buffer composed of 50 mM TES, 1.0 mM $MgCl_2$, 3.3 mM T6P (titrated to pH 7.0 with 1.0 N NaOH) and enzyme preparation in a total volume of 0.5 ml. Linoleic acid was added to the enzyme assays in 1.0 µl 95% ethanol. The mixture was incubated for 30 minutes at 32°C and the reaction halted by the addition of 0.5 ml 10% trichloroacetic acid (W/V). After centrifugation, 0.5 ml of the samples was used for the determination of inorganic phosphate. The data plotted as a Dixon plot show that linoleic acid has a Ki of 13.3 µM.


130
116
101
87
72
58
43
29
14
1/V
160
120
80
40
0
-40
-80
-120
Linoleic (μM)

Part 4

Discussion

4.1 Preamble

The physiological role of the insect hypertrehalosemic hormone has never been elucidated with certainty although from the time of its discovery it has often been cited as an insect analogue of glucagon, a vertebrate pancreatic hormone found in the islet tissue (Scarborough *et al.*, 1984). This comparison is based on the common property of both hormones to increase the concentration of sugar in hemolymph and blood. From a physiological viewpoint there are at least two major systems in insects in which the hypertrehalosemic hormones might participate. These include provision of sugar as a source of energy for the flight muscles and other tissues, and as a source of glucosyl residues for the construction of the new cuticle at the time of moulting. Both processes place heavy demands on the glycogen reserves in the fat body but, more importantly, the requirement for glucosyl residues is variable and depends on the stage of development or the flight status of the insect. It is reasonable to expect that accommodating a high or variable demand for sugar at certain times might require the participation of hormones.

Insect fat body is a complex organ whose function is still poorly understood. It has often been claimed to be

analogous to mammalian liver (Dean *et al.*, 1985) but because of its role in fat storage it could just as easily be likened to vertebrate adipose tissue. The synthesis of trehalose from glycogen in fat body is generally considered to be determined by the activity of glycogen phosphorylase (Steele and Hall, 1985). This view agrees with the finding that phosphorylase is activated by the hypertrehalosemic hormone (Orr *et al.*, 1985). However, this cannot be the whole story since strong activation of phosphorylase can be produced with only negligible increases in trehalose production (McClure and Steele, 1981).

In this study I have obtained evidence to support the hypothesis that production of free fatty acids may be related to the synthesis and release of trehalose from the fat body trophocytes. This conclusion is based on studies which made use of a variety of inhibitors and activators. Much of the information obtained by the use of those agents has been summarized and is presented in tabular form (Table 7). The table is intended to present an overview of the interaction between the various reactions associated with trehalose synthesis.

4.2 Validity of the trophocyte preparation for the study of trehalose efflux

The utilization of intact organs for study *in vitro* may result in variation among samples because of the difficulty

Table 7. Summary of the effect of various effectors[1] on phosphorylase activity, trehalose efflux and free fatty acid accumulation in trophocytes.

Characteristic	BPB	Melittin	NDGA	Indo; Diclo	CCE; CC I; CC II
Phosphorylase activity	no effect	minor increase	minor increase	increase	increase
Phosphorylase[2] activation	no effect	inhibit	no effect	no effect	
Trehalose efflux	decrease	increase	decrease	decrease	increase
[FFA]	minor decrease	increase > 250%	increase	increase	increase ~40%
Activated FFA[3] production	inhibit	not tested	inhibit	inhibit	

[1]Effectors used are, BPB (p-bromophenacyl bromide), phospholipase A_2 inhibitor; Melittin, phospholipase A_2 activator, NDGA (nordihydroguaiaretic acid), lipoxygenase inhibitor; Indo (indomethacin) and Diclo (diclofenac), cyclooxygenase inhibitors.
[2]Activation of phosphorylase by hypertrehalosemic hormone.
[3]Activation of fatty acid efflux by hypertrehalosemic hormone.

in obtaining cockroaches of the same age and nutritional state. The problem can be partly circumvented by using a paired fat body lobe technique whereby one lobe is used as the experimental tissue and the other as the control (Sevala and Steele, 1991). This, however, restricts the use of the fat body to a single comparison: in contrast, the use of disaggregated tissue avoids these problems.

Dispersed cells have been employed routinely in mammalian studies but infrequently with insects although insect cell lines have often been employed (Orr *et al.*, 1988). To date, only a single laboratory appears to have employed dispersed cells for the study of fat body function (Asher *et al.*, 1984).

The trophocytes are considered to be the source of trehalose in the fat body (Keeley, 1985; Steele and Ireland, 1994). It was therefore important to have a preparation of dispersed trophocytes which were as free as possible from other cells.

The dispersion technique used in the present study is based on that described by Steele and Ireland (1994). Like the intact fat body the trophocytes show a time dependent increase in trehalose efflux for approximately 1h. This is similar to the pattern described by McDougall and Steele (1988) for intact cockroach fat body. Furthermore, as this study shows, the increase in trehalose efflux from the trophocytes during this period in response to

hypertrehalosemic hormone is comparable to that obtained with intact fat body (McDougall and Steele, 1988). The trophocytes, therefore, behave in a manner similar to fat body with respect to the synthesis and efflux of trehalose.

4.3 Protein Kinase C

The elevation of inositol trisphosphate (IP_3) levels by the hypertrehalosemic hormones (Brown, 1990) necessitates a consideration of the possible role of protein kinase C (PKC) in this hormonal system. Hydrolysis of phosphatidyl-inositol, which produces inositol trisphosphate (IP_3) and related metabolites, also generates diacylglycerol (DG) (Berridge, 1987), a known activator of PKC (Nishizuka, 1986). Since PKC activity is linked to the generation of inositol phosphates in several systems, the relationship between the hypertrehlaosemic hormones and inositol phosphates in the cockroach suggest that PKC would also be involved in hormonal stimulation of trehalose efflux.

