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INHIBITION OF TUMOUR-INDUCED LIPOLYSIS *IN VITRO* AND CACHEXIA AND TUMOUR GROWTH *IN VIVO* BY EICOSAPENTAENOIC ACID

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Abstract—Stimulation of lipolysis in murine adipocytes in response to a lipid-mobilizing factor produced by a cachexia-inducing murine adenocarcinoma was inhibited by eicosapentaenoic acid (EPA) with a K_i value of 104 μM. The inhibitory effect was strictly structurally specific, since other related fatty acids of both the (n-3) and (n-6) series were ineffective as inhibitors of the lipolytic process. Induction of lipolysis by both salbutamol and ACTH was also inhibited by EPA, suggesting that the effect is exerted on a step central to the process of lipolysis. Lipolysis induced with the tumour lipid-mobilizing factor was associated with a prolonged elevation of the intracellular level of cyclic AMP in adipocytes, in contrast with ACTH and salbutamol. The elevation of adipocyte cyclic AMP in response to the tumour lipid-mobilizing factor and lipolytic hormones was inhibited by EPA. *In vivo*, administration of pure EPA to weight losing mice bearing the MAC16 adenocarcinoma completely prevented weight loss and tumour growth rate. In contrast both the other (n-3) fatty acid present in fish oil, docosahexaenoic acid (DHA), and linoleic acid were ineffective in inhibiting weight loss or the growth of the MAC16 tumour. This suggests that inhibition of tumour lipolytic activity accounts for the anticachectic effect of EPA, and that a correlation may exist between the inhibition of cachexia and the inhibition of tumour growth.

In patients with cancer, weight loss indicates a poor prognosis and a shorter survival time [1]. Numerous metabolic studies have indicated that weight loss associated with cancer cachexia is different from that in simple starvation [2] and although anorexia may be apparent this often develops once weight loss is established [3]. Also in general, weight loss is not effectively reversed by parenteral nutrition [4]. In addition an increased lactate production has been demonstrated in tumour-bearing patients receiving parenteral nutrition [5], suggesting increased metabolic activity with the tumour in response to nutrient uptake.

In order to effectively reverse the cachetic state it is important to know the mechanism by which catabolism of host body tissues take place in the presence of certain tumours. Intervention can then occur at the cellular level in order to overcome the catabolic process. Using a murine model of cancer cachexia (MAC 16) we have demonstrated the presence of circulatory catabolic factors capable of stimulating lipolysis in adipose tissue [6]. Several clinical investigators have noted an increased FFA mobilization in cancer patients before weight loss is observed [7], and we have also demonstrated an enhanced lipid mobilizing activity associated with both tumour and body fluids of patients with clinical cancer cachexia [8]. Chromatographically this material behaves identically to that in the murine model. This material differs from the natural polypeptide hormones in both charge and molecular weight, although it responds to normal metabolic regulation by both insulin and 3-hydroxybutyrate [6]. Both of these agents reduce the extent of weight loss *in vivo* [9, 10]. However, we have recently shown that cachexia in the MAC16 model is more effectively reversed by a diet in which a high proportion of the calories are derived from fish oil, which is rich in the polyunsaturated fatty acids eicosapentaenoic acid, 20:5 (n–3) (EPA) and docosahexaenoic acid, C22:6 (n–3) (DHA) [11]. If the tumour-derived lipid-mobilizing factor is important in the cachectic process this suggests that these essential fatty acids may also interfere with the catabolic activity.

In the present investigation we have determined the effect of the tumour lipid-mobilizing factor on cyclic AMP levels in epididymal adipocyes and the effect of polyunsaturated fatty acids on both lipid-mobilizing activity and cyclic AMP metabolism. In addition, we have tested the antitumour and anti-cachectic activities of the individual polyunsaturated fatty acids, using the MAC16 model *in vivo*.

MATERIALS AND METHODS

Animals. Pure strain NMRI mice were obtained from our own inbred colony, and were fed a rat and mouse breeding diet (Pilsbury Ltd, Birmingham, U.K.) and water *ad lib*. Fragments of the MAC16 tumour were implanted into the flank of female mice (20 g) as previously described [6, 12] and therapy was initiated 10–12 days after transplantation when the tumours became palpable and weight loss had started to occur. This point was chosen to ensure complete tumour take and weight loss. The average tumour volume on initiation of therapy was 96 ± 12 mm^3 and the average weight loss was 5%.

