LIPID MOBILIZATION BY MOUSE STOMACH CARCINOMA #2663

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Submitted to the Faculty of the School of Graduate Studies
of the Medical College of Georgia in Partial Fulfillment
of the Requirements for the Degree of
Master of Science
December 15
1975

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This thesis submitted by Daniel Gerard McChesney has been examined and approved by an appointed committee of the faculty of the School of Graduate Studies of the Medical College of Georgia.

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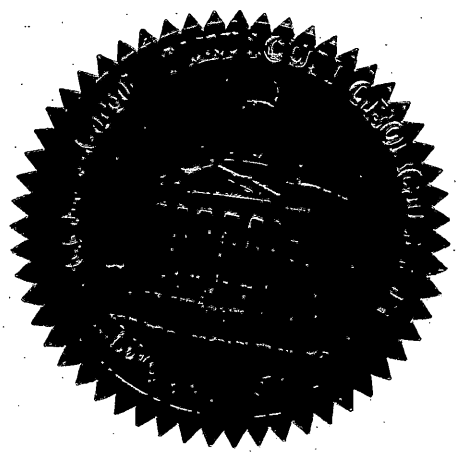

To my Parents and Diana, my wife

ACKNOWLEDGMENTS

I would like to thank Dr. R. A. Liebelt for suggesting this research problem, Dr. T. R. Dirksen for his assistance with the extraction of lipids and Dr. D. F. Scott for his assistance with the assay of cAMP. I would especially like to thank Dr. George Schuster for his assistance and encouragement with this project.

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STATEMENT OF THE PROBLEM

Anorexia and cachexia in cancer patients can cause serious clinical problems. However, it is not well understood how best to alleviate these signs and symptoms. None of the currect theories of how tumors exert their effect on the host's metabolism adequately explain these clinical responses. A possible explanation of the anorexia and cachexia that has not been fully explored is the involvement of lipid mobilization seen with some cancers. It has been reported that in the latter stages of certain types of malignant diseases, of both humans and laboratory animals there is a greater loss of body weight, with an accompanying hyperlipemia, than can be explained by decreased appetite. If this hyperlipemia is produced by substances associated with the tumor and is accompanied by an impairment of the body's ability to utilize the lipids that have been made available, then anorexia and cachexia can be explained by postulating that the hyperlipemia interferes with the body's appetite control mechanism and this, coupled with impaired utilization, allows the body to starve. The mouse stomach carcinoma #2663, a tumor causing hyperlipemia and cachexia provides an excellent model for studying these responses. The present study examines whether the lipid mobilization seen in animals bearing this tumor is a direct response to a lipid mobilizing factor produced by the tumor or is a stimulation of normal physiological lipolysis by its presence.

REVIEW OF THE LITERATURE

The wasting of the human body accompanying the continued growth of a malignant neoplasm as well as a similar phenomenon in experimental animals bearing certain kinds of spontaneous and transplanted tumors is a common observation. Mider (1951) presented evidence indicating that part of the weight loss in rats bearing the transplantable Walker carcinoma 256 could be accounted for by the translocation of nitrogenous substances from normal to neoplastic tissues. It was later reported by Fenninger and Mider (1954) that with continued tumor growth amino acids from the circulation were removed at a more rapid rate than they would be by normal cells, and thus the tumor acted as a 'nitrogen trap.' Early experiments with radioactively labeled amino acids supported the 'nitrogen trap' idea (Lepage et. al., 1952). Later, however, Greenless and Lepage, (1955) found that the trap theory did not apply to early generations of transplanted tumors and that ascites tumors could exchange amino acids with the host. The tumor was thus a 'nitrogen trap' only in a relative sense (Begg, 1958), and the degree of trapping may be related to the type of tumor and the degree of malignancy. The remainder of the wasting of the carcass mass was shown to be the result of continued depletion of the body's fat stores (Haven et. al., 1951; Fenninger and Mider, 1954; Boyd et. al., 1956). Mider (1951), in an attempt to explain this wasting of the carcass,

presented evidence demonstrating a strong positive correlation between energy expenditure and fat loss in tumor bearing animals and thus supported a 'mobilization of energy' concept to explain the loss in lipids. Others suggested that the loss in body lipids resulted from a failure to replace lipids rather than an excessive loss (Jablonski and Olson, 1955). It was generally agreed, however, that while the food intake of tumor bearing animals was usually below that of normal animals and a function of tumor growth, the decreased food intake could not satisfactorily explain the observed lipid depletion (Mider et. al., 1949; Begg and Lotz, 1956).