The present study suggests that protein kinase C is not involved in hormonal stimulation of trehalose efflux since the PKC inhibitors sphingosine (Hannun *et al.*, 1986) and H7 (Ito *et al.*, 1988) did not modify the action of the hormones. This conclusion is supported by the data showing that 1-oleoyl-2-acetylglycerol (OAG), a known activator of protein kinase C (Kroll *et al.*, 1988) neither stimulated trehalose efflux from unchallenged fat body nor that from

fat body treated with hormone. Furthermore, incubation of fat body with OAG in conjunction with the Ca^{2+} ionophore, A23187, to ensure that sufficient Ca^{2+} for the activation was present also failed to stimulate trehalose efflux. The failure of OAG to simulate trehalose efflux is strong evidence that PKC is probably not involved in hormonal control of trehalose efflux.

4.4 Location of lipolytic activity

The evidence suggests that an important step in the action of the hormones is the release of fatty acids due to a reaction catalysed by a lipase. The presence of large reserves of triacylglycerol in the fat body together with significant amounts of phospholipid associated with the membrane fraction of the tissue (Bailey *et al.*, 1975) suggests that either group of lipids could be the source of the fatty acids. Regardless of the source it is important to demonstrate that the lipolytic system responsible for the appearance of the fatty acids is responsive to the hormones that increase trehalose production.

Lipases that hydrolyse triacylglycerol, diacylglycerol and monoacylglycerol have been found in the fat body of the locust, *Locusta mirgratoria* (Tietz and Weintraub, 1978). A triacylglycerol lipase has also been demonstrated in the desert locust, *Schistocerca gregaria* (Spencer and Candy, 1976). Triacylglycerol lipase, including that in the fat

body of the American cockroach, *Periplaneta americana* (Hoffman and Downer, 1979), appears to be a characteristic feature of that tissue.

In general, triacylglycerol lipases are activated by cyclic AMP dependent protein kinases (Pines *et al.*, 1981; Egan *et al.*, 1992). Because extracts of the corpora cardiaca have been shown to increase cAMP in the fat body by activation of adenylate cyclase (Gade, 1977; Hanaoka and Takahashi, 1977), and because the gland extracts also decrease triacylglycerol in the tissue (Goldsworthy *et al.*, 1972) it is possible that a cAMP dependent triacylglycerol lipase may be activated to release fatty acids.

A second line of evidence that a neutral triacylglycerol lipase is not responsible for the appearance of free fatty acids in hormone treated trophocytes is the tissue distribution of the lipolytic activity. Triacylglycerol lipases are present in the cytosol or associated with the microsomal membranes. Pines *et al.*, (1981) found triacylglycerol lipase in both the cytosol and the microsomal fraction of fat body homogenates. Spencer and Candy (1976) also found triacylglycerol lipase in the soluble fraction of fat body homogenate. That these are unlikely to be lipases responsible for the appearance of fatty acids during trehalose efflux was suggested by the observation that the lipolytic activity was associated with membranes which included primarily the heavier plasma

membranes. The fact that isolated plasma membranes produce the same fatty acids as do hormone challenged trophocytes suggests that the release of fatty acids in the trophocytes is not due to a neutral triacylglycerol lipase.

The data suggest that a possible site of regulation of fatty acid production is the reaction catalysed by phospholipase A_2. This conclusion is supported by the finding that p-bromophenacyl bromide, an inhibitor of PLA_2 (Chang *et al.*, 1987; Bernard *et al.*, 1994; Hargreaves *et al.*, 1994) not only blocks trehalose efflux due to either of the hormones but also decreases free fatty acid production by the trophocytes.

Stimulation of fatty acid production in trophocytes treated with melittin also suggests that the fatty acids could be released by the action of phospholipase A_2. Melittin, an activator of PLA_2 (Mollay and Kriel, 1974; Choi *et al.*, 1992; Cooper and Bomalaski, 1994), increases the same fatty acids intracellularly as do the hypertrehalosemic hormones. This suggests that melittin may act like the hormone. The evidence however, does not support this interpretation since CC I is unable to release fatty acids from isolated trophocyte membranes, presumably because certain co-factors are absent. Melittin releases fatty acids from the isolated membrane because of its ability to directly activate phospholipase A_2 (Mollay and Kriel, 1974).

Phospholipase A_2 releases fatty acids esterified at the

sn_2 position (Murakami, 1993) from several membrane components including phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine and phosphatidic acid (Broekman, 1986). In mammalian cells the fatty acid esterified at that position is usually arachidonic acid (Murakami, 1993). This is also true for insects where AA is also esterified at the sn_2 position (Ragab *et al.*, 1987; Sauer, 1993; Stanley-Samuelson, 1993) and is acted upon by PLA_2 (Stanley-Samuelson, 1993). The finding that the release of fatty acids in activated trophocytes can be blocked by PLA_2 inhibitors shows that this is the most likely origin of the fatty acids.

The release of fatty acids other than AA by PLA_2 is not unique to trophocytes. In rat platelets and human polymorphonuclear cells phospholipase A_2 acts equally to release arachidonic or oleic acid (Schalkwijk *et al.*, 1990). Human osteoarthritic synovial fluid PLA_2 also shows a similar lack of specificity for linoleic or arachidonic acid (Parks *et al.*, 1990). This suggests that trophocyte phospholipase A_2 is not especially specific for the fatty acids that it removes.

The major portion of the fatty acid complement of insects is represented by eight fatty acids consisting of the saturated fatty acids, myristic, palmitic and stearic acids, the monounsaturated fatty acids, myristoleic, palmitoleic and oleic acids, and the polyunsaturated fatty

acids, linoleic and linolenic acids (Downer, 1985). Palmitic, stearic, oleic and linoleic acids are the major fatty acids esterified to phospholipids in the American cockroach nymph (Kinsella, 1966). Arachidonic acid is also present in trace amounts in most insects, but usually represents less than 0.1% of the total phospholipid (Stanley-Samuelson, 1993). It is therefore not surprising that palmitic, stearic, oleic and linoleic acids can be detected but not AA.