Chemicals. Linoleic acid and DHA (both 99% pure) were purchased from the Sigma Chemical Co. (Poole, U.K.), and EPA (95% pure) was purchased from Peninsula Laboratories Europe Ltd (Merseyside, U.K.).

Preparation of lipid-mobilizing activity from the MAC16 tumour. MAC16 tumours were obtained from mice that had lost up to one-third of their original body weight, and were homogenized at 4° in Krebs–Ringer buffer at a concentration of 0.2 g/mL. The homogenate was then centrifuged for 10 min at 600 *g* to remove debris, and the supernatant was fractionated using Sephadex G150, Biogel P4 and hydrophobic chromatography using a C18 column, and used for lipolysis studies [13].

Test for inhibitory activity. Adipocytes were prepared from finely chopped epididymal fat pads of MRI mice by incubation with a collagenase solution (2 mg/mL) in Krebs–Ringer bicarbonate buffer, pH 7.2, for 2 hr at 37°. The Krebs–Ringer buffer also contained 0.55 mM D-glucose and 30 g/L bovine serum albumin, which were added fresh on the day of the experiment. Adipocytes were then washed three times with the Krebs buffer and incubated with or without the potential inhibitor at a concentration of adipocytes of 1.5 to 2.0 × 10^5 cells/mL. The effect of the inhibitor on both basal and stimulated lipolysis was determined in each case. The adipocyte mixture was gassed for 2 min with 95% O_2: 5% CO_2 mixture, mixed and incubated for 2 hr at 37°, after which time the glycerol content of 0.5 mL samples was determined by the method of Wieland [14]. Control samples containing adipocytes alone were also analysed to measure any spontaneous glycerol release.

Glycerol determination. To 0.5 mL of the incubation buffer was added 0.5 mL of 10% (w/v) perchloric acid and the mixture was shaken to ensure total deproteinization. The precipiated protein was sedimented by centrifugation (450 *g*, 10 min); the supernatant was aspirated, neutralized with KOH and the insoluble potassium perchlorate sedimented by centrifugation. The glycerol content of the supernatant was determined enzymatically by the conversion of NADH into NAD measured spectrophotometrically at 340 nm [14].

Determination of cyclic AMP. Adipocytes (1 mL; 10^5/mL) were incubated for 10 min at 37° with various additions. The reaction was terminated by the addition of the mixture to 1 mL of cold 5% trichloroacetic acid, mixed, and after the insoluble material was sedimented the supernatant was extracted five times with water-saturated ether. The aqueous phase was lyophilized and dissolved in 0.2 mL of the assay buffer. The concentration of cyclic AMP was determined with a commercial assay kit (Amersham International, Amersham, U.K.).