Several authors have described a hyperlipemia in animals bearing various types of transplanted tumors (Green and Jenkinson, 1934; Haven et. al., 1951; Liebelt et. al., 1974). Biochemical studies have been conducted by several investigators in an attempt to determine the cause of the observed hyperlipemia, and varying hypotheses have resulted. Haven proposed that the hyperlipemia was the result of either a mobilization of lipids for energy or to meet the demand for unsaturated fatty acids (Haven et. al., 1951). Begg (1955) felt that a triggering mechanism might initiate the hyperlipemia and suggested that a pituitary factor such as adipokinin might be released and remain present in substantial amounts. Haven and Bloor (1956) expanded Begg's (1955) basic idea to say that the tumor itself might release a hormonal factor which in turn would cause the release of a pituitary factor responsible for the mobilization of fat. In 1956 Begg and Lotz presented evidence for a fourth possible cause of the observed hyperlipemia by demonstrating that tumor

bearing rats had a lower level of lipoprotein lipase activity in their blood and that there was a delay in the removal of an intravenous oil emulsion from the blood of these rats. Begg and Lotz (1957) were able to demonstrate that the low activity of lipoprotein lipase observed in tumor bearing rats could not be explained by starvation or anemia. To date, a definitive statement cannot be made as to the exact cause of the hyperlipemia and the proposal by Haven and Bloor (1956) that several mechanisms may be involved seems to best describe the situation.

In an attempt to explain the clinical findings of cachexia and anorexia in patients with cancer, Ewing, in 1934, suggested that a toxic substance was produced by the tumor. Ewing's idea remained dormant until the 1940's when Greenstein and co-workers (Greenstein, 1942; Greenstein and Andervant, 1943; Greenstein et. al., 1942) demonstrated conclusively that an observed decrease in liver catalase activity was specifically attributable to the presence of the growing tumor and not to any secondary cause to which the tumor's presence might give rise (Nakahara and Fukuoka, 1958). Nakahara and Fukuoka in 1948 were able to isolate by alcohol precipitation a fraction from human tumor tissue which, when injected into mice, produced a marked decrease in liver catalase activity. The presence of this 'toxohormone' was subsequently demonstrated by several investigators in all tumors studied as well as in the urine of patients with cancer (Greenfield and Meister, 1951; Oksushima, 1952; Kawamorita et. al., 1951; Osawa, 1954). Attempts by Yunoki and Griffin (1961) to further purify this toxohormone were only partially successful and the in vitro activity of toxohormone was never demon-

strated . In 1963 Kampschmidt and Schultz demonstrated that injection of homogenates from normal rat liver or spleen into rats caused a decrease in liver catalase activity comparable to that obtained with homogenates of tumor tissue from NIH Novikoff, Noble Novikoff, or Walker 256 carcinoma. The only preparation that caused a greater decrease in liver catalase activity of normal rats was a Walker 256 carcinoma contaminated with Salmonella typhimurium. Similar results were obtained when toxohormone preparations from the various tissues were injected into mice. These results, coupled with the demonstration by other investigators of the ability of many substances to lower liver catalase activity, advise caution when evaluating toxohormone activity (Kampschmidt et. al., 1962; Riley, 1959; Adams, 1961).