Detection of arachidonic acid may have been limited by the small amount of tissue available. The fatty acids were routinely determined in 50,000-100,000 trophocytes but even samples of more than a million cells did not enable AA to be detected suggesting that indeed very little was present. AA has been demonstrated in lipid extracted from 1000 houseflies (Wakayama *et al.*, 1986a). AA comprised 0.04% of the total fatty acids in the phospholipid fraction in newly emerged males and 0.02% in each of the triacylglycerol and free fatty acid fractions. In tobacco hornworm hemocytes, only trace amounts of AA were detected in total lipid from the hemolymph of ten larvae (Ogg *et al.*, 1991). Using one thousand malpighian tubules from the same species Petzel *et al.* (1993) showed that 7.8 percent of the total lipid contained AA, of which 5.6 was in the phospholipid fraction. The detection of AA in the cockroach was made possible using sufficient starting material to provide 500 mg of total

Folch extracted lipid (Kinsella, 1966). From this extract only 0.65 percent was determined to be fatty acids consisting of chain length of 20-24 carbons. These studies are consistent with the failure to detect AA in the present study because of the small amount of tissue available.

The release from a membrane preparation of fatty acids having the same composition as that released in intact trophocytes suggests that their release from lipids in large storage vacuoles is unlikely. Since a large fraction of the membrane mass is composed of phospholipids, the release of fatty acids from the membranes supports the hypothesis that phospholipase A_2 is involved.

The ability of the trophocytes to synthesize AA from linoleic acid in the presence of hormone probably explains why large quantities of AA are not found esterified to phospholipids. Thus the trophocytes are able to generate AA on demand by synthesis rather than release from the membranes. For this reason large reserves of AA, which may be employed for signal transduction, are not found esterified to phospholipids in the fat body.

4.5 Control of free fatty acid concentration

4.5.1 Intracellular free fatty acids

The only fatty acids in the trophocytes whose levels were observed to change following treatment with either CC extract, CC I or CC II hormones were palmitic, stearic,

oleic and linoleic acids. A similar release of fatty acids has been noted in activated blood platelets where arachidonic, stearic and oleic acids are released (Broekman, 1986).

Free fatty acids are elevated in trophocytes treated with the synthetic hormones CC I and CC II. Of the four fatty acids released oleic acid is present in the highest concentration, followed by stearic and palmitic acids whose levels are similar, and linoleic acid with the lowest concentration. Quantitatively the release of fatty acids by the synthetic hormones and crude CC extract is similar, the only exception being that palmitic acid is not significantly elevated by crude CC extract. The inability of CC extract to increase intracellular palmitic acid suggests that this fatty acid may be utilized as quickly as it is released. Since CC extract has been shown to increase intracellular cAMP (Hanoaka and Takahashi, 1977; Gade, 1977; Orr *et al.*, 1985) whereas the hypertrehalosemic hormones do not (Orr *et al.*, 1985), the difference in palmitic acid levels may be due to a cAMP mediated process which is yet to be discovered in the fat body.

A maximal effect of crude hormone prepared from the CC was obtained with approximately 0.1 gland pair equivalents per ml. This suggests that the effect on cytosolic fatty acids may be physiologically significant, a view which is supported by the finding that 15 pmol/ml of CC II maximally

increases intracellular fatty acids. This amount of CC II appears to be within the range that could be provided by the corpus cardiacum (Scarborough *et al.*, 1984).

When trophocytes are treated with CC II the fatty acids reach a maximal level within 15 min following which the concentration continues to increase slowly for up to an hour, at which time the fatty acids attain their highest concentration. This suggests that an elevated level of lipase activity is maintained for at least 15 min.

4.5.2 Free fatty acids in the hemolymph

Injecting cockroaches with CC I and CC II increases fatty acid levels in the hemolymph. This appears to contradict the data showing that neither of the hormones promote the release of fatty acids from the trophocytes into the incubation medium in vitro. A possible reasor for this discrepancy is the absence of proteins in the incubation medium such as those described for the honey bee, *Apis mellifera* (Robbs *et al.*, 1985), which would bind the fatty acids. A significant effect on hemolymph fatty acids was obtained with the injection of 15 pmol of CC II whereas 100 pmol of CC I was required. This difference may be of physiological significance. It would be of interest to learn more about the possible physiological importance of the release of free fatty acids to the hemolymph.

4.6 Activation of PLA_2

The data showing that CC I promotes production of fatty acids and that this can be decreased by the action of ρ-bromophenacyl bromide is evidence that PLA_2 is activated by the hormone. This action by a hormone is by no means unique. Other examples include the action of bradykinin on the endothelial cells of porcine aorta (Kaya *et al.*, 1989), epidermal growth factor in renal mesanglial cells (Goldberg *et al.*, 1990), parathyroid hormone in the proximal tubules of rat kidneys (Ribeiro *et al.*, 1994), gonadotropin-releasing hormone acting on primary cultures of rat anterior pituitary cells (Chang *et al.*, 1988) and gamma-thrombin on rabbit platelets (Touqui *et al.*, 1989). These findings suggest that activation of phospholipase A_2 by hormones or similar factors occurs widely in animal tissues.

In several systems, phospholipase A_2 is activated by Ca^{2+} and this leads to the release of arachidonic acid from phospholipids (Irvine, 1982; Withnall, 1984; Baron and Limbird, 1988; Murakami, 1993; Lloret and Moreno, 1994; Faili *et al.*, 1994). There is good evidence that IP_3 is responsible for an increase in intracellular Ca^{2+} (Berridge and Fain, 1979). This hypothesis is consistent with the data for tick salivary gland phospholipase where the enzyme is activated by micromolar calcium with half-maximal activation occurring at 0.5 μM (Sauer *et al.*, 1993). However, the possibility that trophocyte phospholipase A_2 is

activated by Ca^{2+} is unlikely since treatment of the fat body with the calcium ionophore A23187 does not stimulate trehalose efflux (McClure and Steele, 1981) even though activation of phosphorylase, a calcium dependent mechanism, does occur. Since A23187 has been shown to activate phospholipase A_2 in several cell types (Sweatt *et al.*, 1986; Billah and Lapentina, 1982; Rittenhouse, 1984) its inability to stimulate trehalose efflux, which may require the release of fatty acids, suggests that calcium may not activate PLA_2 in trophocytes.