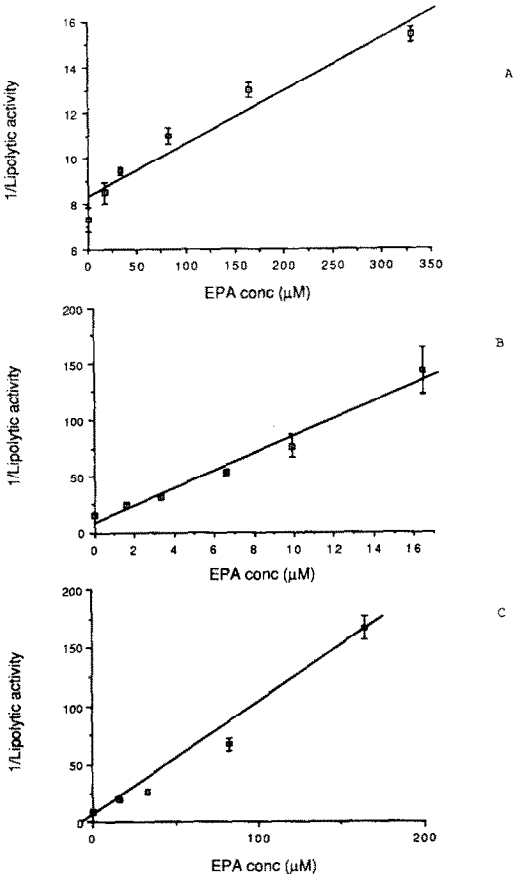

Fig. 1. Dixon plots for the inhibition of lipolytic activity isolated from the MAC16 tumour (50 μL; 1 μL caused the release of 1.6 nmol glycerol/10^5 adipocytes) (A), salbutamol, 0.165 mM (B) and ACTH, 25 I.U. (C) by EPA. MAC16 lipolytic activity is expressed as μmol glycerol released from murine epididymal adipocytes per hr per mg tumour protein while that induced by salbutamol and ACTH is expressed as μmol glycerol released per 10^5 adipocytes per hr (A). Results are expressed as mean ± SE and the number of experiments performed was three to four.

RESULTS

Absolute structural specificity was shown in the inhibition of lipid-mobilizing activity extracted from the MAC16 tumour by a range of unsaturated fatty acids. Thus, while EPA was effective in inhibiting lipolytic activity (Fig. 1A), other fatty acids of the (n–3) series including docsahexaenoic, (DHA), linolenic, octadecatectraenoic and *trans* 3- and *trans* 2-hexenoic acids were ineffective at concentrations up to 0.3 mM. Unsaturated fatty acids of the (n–6) series including linoleic, eicosatrienoic, arachidonic and 3-octenoic acid were also ineffective at concentrations up to 0.3 mM as was palmitoleic acid. EPA was also effective in inhibiting the lipolytic effect of salbutamol (Fig. 1B) and ACTH (Fig. 1C) and the effective inhibitory concentration for both salbutamol (IC_{50} 16.5 μM) and ACTH (IC_{50} 41 μM) mediated lipolysis were similar to that for the tumour lipolytic factor (IC_{50} 40 μM). These results suggest that EPA is a general inhibitor of lipolytic stimuli, and further that the inhibitory effect is highly structurally specific, and may be exerted either at the level of binding of the lipolytic factor to the receptor on adipocytes or in the production of a second messenger.

Lipolysis in adipocytes induced by lipolytic hormones is thought to be mediated via an elevation in intracellular cyclic AMP and subsequent activation of a protein kinase, which then activates an inactive form of triglyceride lipase [15]. The results in Fig.

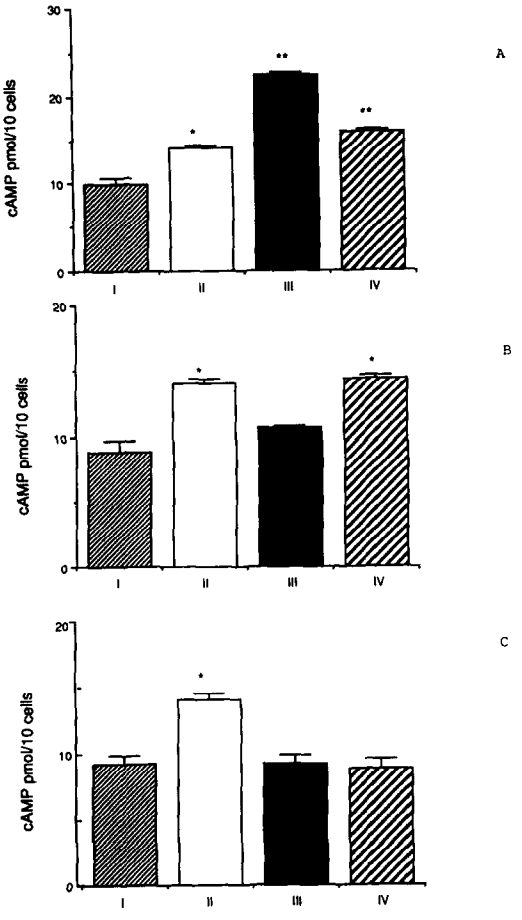

Fig. 2. Level of cyclic AMP in epididymal adipocytes and incubation medium 10 min (A), 1 hr (B) and 2 hr (C) in control cells (I), cells treated with 50 μL of the MAC16 tumour lipolytic factor (II) (1 μL caused the release of 1.6 nmol glycerol/10^5 adipocytes) 25 I.U. ACTH (III) and 0.165 mM salbutamol (IV). Results are expressed as means ± SE and are the average of three to four experiments. *P < 0.05, *P < 0.001 compared with control cells.