The importance of tumor-elaborated, biologically active substances that affect the host's physiology has been well documented (Lipsett, 1964). Evidence suggests that neoplastic cells have the capacity to synthesize highly complex molecules of considerable specificity. Since each cell presumably is endowed with DNA containing the same information, it appears that the neoplastic transformation of the cell permits synthesis of proteins and peptides that are ordinarily repressed (Lipsett, 1964). In 1960 Liebelt, Lay and Davis reported that mice of the CE strain bearing the spontaneous mammary tumor CE 1460 had a marked rise in leukocytes. In 1963 Delmonte and Liebelt postulated that this granulocytosis was related to the presence of the CE 1460 mammary tumor and not dependent on the host. They were able to demonstrate this by the fact that a marked

granulocytosis was observable in F-1 hybrids (Balb/C x CE) receiving the CE 1460 mammary tumor but not those receiving the Balb/C 2301 mammary tumor. This led Delmonte, Starbuck and Liebelt to postulate the presence of a granulocytosis promoting factor (GPF). In 1965 Delmonte and Liebelt reported partial purification of this GPF. In this form it was active in mice at a dose of 0.8 micrograms per gram of body weight. Further studies by Delmonte and Liebelt in 1968 demonstrated the presence of a GPF in mouse mammary tumor CE 1460, normal mouse, rabbit and bovine kidney, and blood. They were also able to demonstrate that the granulocytosis promoting factor contained no protein or phosphorus, had a molecular weight of less than 2,000, and was specific for the granulocyte series of white blood cells. These findings suggest that the GPF may be a naturally occurring granulopoietic substance of mammalian tissues that is being elaborated in greater than normal amounts by the CE 1460 tumor or its presence. In 1974 Liebelt and co-workers reevaluated their earlier results and, with the addition of new data, concluded that the capacity of a tumor to stimulate granulocytosis is primarily a function of the genetics of the host of origin rather than the type of tissue in which the tumor occurs. It has not yet been possible to demonstrate that tumor associated granulocytosis is exclusively tumor specific.

Numerous cases have been reported in which syndromes caused by endocrine disorders such as hypercalcemia, hypoglycemia, erythremia, hyperthyroidism, hyperserotonemia and Cushing's syndrome have been associated with tumors of nonendocrine origin. In these cases it has been demonstrated clinically and supported

by laboratory results that tumor cells of various tissue origins not normally associated with the production of these hormones are responsible for the observed endocrine disorders. In 1968 O'Neal et. al. reported that six out of fifteen cases of adrenocorticotrophic hormone-secreting tumors observed also secreted additional hormonal substances. Among these were gastrin, melanotropin, 5-hydroxytryptamine, parathoromone, vasopressin, norepinephrine, and glucagon. More recently Kubata (1973) reported the isolation and partial purification of a hypoalbuminemic substance from Ehrlich solid carcinoma, and Nair and De Ome (1973) reported the presence of a growth-stimulating factor released by cultured mouse mammary tumor cells.

Recently several hypotheses have been advanced to account for the cachexia and anorexia observed in patients with cancer. One suggests that alterations in the metabolic patterns of the host and changes in various metabolic equilibria and concentrations of metabolites might be the result of allosteric transitions, activations, or inactivations of various enzymes in the tissue of the host (Theologides, 1972). The activity of various enzymes is modified by many kinds of small molecules, especially polypeptides, (Monod et. al., 1963), and thus the tumor, by producing low molecular weight metabolites, might throw the metabolism of the host into a chaotic state. Under these conditions, usual biochemical reactions might be activated or inactivated without a purpose or without control (Theologides, 1972). The result for the host would be an increased energy expenditure and an inability to lower the basal metabolic rate despite semistarvation. This

process could eventually lead to cachexia and anorexia.