There are several examples of PLA_2 that do not employ calcium as an activator (Cao *et al.*, 1987; Hanzen *et al.*, 1990; Rao and Subrahmanyam, 1969). The tobacco hornworm PLA_2 appears to be either calcium-independent or requires very low levels of free calcium (Stanley-Samuelson, 1993). Activity of this PLA_2 is dependent on homogenate protein concentration, time, temperature and pH (Stanley-Samuelson, 1993). The proteins responsible include the GTP-binding G-proteins and/or, receptors on the cell membranes. Although several examples of receptor-mediated activation of PLA_2 are known (Sweatt *et al.*, 1986; Garcia *et al.*, 1994) there is no substantive evidence to show that activation of PLA_2 in trophocytes is by means of a receptor mediated mechanism. Nevertheless, the model remains an attractive possibility.

4.7 Role of free fatty acids in trehalose efflux

The stimulation of trehalose efflux by fatty acids suggests that they are part of the hormonal mechanism that controls trehalose efflux. Inhibition of hormone activated trehalose efflux by mepacrine supports the idea that PLA_2 is involved. This view is based on the knowledge that mepacrine is an inhibitor of PLA_2 (Fan, 1994; Jancinova *et al.*, 1994; Schaad *et al.*, 1987; Chang *et al.*, 1987) as is ρ-bromophenacyl bromide (Bernard *et al.*, 1994; Hargreaves, 1994). The results confirm that treatment of the trophocytes with the PLA_2 inhibitors leads to a decrease in the concentration of free fatty acids and that this is accompanied by a reduction in trehalose synthesis. Conversely, stimulation of trehalose synthesis by the phospholipase A_2 activator melittin increases the concentration of free fatty acids and coincident with this an increase in trehalose efflux.

The failure of palmitic acid to increase trehalose efflux suggests that stimulation of trehalose efflux is dependent on either fatty acid chain length (C_{18} or longer) or the presence of one or more double bonds. Some systems are affected by carbon chain length, whereas, others are dependent on the degree of unsaturation of the fatty acid. Stimulation of Cl^{-1} transport in rat pancreatic secretory granules by fatty acids is dependent on carbon chain length and, to a lesser extent, on the degree of unsaturation

(Gasser and Holda, 1994). An increase in intracellular Ca^{2+} in thyroid cells by fatty acids, on the other hand, is dependent on the degree of unsaturation. The unsaturated fatty acids, oleic and arachidonic acid, stimulate intracellular Ca^{2+} levels, whereas, long chain saturated fatty acids do not (Ekokoski *et al.*, 1994).

The cause of the stimulatory effect of fatty acids on trehalose efflux by fatty acids is not known. Several studies suggest that oxidation of lipid is necessary to provide the energy for trehalose synthesis. Trehalose synthesis requires uridine 5'-diphosphoglucose (UDPG) as a glucosyl donor (Candy and Kilby, 1961). Because the sum of UDPG and its degradation product UDP remains constant UDPG must be regenerated from UDP. This requires ATP. The source of energy utilized for the generation of ATP for this purpose has yet to be determined, although several studies suggest that it may be lipid. The increase in lipid oxidation (McDougall and Steele, 1988) and the decrease in the respiratory quotient of the fat body *in vitro* following the addition of aqueous extracts of *corpora cardiaca* (Wiens and Gilbert, 1967) point to this reserve. A decrease in glucose utilization with a concomitant decrease in glycolysis following treatment with CC extract also suggests that lipid is the source of energy. The demonstration that hormonal stimulation of trehalose efflux could be inhibited by the ß-oxidation inhibitor pent-4-enoic acid (PEA) also

suggests that fatty acid oxidation may be the source of energy for trehalose synthesis (McDougall and Steele, 1988).

Because of its size trehalose diffuses through membranes very slowly (Knowles, 1975). It is interesting therefore to speculate on the possibility that fatty acids may play a role in the transport of trehalose across the membrane. Because the increase in fatty acids in the trophocytes follows a pattern which is similar to the efflux of trehalose from the cell it is of interest to consider that these two events may be related. There are, for example, several fatty acids which affect membrane fluidity. Cis-unsaturated fatty acids, including linoleic and arachidonic acids, have been shown to increase membrane fluidity in platelets (Kitigawa, 1985). It is possible, therefore, that these fatty acids change the fluidity of membranes making them more permeable to trehalose and thereby facilitating its release from the cell.

It seems unlikely that energy is required to move trehalose out of the cell because of an unfavourable concentration gradient. Using a value of 30 μm for the diameter of the trophocyte, the concentration of intracellular trehalose in the resting cell is estimated to be 19.1 mM and increases to 28.3 mM in hormone challenged cells. Since the concentration of trehalose in the medium was found to be approximately 6 mM it seems clear that trehalose is moving down the concentration gradient out of

the cell. This conclusion is supported by the study of Jungreis and Wyatt (1972) who showed that the concentration of trehalose in the fat body was significantly higher than that in the hemolymph. This also suggests that efflux of trehalose occurs by diffusion down the concentration gradient.