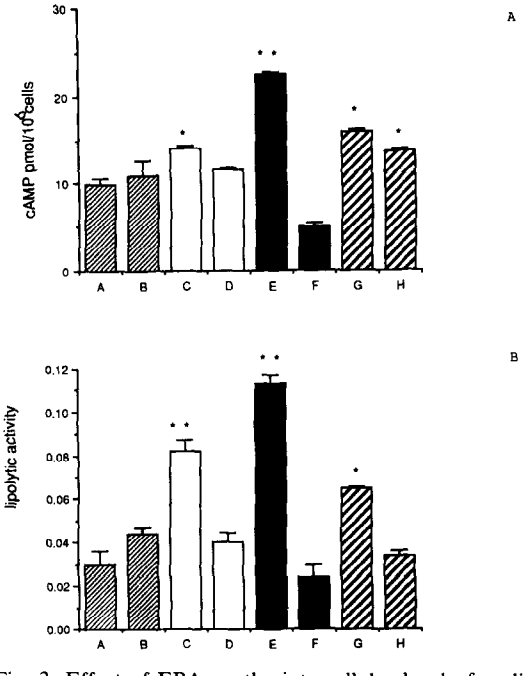

Fig. 3. Effect of EPA on the intracellular level of cyclic AMP (A) and the induction of lipolysis (B) in epididymal adipocytes after treatment with the MAC16 tumour lipolytic factor, ACTH or salbutamol. The results are expressed as mean ± SE and are the average of three to four experiments. Measurements of cyclic AMP levels were made after 10 min incubation, while glycerol release was measured after 2 hr and is expressed as μmol glycerol per 10^5 adipocytes per incubation. (A) Control cells; (B) 330 μM EPA; (C) MAC16 tumour lipolytic factor (50 μL); (D) MAC16 tumour lipolytic factor (50 μL) + 330 μM EPA; (E) 25 I.U. ACTH; (F) 25 I.U. ACTH + 330 μM EPA; (G) 0.165 mM salbutamol; (H) 0.165 mM salbutamol + 330 μM EPA. *P < 0.05, **P < 0.001 compared with control cells.

2A show an elevation in the intracellular level of cyclic AMP in murine epididymal adipocytes 10 min after treatment with the MAC16 lipid-mobilizing factor, which was of a comparable magnitude to that induced by 0.165 mM of the β-adrenergic agonist salbutamol, but was less pronounced than that produced by 25 I.U. of ACTH. The increase in cyclic AMP produced by the three agents paralleled their ability to stimulate glycerol release from adipocytes (Fig. 3B). The effect of the tumour lipolytic factor on cyclic AMP production differed from that induced by salbutamol and ACTH in that production continued for up to 2 hr of incubation, whereas the stimulatory effect of ACTH was not evident at 1 hr (Fig. 2B) and the effect of salbutamol had disappeared by 2 hr (Fig. 2C). Both glycerol release from adipocytes and the increase in intracellular cyclic AMP in response to the tumour lipolytic factor, salbutamol and ACTH were effectively inhibited by EPA (Fig. 3A and B). EPA was more effective in inhibiting both cyclic AMP production and glycerol release in adipocytes in response to ACTH than to either salbutamol or the tumour lipolytic factor.