Human patients and tumor bearing animals characteristically show a striking depletion of body lipid stores and a subsequent hyperlipemia during the course of tumor growth (Begg, 1958), and as previously stated, the physiological mechanisms involved in this tumor-host relationship are not fully understood. It has been suggested that the neoplastic cells are elaborating a lipid mobilizing substance and the resulting hyperlipemia during the depletion of the lipid component of the fat depots influences food intake regulation (Haven et. al., 1951; Begg, 1955; Liebelt et. al., 1973). Evidence supporting the existence of a lipid mobilizing substance in the urine and serum of animals after fasting has been provided by several investigators (Weil and Stetten, 1947; Chalmers et. al, 1960; Stevenson and Beaton, 1965; Curtis-Prior and Hanley, 1973). Lipid mobilizing activity also has been demonstrated for extracts of experimental tumors (Costa and Holland, 1962; Liebelt et. al., 1971,1974) and for extracts of the midbrain of sheep and calves (Shah et. al.,1972; Hollett, 1968). The lipid mobilizing substance from midbrain has been tentatively identified as isoprenaline (Shah et. al., 1972). One of the more recent works supporting the existence of a lipid mobilizing substance in serum is that of Curtis-Prior and Hanley (1973). Their results indicate that as the amount of serum from fasted or fed rats in the incubation medium in which adipose cells were incubating was increased, the release of glycerol from the cells also rose and eventually reached a peak ten to fifteen times above the basal level. They also reported that when beta-adrenergic blocking agents were

added prior to the addition of serum, the release of glycerol from adipose cells was progressively inhibited. From these results they concluded that rat serum contained one or more substances capable of inducing in vitro hydrolysis of triglycerides in adipose cells and that this substance mediates its effect through beta-adrenergic receptors. Workers who previously observed the lipolytic action of serum (Mosinger et. al., 1962; Burns et. al., 1967) have regarded the effect as artifactual. The possibility that the lipolytic action of the serum was artifactual was considered by Curtis-Prior and Hanley (1973) and ruled out when the appropriate controls were performed. Their results do not offer any insight into the identity of the substance, nor do they exclude the possibility that the serum stimulated lipolysis is due to catecholamines or other known hormones with lipolytic action. They do, however, indicate the presence of a lipid mobilizing substance that seems to exert its effect through beta-adrenergic receptors. In 1971 Liebelt et. al. reported evidence for a lipid mobilizing substance that was apparently being produced by a CBA mouse stomach carcinoma. Costa and Holland (1962) had reported a similar finding for a different tumor line in Swiss Ha/ICR mice. In the study conducted by Liebelt (1971), the CBA #2663 stomach carcinoma was transplanted by the trochar method (Liebelt and Liebelt, 1967) to four groups of mice and allowed to grow. The results demonstrated the following: 1) mice made obese by goldthioglucose (GTC) lost body lipid faster than the non-obese mice but the percent of lipid mobilized was comparable: 2) a linear relationship existed between tumor

weight and total body lipid for both non-obese hosts; 3) GTC obese F_1 hybrid mice (CBA^- x C57Bl) carrying the CBA^- stomach tumor mobilized twice as much total carcass lipid/day as mice carrying the C57Bl fibrosarcoma. The results of these experiments, in conjunction with preliminary studies (Liebelt et. al., 1974) revealing that the total plasma neutral lipids in similar F_1 hybrids bearing the CBA tumor at one centimeter in size were three times as great as the plasma neutral lipid in F_1 hybrids bearing the C57Bl tumor at one centimeter in size. This indicates that the CBA stomach tumor has a better capacity to cause lipid mobilization than the C56Bl tumor. These facts suggest a biological uniqueness for the CBA tumor that could be accounted for by a difference in production of a postulated lipid mobilizing substance. When this study was expanded (Liebelt et. al., 1974), it was decided to attempt to grow the CBA stomach tumor in cell culture in order to obtain a more homogenous preparation for the isolation of the lipid mobilizing substance. This approach was taken because of the complications encountered in isolating the granulocytosis promoting factor from the CE 1460 mammary carcinoma (Delmonte and Liebelt, 1965). The tumor was established in cell culture and a preliminary study in which the 0.5 ml. of fresh culture medium homogenized cultured cells or medium in which the CBA cells had grown for forty-eight hours was injected into CBA male mice twice daily for four days. At the end of the four days the animals were killed and the weight of the inguinal and gonadal fat depots were determined. The results indicated that the forty-eight hour culture medium had a significantly better capacity to reduce

body lipid stores as compared to controls and the homogenized cells had an even greater lipid depleting capacity. These data were consistent with the presence of a lipid mobilizing substance associated with the CBA tumor.