An interesting possibility is that fatty acids and not IP_3 are responsible for the increase in Ca^{2+} uptake into hypertrehalosemic hormone treated trophocytes. Fatty acids have been shown to increase intracellular Ca^{2+} in thyroid cells (Ekokoski *et al.*, 1994). Treatment of these cells with the unsaturated fatty acids, linoleic and arachidonic acid, led to a dose-dependent increase in intracellular Ca^{2+}. An effect of fatty acids on Ca^{2+} flux through membranes has also been demonstrated by Utsumi *et al.*(1985), who showed that unsaturated fatty acids, arachidonic, linolenic, linoleic and oleic acids stimulated Ca^{2+} uptake into liposomes. A dose-dependent increase of intracellular Ca^{2+} by oleic acid was also demonstrated in alveolar epithelial monolayer cells in rats (Wang *et al.*, 1994). Intracellular calcium in these cells was also increased by stearic, linoleic and linolenic acids. These fatty acids include those that stimulate trehalose efflux from trophocytes thus suggesting a link with Ca^{2+}. This could explain the activation of phosphorylase and the subsequent synthesis of trehalose. Although the increase in IP_3 in

hypertrehalosemic hormone treated trophocytes has been linked to the influx of Ca^{2+} (Brown, 1990), there are no data to show that both events are causally linked. It will therefore be of interest to know whether the Ca^{2+} influx could be due to the increase in concentration of fatty acids in the hormone challenged fat body.

4.8.1 Control of phosphorylase activity

The rate-limiting step in the pathway between glycogen and the formation of trehalose stimulated by hypertrehalosemic hormones is the step catalysed by phosphorylase (Steele and Hall, 1985). Numerous studies have shown that the hypertrehalosemic hormones activate phosphorylase and that this results in the stimulation of trehalose efflux (Steele, 1963; Wiens and Gilbert, 1967; Goldsworthy, 1970). The results of the present study are in agreement with the earlier work and show that both crude and synthetic hypertrehalosemic hormones more than double the activity of glycogen phosphorylase in trophocytes.

The most notable conclusion to be drawn from the data in Table 7 is an apparent correlation between an increase in intracellular free fatty acids and an increase in the activity of the phosphorylase. It is well established that mammalian adipose tissue responds to various hormones, including glucagon, epinephrine, ACTH and vasopressin, by activating lipolysis and consequently increasing fatty acid production (Rizack, 1961; Vaughan *et al.*, 1964; Steinberg

and Khoo, 1977). Although this would seen to be analogous to the situation in the cockroach fat body the evidence does not support this view. The activation of hormone-sensitive lipase has been shown to be mediated by a cyclic AMP-dependent protein kinase mechanism (Steinberg and Khoo, 1977); Rizak, 1964). Since neither CC I and CC II, both of which increase the production of free fatty acids have the ability to stimulate cyclic AMP synthesis in the fat body (Orr *et al.*, 1985) this system cannot be implicated in the activation of the lipolytic system.

Interestingly, the same hormones that increase lipolytic activity in rat adipose tissue also activate phosphorylase (Vaughan, 1960; Lawrence and Larner, 1977) although these authors suggest that any relationship between lipolysis and phosphorylase activation is an indirect one. Concomitant activation of phosphorylase and lipolytic activity can be demonstrated in the locust. The fat body of this species contains a lipase that hydrolyses tri- and diacylglycerol and for which evidence has been presented to show that it is activated by a cyclic AMP dependent protein kinase system (Pines and Applebaum, 1978; Pines *et al.*, 1981). AKH, in addition to its activation of fat body lipase, also activates phosphorylase (Van Marrewijk *et al.*, 1983). Despite these similarities in the hypertrehalosemic hormones of the cockroach it seem unlikely that activation of phosphorylase in that species can be explained by a

similar mechanism since, as already noted, neither CC I nor CC II appears able to stimulate the synthesis of cyclic AMP (Orr *et al.*, 1985).

The effectors that increase the activity of phosphorylase in the unchallenged tissue and increase the fatty acid concentration (Table 7) each have a different mode of action. Melittin activates PLA_2 directly (Malloy and Keirl, 1974; Choi *et al.*, 1992), NDGA is an inhibitor of lipoxygenase (Needleman *et al.*, 1986), indomethacin and diclofenac are inhibitors of cyclooxygenase (Schaad *et al.*, 1987), and both the crude and synthetic hypertrehalosemic hormones are believed to act via the phosphatidylinositol system (Brown, 1990). Of all the effectors tested only ρ-bromophenacyl bromide failed to increase the basal activity of phosphorylase. Not surprisingly, the inhibitor of PLA_2 also failed to increase the level of free fatty acids in the trophocytes. These results clearly suggest that an increase in free fatty acids could be a signal that initiates the activation of phosphorylase. When these effectors were tested against tissue that had been challenged with natural or synthetic hormone very different results were obtained. Neither BPB, NDGA nor indomethacin had an effect on phosphorylase activation due to natural or synthetic hypertrehalosemic hormone (Table 7). This was consistent with the observation that the same inhibitors blocked the increase in fatty acids that normally accompanies the action

of the hormone (Table 7). Melittin, however, in contrast to the other agents tested had an inhibitory effect on the activation of phosphorylase by the hormone (Table 7). Whether this is due to an over-production of fatty acids is not known. All of these results support the idea that activation of phosphorylase is a consequence of an increase in fatty acid levels.

The action of melittin as an inhibitor of phosphorylase activation is interesting because of its stimulatory effect on fatty acid accumulation. Because fatty acids increase Ca^{2+} accumulation in thyroid cells (Ekokoski *et al.*, 1994) and because there is also evidence that the hypertrehalosemic hormones increase intracellular Ca^{2+} (McClure and Steele, 1981) it is possible that both agents acting together might produce unusually high levels of intracellular Ca^{2+} resulting in inhibition of phosphorylase kinase in contrast to the activation that occurs at low concentrations as demonstrated by Ashida and Wyatt (1979).

Activation of phosphorylase by indomethacin was at least as effective as that obtained with hormone preparations but markedly less than that obtained with NDGA. Since the increase in fatty acids provoked by each inhibitor was similar there is no apparent reason for the greater activation of phosphorylase obtained with indomethacin. A possible cause is that as in rabbit uterus, indomethacin might inhibit the phosphodiesterase responsible for the

hydrolysis of cyclic AMP (Zor *et al.*, 1976) thus allowing the cyclic nucleotide to accumulate. This would explain the additional activation of phosphorylase since it has been shown that cyclic AMP activates phosphorylase in cockroach fat body (Steele, 1964).