The effect of oral administration by gavage of pure fatty acids to weight losing mice bearing the MAC16 adenocarcinoma on host weight loss and tumour growth rate is shown in Figs 4 and 5, respectively. The dose of EPA was chosen to correspond to that expected from the maximally effective dose of fish oil [11]. In all cases there was no significant difference in the food and water intake between the control and fatty acid treated groups. Animals were sham dosed for 7 days prior to the initiation of the experiment to reduce trauma and subsequent weight loss. The length of time of dosing was limited by the cost of the pure fatty acids. Of the fatty acids administered only EPA was effective in completely preventing weight loss, while DHA and linoleic acid at the same dose level were ineffective (Fig. 4). While EPA was non-toxic, DHA showed marked signs of toxicity, as evidenced by an increased weight loss compared with the control at day 5 (Fig. 4) and the experiment had to be terminated after three oral doses. In addition to the anticachectic effect, EPA also produced marked tumour cytostasis. In contrast to the antiproliferative effect of EPA the related DHA caused a slight

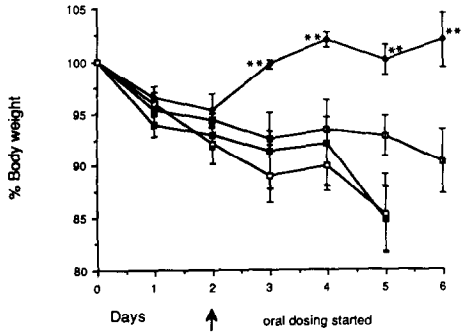

Fig. 4. Effect of oral dosing with pure fatty acids on the body weight of female NMRI mice bearing the MAC16 tumour. Mice (20 g) were dosed orally with either 100 mg EPA/day/mice (●), 100 μL 0.9% saline/day/mouse (⊡), 100 mg DHA/day/mouse (■) or 100 mg linoleic acid/day/mouse (□). Treatment was initiated 14 days after tumour transplantation when weight loss became apparent (average weight loss 5%). Body weights were measured daily and recorded as a percentage of the body weight prior to oral dosing. Results are expressed as mean ± SE. There was no significant difference in food and water intake. **P < 0.001 compared with saline controls.

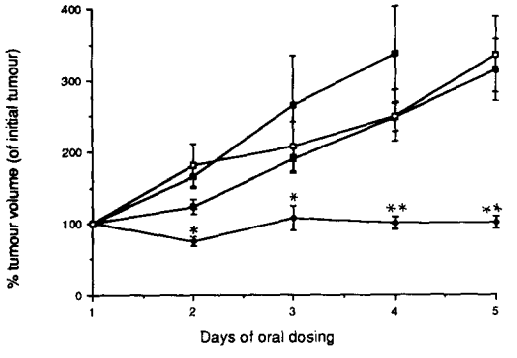

Fig. 5. Effect of oral dosing with pure fatty acids on the growth of the MAC16 adenocarcinoma in female NMRI mice. Mice (20 g) were dosed orally with either 100 mg EPA/day/mouse (●), 100 μL 0.9% saline/day/mouse (⊡), 100 mg DHA/day/mouse (■) or 100 mg linoleic acid/day/mouse (□). The average initial tumour volume on initiation of therapy was 96 ± 12 mm^3. Tumour volumes were measured daily by means of calipers and recorded as a percentage of the tumour volume prior to oral dosing. Results are expressed as mean ± SE. *P < 0.005; **P < 0.001 compared with saline controls.

stimulation of proliferation while linoleic acid was ineffective in controlling tumour proliferation.

These results confirm that the antitumour and anticachetic effect of fish oil is due to the presence of EPA, which appears to have special properties not shown by the other polyunsaturated fatty acids.

DISCUSSION

We have identified a lipid-mobilizing factor uniquely associated with cachexia in both an animal model [6] and in humans with clinical cancer cachexia [13] which appears to be distinct from the natural lipolytic hormones. The role of this material in the production of the cachectic state is suggested by its effect in reducing body weight of animals (unpublished results) and would be strengthened if inhibitors of the cachectic process were also inhibitors of the lipid-mobilizing activity. We have recently shown that a diet isocaloric and isonitrogenous with the normal diet, but in which an increasing proportion of the carbohydrate calories are replaced by lipids from fish oil, is highly effective in inhibiting the weight loss associated with the MAC16 tumour as well as reducing tumour growth [11]. This suggests that one or more of the components of the fish oil may be effective inhibitors of the lipolytic process.