In an attempt to determine the mechanisms of lipolysis in adipose tissue, several investigators have examined the role of adenosine 3'5'-cyclic monophosphate (cAMP) in lipolysis. In 1962 Mossinger and Kujalova reported that following the addition of epinephrine to adipose tissue incubated in Krebs-Ringer phosphate buffer plus albumin that there was a marked increase in the free fatty acid (FFA) in the media. Butcher et. al. in 1965, reported that cAMP levels increased in adipose tissue incubated in Krebs-Ringer bicarbonate buffer plus albumin in the presence of epinephrine and that this increase in cAMP occurred prior to an increase in lipolysis, as determined by a release of free fatty acids into the medium. Other investigators (Weiss et. al., 1966; Humes et. al., 1968) have since confirmed the findings of Butcher. In 1968 Butcher et. al. demonstrated that ability of various substances, known to be lipolytic, such as adrencorticotrophic hormone, thyroid stimulating hormone and glucagon to increase cyclic AMP levels. Several reviews detailing the effects of these various substances on cyclic AMP are available (Robison et. al., 1971a and Robison et. al., 1971b). More recently Huttunen and Steinberg (1971) reported results that are consistent with the view of Butcher et. al. (1965). They also presented evidence that the hormonal activation of adipose tissue lipase is mediated by cyclic AMP in the presence of ATP and Mg^{++}, that cyclic AMP

acts via a protein kinase, and that the conversion of lipase from an inactive to an active form is linked to enzyme phosphorylation. Their work was done with rat adipose tissue lipase and rabbit skeletal muscle protein kinase. In 1974 Khoo and his co-workers were able to add further proof to the findings of Huttunen and Steinberg by demonstrating that a partially purified hormone sensitive triglyceride lipase from human adipose tissue was activated by the addition of cyclic AMP, ATP, and Mg++. They further demonstrated that the addition of a protein kinase inhibitor from rabbit skeletal muscle at zero time completely inhibited activation, that this inhibition could be prevented by prior addition of excess exogenous protein kinase, and that addition of protein kinase inhibitor during the activation suggested that the kinase might be acting directly on the lipase. Further purification of the lipase fraction yielded a fraction free of protein kinase. They were able to demonstrate that lipase activation in this fraction was absolutely dependent on the addition of exogenous protein kinase. From their data and that of Huttunen and Steinberg, Khoo and his associates proposed that adenyl cyclase was stimulated by a lipolytic substance causing the conversion of ATP to cAMP. The cAMP thus formed in the presence of ATP and Mg++ and a protein kinase allows the phosphorylation by the kinase of the nonactive, hormone sensitive lipase, converting it to an active triglyceride lipase. Thus, it appears at present that a variety of substances produced by the body can stimulate lipolysis by causing an increase in cAMP which allows the activation of a triglyceride lipase. Therefore, it is theoretically possible

for tumors to produce substances that mediate lipolysis via cAMP.

Anorexia and cachexia in cancer patients can cause serious problems and none of the current theories of how tumors exert their effect on the host's metabolism adequately explain these clinical responses. A possible explanation of the anorexia and cachexia that has not been fully explored is the involvement of lipid mobilization seen with some cancers. It has been reported that in the latter stages of certain types of malignant diseases, of both human and laboratory animals, there is a greater loss of body weight, with accompanying hyperlipemia, than can be explained by decreased appetite. The present study examines whether the cachexia and lipid mobilization seen in animals bearing the mouse stomach carcinoma #2663 is a direct response to a lipid mobilizing factor produced by the tumor or is a stimulation of normal physiological lipolysis by the presence of this tumor.

MATERIALS AND METHODS

Materials

A. Animals

The CBA⁻/Ki mice used in the study were a gift from Dr. A. G. Liebelt. Male CBA⁻ mice, twelve to fourteen weeks of age, were used for all *in vivo* studies. Male and female CBA⁻ mice, eight to sixteen weeks old, were used for *in vitro* studies. All mice were housed in a controlled environment at 72±1° F. and received water and standard lab chow *ad libitum*.