Phosphorylase activation in cockroach fat body by either of the hypertrehalosemic hormones requires Ca^{2+} in the extracellular medium bathing the fat body (McClure and Steele, 1981). Similarly, in rat liver and adipose tissue, epinephrine, nor-epinephrine, vasopressin and angiotensin have been shown to cause formation of phosphorylase a but without an increase in cyclic AMP or the activation of cyclic AMP-dependent protein kinase, or covalent activation of phosphorylase kinase (Keppens and DeWulf, 1975, 1976: Lawrence and Larner, 1977). The evidence strongly supports the idea that phosphorylase kinase is activated directly by Ca^{2+} (Khoo and Steinberg, 1975; Shimazu and Amokawa, 1975). Although a role for fatty acids as a mediator of Ca^{2+} uptake into trophocytes is still speculative, the relationship between fatty acid levels and phosphorylase activity makes the hypothesis an attractive one, especially since linoleic acid and arachidonic acid have been shown to increase Ca^{2+} uptake into thyroid cells (Ekokski *et al.*, 1994).

4.9 Control of trehalose-6-phosphatase activity

The removal of phosphate from trehalose-6-phosphate is

an important reaction that is likely to determine the rate at which trehalose is released from the cell. This study shows that trehalose-6-phosphatase is inhibited by oleic, linoleic and arachidonic acids, the inhibition being positively correlated with degree of unsaturation of the fatty acids. The saturated fatty acids, palmitic and stearic were without effect on the enzyme. This result is similar to that obtained for rat liver microsomal glucose-6-phosphatase which is inhibited by linoleic and linolenic acids whereas palmitic, stearic and elaidic acid are without effect (Hill *et al.*, 1983). The concentration at which the fatty acids are most inhibitory to the microsomal glucose-6-phosphatase is approximately 18 μM, a Ki value which is similar to the value of 13.3 μM obtained for trophocyte trehalose-6-phosphatase using linoleic acid as the inhibitor. The regulation of both enzymes by fatty acids would thus be expected to have similar effects on sugar efflux, and also on the accumulation of G6P in liver and T6P in trophocytes.

The data suggest that fatty acids may be important regulators of trehalose synthesis. At low concentration the fatty acids have a stimulatory effect on trehalose efflux from trophocytes but as the concentration approaches 30 μM, particularly that of linoleic or arachidonic acid, they become inhibitory. These concentrations are close to the Ki value for the inhibition of trehalose-6-phosphatase by

linoleic acid and are those that would be expected to occur under biological conditions. When the concentration of linoleic and arachidonic acids is increased to 100 μM the efflux of trehalose decreases below the control values. The decrease in trehalose efflux may be attributed to the inhibition of trehalose-6-phosphatase.

In general most enzymatic reactions are affected by their products whereby an accumulation of end-product leads to feed-back inhibition of the enzymes responsible for synthesis of the product. In *Hyalophora cecropia* fat body trehalose has a significant inhibitory effect on the trehalose-6-phosphate synthase reaction (Murphy and Wyatt, 1965). Furthermore the same authors demonstrated that T6P was equally inhibitory, if not more so, to the T6P synthase reaction. Thus the action of trehalose as a feedback inhibitor of the T6P synthase reaction, acting in concert with the inhibitory effect of the fatty acids, constitutes a powerful mechanism for the regulation of trehalose efflux from the fat body.

4.10 Origin of arachidonic acid

Several pieces of evidence suggest that arachidonic acid is a fatty acid that would serve more than one purpose in hormonal control of trehalose production. The likelihood that it plays a primary role is indicated by the fact that it stimulates trehalose production by the trophocytes.

Also, inhibitors that normally block AA metabolism have a marked effect on trehalose efflux. The failure to detect arachidonic acid does not rule out the involvement of this fatty acid in the trehalose efflux mechanism. The inability to detect the fatty acid is probably because arachidonic acid is present in very low concentrations (often < 0.1% of phospholipid fatty acids; Stanley-Samuelson, 1993). Since stearic, oleic and linoleic acids stimulate trehalose efflux in a manner similar to arachidonic acid, it is postulated that these fatty acids may be converted to AA, and that the metabolism of AA gives rise to active metabolites. The demonstration in this study that linoleic acid can be converted to AA by fat body challenged with CC I suggests that this hypothesis may be correct. The detection of AA in cells incubated with linoleic acid, challenged with CC I and treated with indomethacin is most likely because AA is ordinarily utilized as quickly as it is synthesized. The accumulation of arachidonic acid in CC I challenged fat body which had been pre-incubated with indomethacin but not in fat body incubated with indomethacin alone, suggests that the hormone increases the availability of precursors for the synthesis of AA. The accumulation of AA in indomethacin treated fat body suggests that AA is metabolized via the cyclooxygenase pathway, and that inhibition of this enzyme is responsible for the accumulation of the fatty acid.

The conversion of linoleic acid into arachidonic acid

also suggests that the cockroach can convert palmitic, stearic and oleic acids to arachidonic acid since each of these acids can be converted to linoleic acid (Stanley-Samuelson *et al.*, 1988). The data offer good evidence that the cockroach can convert linoleic acid into arachidonic acid and that the rate of its formation is increased by the hypertrehalosemic hormones. Hormonal stimulation of arachidonic acid synthesis from linoleic acid is novel. This newly discovered activity may also occur in other insect species which have low arachidonic acid content in the membrane phospholipids and have the ability to convert fatty acids such as oleic and linoleic acids to arachidonic acid.