Since fish oil is mainly composed of the fatty acids EPA and DHA the individual fatty acids were tested as inhibitors of the tumour lipolytic activity. Inhibition of lipolysis proved to be highly structurally specific in that out of a series of unsaturated fatty acids of both the (n–3) and (n–6) series only EPA inhibited the stimulatory effect of the tumour lipolytic factor, suggesting that the anticachectic effect of a fish oil diet is due solely to the presence of EPA. This has now been confirmed by oral administration of the pure fatty acids to weight losing mice bearing the MAC16 adenocarcinoma. Thus, while EPA completely inhibited weight loss at a dose of 5 g/kg, neither DHA nor linoleic acid at a similar dose level had any anticachectic activity, and in fact weight loss was enhanced by both DHA and linoleic acid. No toxicity or other adverse effects were noted at this dose level of EPA, which was chosen to compare with a diet in which 50% of the calories were derived from fish oil, which should have given an equivalent dose of EPA. Since animals treated with the other fatty acids received an equivalent caloric intake to those on EPA, these results suggest that, at least in animals bearing the MAC16 tumour, cachexia is due to the metabolic effect of the tumour via the production of a lipid-mobilizing factor. Since, we have recently identified a similar material in cachectic cancer patients [8] this suggests that cachexia in humans may also be due to a metabolic abnormality.

The antilipolytic effect of EPA was also shown against lipolysis stimulated by both ACTH and the β-adrenergic agonist, salbutamol. Since EPA is effective in inhibiting the stimulation of lipolysis by a range of agents having different cell surface receptors it suggests that the effect may be exerted on a process common to all lipolytic agents.

Lipolysis in adipocytes is thought to be exerted through the intracellular mediator cyclic AMP formed in response to activation of adenylate cyclase through binding of the hormone to its receptor [16]. Thus, we have shown that cyclic AMP levels in adipocytes are elevated in response to the lipolytic effects of ACTH and salbutamol. Lipolysis induced by the tumour lipolytic factor is also associated with an elevation of the intracellular level of cyclic AMP in adipocytes, although unlike the effects of ACTH and salbutamol, a prolonged stimulation of cyclic AMP production is observed in a similar manner to that found with bacterial toxins [17].

Stimulation of adipocyte cyclic AMP levels by the tumour lipolytic factor, ACTH and salbutamol was

inhibited by EPA, suggesting that the effect is exerted at a step common to all three agonists. Changes in fatty acid composition of cell membranes may be associated with changes in fluidity and thus in adenylate cyclase activity, although since EPA is the only fatty acid effective in the process it suggests a more specific effect. Atria from rats fed a cod liver oil supplemented diet, which resulted in a marked increase in the membrane content of the (n–3) fatty acids, EPA and DHA, have been shown to have a significantly lower basal level of cyclic AMP and a lower noradrenaline stimulated level [18]. This increase in the content of (n–3) fatty acids would lead to an increase in trienoic prostaglandins (PG_3). Both the (n–3) fatty acids and metabolites have a competitive and inhibitory effect on the metabolism of linoleic and arachidonic acid and can reduce the synthesis of dienoic prostaglandins [19]. However, in adipocytes PGE_2 is an inhibitor of lipolysis [20] and we have previously shown that indomethacin, also an inhibitor of the cyclooxygenase, is not an inhibitor of the tumour lipolytic factor [6].

Marine oils rich in (n–3) fatty acids have been shown to reduce plasma triglycerides and this effect has been attributed to an inhibition of hepatic triglyceride synthesis [21]. This study suggests that (n–3) fatty acids would produce a reducton in free fatty acid release in response to lipolytic hormones, thus reducing substrate availability for hepatic triglyceride synthesis. The antilipolytic effect of EPA would explain its ability to reverse experimental cancer cachexia and its effectiveness in postburn weight loss [22]. Since EPA was the only polyunsaturated fatty acid capable of inhibiting the growth of the MAC16 adenocarcinoma this suggests a relationship between the inhibition of cachexia and the inhibition of tumour growth. *In vitro* studies show both EPA and DHA to be equally cytotoxic to MAC16 cells, suggesting that the *in vivo* antitumour effect arises from a mechanism other than direct cytotoxicity.

Thus, the present study has shown unequivocally that the antitumour effect of a fish oil diet is due to the presence of EPA and that DHA has no antitumour activity. Since fish oil concentrates contain only about 19% EPA this suggests that further clinical trials should be carried out with the pure material, which would enable higher concentrations of the active drug to be administered.