4.11 The role of arachidonic acid metabolites

Inhibition of hormone activated trehalose efflux by the cyclooxygenase and lipoxygenase inhibitors suggests that metabolites of arachidonic acid may be involved in the activation mechanism. The demonstration that synthesis of arachidonic acid can be synthesized from linoleic acid also supports this argument as does the accumulation of arachidonic acid in indomethacin treated tissue. The finding that exogenous prostaglandin $F_{2\alpha}$ stimulates trehalose efflux supports this concept, particularly since Murtaugh and Denlinger (1982) have shown PGE_2 and $PGF_{2\alpha}$ in the cockroach. The evidence therefore supports the idea

that prostaglandins may be important in hormonal activation of trehalose efflux.

This study shows that stearic, oleic and linoleic acids stimulate trehalose efflux from trophocytes. Since these fatty acids could give rise to arachidonic acid the inhibition of hormone stimulated trehalose efflux by cyclooxygenase and lipoxygenase inhibitors suggests that metabolites of arachidonic acid rather than the fatty acid itself may be the activator of trehalose efflux. If this interpretation is correct inhibition of arachidonic acid oxidation should lead to an accumulation of fatty acids. This was found to occur in trophocytes pre-incubated with indomethacin and challenged with CC I. Similar results were obtained with trophocytes challenged with CC II. These results support the hypothesis that arachidonic acid originating from fatty acids derived from membrane phospholipids is the source of metabolites which play a role in the stimulation of trehalose efflux.

The trophocytes contain products which can be detected by their absorbance at 192 nm. These substances are increased significantly in trophocytes challenged with the hypertrehalosemic hormones but inhibited by indomethacin, a cyclooxygenase inhibitor (Wakayama *et al.*, 1986c; Wagner *et al.*, 1995; Wasner *et al.*, 1994), suggesting that these products may arise as a consequence of cyclooxygenase activity. Formation of these products in hormone challenged

trophocytes treated with BPB, an inhibitor of PLA_2 (Hargraves *et al.*, 1994; Bernard *et al.*, 1994) was also blocked, suggesting that the unknown substances are also dependent on phospholipase A_2 activity. This is additional support for the idea that the unknown substances may be of phospholipid origin. The products elute from the HPLC in the regior. where prostaglandins appear (Henke, *et al.* 1984; Kuksis, 1987), thus attenuation of the peaks by inhibitors of fatty acid release (BPB) and metabolism (indomethacin), suggests that the unknown substances may be prostaglandins or similar substances. The stimulation of trehalose efflux from trophocytes which had been challenged with the material eluted from the HPLC further supports the notion that the eluted material is prostaglandin-like. Stimulation of trehalose efflux by $PGF_{2\alpha}$ also supports the idea that this may be one of the metabolites. The loss of biological activity by heating the active material to 100°C (data not shown) supports the hypothesis that the active material could be prostaglandin-like, since prostaglandins are also heat sensitive (Srivastava and Clausen, 1973).

The weak effect of acetylsalicylic acid on hormonal stimulation of trehalose efflux when compared to the much stronger effect on PG biosynthesis in the spermatophore of the cricket, *Teleogryllus commodus* (Tobe and Loher, 1983) suggests that there are important species differences in cyclooxygenase activity. Wakayama *et al.* (1986) also found

that several anti-inflammatory drugs, naproxen, acetaminophen, aspirin and indomethacin were all effective PG synthase inhibitors in house fly. Indomethacin, however, did not inhibit PG synthesis in the preparation from male reproductive tracts of the house cricket (Destaphano *et al.*, 1974). Stanley-Samuelson and Ogg (1994) has hypothesized that differential sensitivities to the same inhibitors indicate that the PG synthase systems substantially differ among animals and that putative inhibitors should be characterized for each system.

It would have been predicted that the lipoxygenase inhibitor NDGA would increase fatty acid levels in trophocytes treated with CC I. Instead they were significantly decreased. This is opposite to that which would have been expected had the inhibitor been specific in its action on lipoxygenase. The significant inhibition by NDGA of fatty acid release suggests that it may have blocked the release of fatty acids by inhibition of PLA_2 itself in addition to any inhibition of lipoxygenase that it might have had. NDGA has been shown to inhibit PLA_2 in intervertebral disc in humans (Carabaza *et al.*, 1993) and in zymosan-stimulated mouse peritoneal macrophages (Balisinde *et al.*, 1992). The effect of NDGA on trehalose efflux might therefore be more attributable to inhibition of PLA_2 rather than lipoxygenase.

The results of this study could be interpreted to mean

that prostaglandins or other eicosanoids stimulate trehalose synthesis rather than efflux. This conclusion is supported by work showing that prostaglandin E_2, $F_{2\alpha}$ and D_2 stimulate glycogen phosphorylase in rat liver (Haussinger *et al.*, 1988) or that phosphorylase is activated in rat liver by an increase in intracellular Ca^{2+} in response to prostaglandins (Gomez-Foix, 1989). In trophocytes, the activation of glycogen phosphorylase is also dependent on calcium, which likely follows an increase in IP_3 in response to the hypertrehalosemic hormones (Brown, 1990). The fact that the ability of the hormones to activate phosphorylase is not affected by BPB or indomethacin, suggests that fatty acids, and fatty acid metabolites, are not involved in the activation of phosphorylase. The prostaglandins, therefore, must have their site of action elsewhere.

Stimulation of trehalose efflux by prostaglandin $F_{2\alpha}$ suggests that it can activate the entire cascade of events that include synthesis and release of trehalose. However, this may be more apparent than real since the same effect could be achieved by a change in the permeability of the trophocyte membrane alone. Prostaglandins are known to be involved in glucose uptake stimulated by insulin (Wasner *et al.*, 1994). Glucose uptake differs from the release of trehalose in that the direction of transport is in the opposite direction. The important point, however, is that although glucose transport through the membrane is

facilitated by glucose transporters the transport of trehalose, which is significantly less permeable than glucose (Knowles, 1975), may make use of a similar mechanism. Interestingly, the uptake of glucose in rat adipocytes is significantly inhibited by indomethacin (Wasner *et al.*, 1994), a situation which is reflected by the trophocytes where treatment with indomethacin leads to a significant reduction in trehalose efflux.