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REFERENCES

1. De Wys W, Management of cancer cachexia. *Semin Oncol* **12**: 271–280, 1985.
2. Brennan MF, Uncomplicated starvation versus cancer cachexia. *Cancer Res* **37**: 2359–2364, 1977.
3. Rich AJ, Nutritional status in cancer. *Anticancer Res* **7**: 271–280, 1987.
4. Heber D, Byerley LO, Chi J, Grosvenor M, Bergman RN, Coleman M and Chlebowski RT, Pathophysiology of malnutrition in the adult cancer patient. *Cancer* **58**: 1867–1873, 1986.
5. Holroyde CP, Myers RN, Smink RD, Putnam RE, Paul P and Reichard GA, Metabolic response to total parenteral nurition in cancer patients. *Cancer Res* **37**: 3109–3144, 1977.
6. Beck SA and Tisdale MJ, Production of lipolytic and proteolytic factors by a murine tumor-producing cachexia in the host. *Cancer Res* **47**: 5919–5923, 1987.
7. Costa G, Bewley P, Aragon M and Siebold J, Anorexia and weight loss in cancer patients. *Cancer Treat Rep* **65**: 131–137, 1981.
8. Groundwater P, Beck SA, Barton C, Adamson C, Ferrier IN, Tisdale MJ, Alterations of serum and urinary lipolytic activity with weight loss in cachectic cancer patients. *Br J Cancer*, in press.
9. Beck SA and Tisdale MJ, Effect of insulin on weight loss and tumour growth in a cachexia model. *Br J Cancer* **59**: 677–681, 1989.
10. Tisdale MJ, Brennan RA and Fearon KC, Reduction of weight loss and tumour size in a cachexia model by a high fat diet. *Br J Cancer* **56**: 39–43, 1987.
11. Tisdale MJ and Dhesi JM, Inhibition of weight loss by n–3 fatty acids in an experimental cachexia model. *Cancer Res* **50**: 5022–5026, 1990.
12. Bibby MD, Double JA, Ali SA, Fearon KCH, Brennan RA and Tisdale MJ, Characterization of a transplantable adenocarcinoma of the mouse colon producing cachexia in recipient animals. *J Natl Cancer Inst* **78**: 539–545, 1987.
13. Beck SA, Catabolic factors in tumour-induced cachexia. PhD Thesis, Aston University, U.K., 1989.
14. Wieland O, Glycerol UV Method. In: *Methods of Enzymatic Analysis* (Ed. Bergmeyer HU), pp. 1404–1409. Academic Press, London, 1974.
15. Fain JN, Biochemical aspects of drug and hormone action on adipose tissue. *Pharmacol Rev* **25**: 67–118, 1973.
16. Butcher RN, Baird CE and Sutherland EW, Effects of lipolytic and antilipolytic substances on adenosine 3′,5′-monophosphate levels in isolated fat cells. *J Biol Chem* **248**: 1705–1710, 1968.
17. Sharp GWG and Hynie S, Stimulation of intestinal adenyl cyclase by cholera toxin. *Nature* **229**: 266–269, 1971.
18. Laustiola K, Salo MK and Metsa-Ketela T, Altered physiological responsiveness and decreased cyclic AMP levels in rat atria after dietary cod liver oil supplementation and its possible association with an increased membrane phospholipid n–3/n–6 fatty acid ratio. *Biochim Biophys Acta* **889**: 95–102, 1986.
19. Corey EJ, Shik C and Cashman JR, Docosahexaenoic acid is a strong inhibitor of prostaglandin but not leukotriene biosynthesis. *Proc Natl Acad Sci USA* **80**: 3851–3584, 1983.
20. Anderson NH, Biological aspects of prostaglandins. *Arch Int Med* **133**: 30–50, 1974.
21. Harris WS, Fish oil and plasma lipid and lipoprotein metabolism in humans: a critical review. *J Lipid Res* **30**: 785–807, 1989.
22. Trocki O, Heyd TJ, Waymack P and Alexander JW, Effects of fish oil on postburn metabolism and immunity. *J Parenteral and Enteral Nutr* **11**: 521–528, 1987.