Although the possibility that trehalose is transported by a mechanism similar to that for glucose is not certain there is reason to believe that the release of trehalose is dependent on changes in the permeability of the membrane. Jungreis and Wyatt (1972) have shown that the permeability of silkmoth pupa fat body membranes to trehalose is dependent on the condition and stage of development of the fat body. It is interesting therefore that prostaglandins $F_{2\alpha}$ have been shown to increase the fluidity of mouse liver microsomal membranes during pregnancy (Dave *et al.*, 1983). Also, prostaglandin E_1 fluidizes membranes isolated from rat brain (Heinonen *et al.*, 1982). The effect of prostaglandin is to fluidize the surface and intermediate lipid layers of the synaptosomal membranes. Prostaglandins have also been shown to increase endometrial vascular permeability in the rat uterus (Kennedy, 1983). The release of trehalose in the hormone stimulated trophocytes may, therefore, be facilitated by prostaglandin-like metabolites produced by

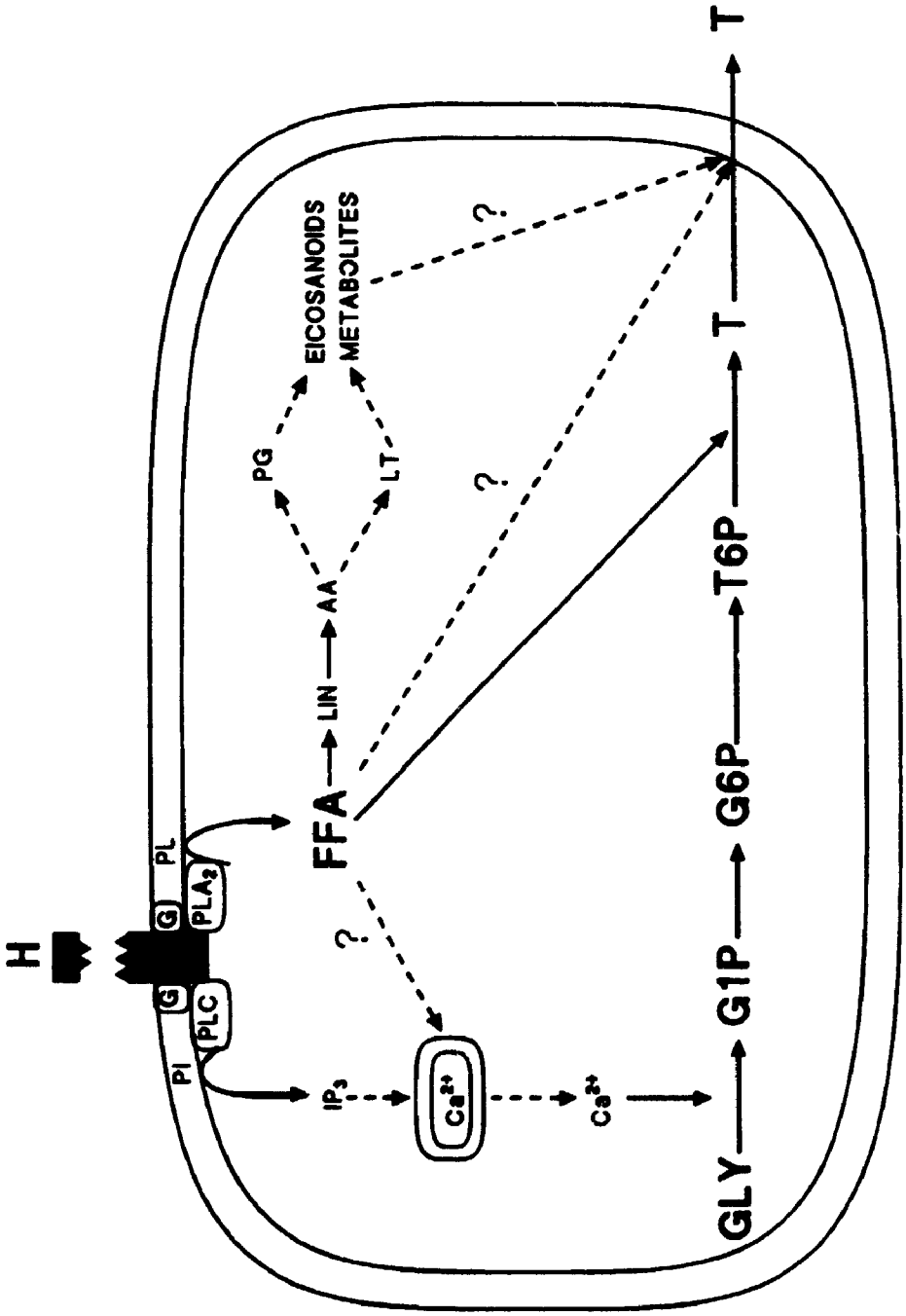
the trophocytes.

4.12 Model for hypertrehalosemic hormone stimulated trehalose efflux mechanism.

The model in Fig. 46 suggests that the hypertrehalosemic hormones act at a single receptor to activate two distinct pathways. These are the phosphatidyl inositol system which increases intracellular Ca^{2+} and the PLA_2 system associated with the plasma membrane. The latter system when activated by hormones increases the concentration of unsaturated fatty acids which are postulated to control the activity of trehalose 6-phosphate and to give rise to eicosanoids which may be important in regulating trehalose permeability of the plasma membrane.

Figure 46. A model to describe the action of hypertrehalosemic hormone in *Periplaneta americana* trophocytes.

Abbreviations used are, AA, arachidonic acid; CC, hypertrehalosemic hormone; FFA, free fatty acids; G, GTP-binding protein; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; GLY, glycogen; IP_3, inositol trisphosphate; LIN, linoleic acid; LT, leukotrienes; PG, prostaglandins, PLA_2, phospholipase A_2; PLC, phospholipase C; T, trehalose; T6P, trehalose-6-phosphate.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Part 5
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