B. Chemicals and Equipment

Minimal essential medium (MEM), Waymouth's 752/1 medium, L-glutamine, fetal bovine serum (FBS) and trypsin were obtained from Grand Island Biologicals (Grand Island, N. Y.). Streptomycin sulfate was purchased from the Charles Pfizer Company (New York, N. Y.) and potassium penicillin-G from E. R. Squibb and Sons (New York, N. Y.). Heptane, tetrabutylammonium hydroxide titrant (25% in methanol) and X-omat RP-14 X-ray film were supplied by the Eastman Kodak Company (Rochester, N. Y.). Bovine serum albumin (BSA) fraction V, L-epinephrine and thymolphthalein were purchased from the Sigma Chemical Company (St. Louis, Mo.). Aquasol, 1-^{14}C acetic acid, sodium salt (60 Ci/mmole), adenosine 3'5'-monophosphate, ammonium salt (25 Ci/mmole) were purchased from New England Nuclear (Boston, Mass.). U-80 insulin was purchased from Eli Lilly Company (Indianapolis, Ind.).

All other chemicals were purchased from the Fisher Scientific Company(Atlanta, Ga.). The Sonifier Cell Disruptor was purchased from Heat Systems-Ultrasonics (Plainview, N. Y.), and the Polytron homogenizer from Brinkmann Instruments (Lucerne, Switzerland).

Methods

A. Propogation of CBA #2663 stomach carcinoma in culture and in CBA/Ki hosts

The tumor cells were grown in Waymouth's 752/1 medium supplemented with 10% (v/v) fetal bovine serum, sodium bicarbonate (175 mg/dl), L-glutamine (4.4 mM), potassium penicillin-G (89units/ml.) and streptomycin sulfate (89 µg/ml.) at 37°C under an atmosphere of 95% air, 5% CO_2. U-80 insulin (.266 units/ml.) was originally used in the medium; however, since passage 20, insulin has been removed and is no longer required for growth. The removal of insulin did not change the lipid mobilizing properties of the tumor. Approximately 2.5×10^6 tumor cells in 0.2 ml. of Hanks balanced buffered salt solution from every third passage were inoculated into CBA/Ki hosts, and tumor growth examined. The weight of the inguinal and gonadal fat depots was determined at the end of twenty-six days and used as an index of lipid mobilization (Liebelt, 1963). A decrease in weight of the fat depots over that of controls was interpreted as increased lipid mobilization.

B. Radioactive labeling of adipose tissue and extraction of lipid.

Adipose tissue was labeled by incubating pieces of tissue for two hours in minimal essential medium (MEM) supplemented with sodium bicarbonate (175 mg/dl), potassium penicillin-G (89 units/ml.), streptomycin sulfate (89 µg/ml.), 5% (v/v) fetal bovine

serum and 1-^{14}C acetate (2.5 μCi/ml.). The amount of radioactivity incorporated into triglycerides, diglycerides and phospholipids was determined by extracting the adipose tissue with chloroform and methanol (Folch et. al., 1957). Chromatography was performed on Whatman SG 81 silica gel loaded paper in a solvent system containing heptane, 2,6 dimethyl 4 heptanone and acetic acid (85: 15: 1) by volume. The chromatography paper was removed after development and the solvent allowed to evaporate. The chromatography paper was then placed in a holder with X-ray film and the film exposed for two weeks. At the end of this time the film was developed and the various classes of lipids containing radioactivity were visualized by the radioautography. These areas were cut from the chromatogram and the radioactivity determined on a Packard 3320 liquid scintillation spectrometer using Aquasol as the cocktail.

C. Preparation of cell homogenates and extracts.

To prepare cell homogenates and extracts, CBA⁻ stomach carcinoma #2663 tumor cells were harvested by scraping bottles containing a small amount of medium with a rubber policeman and then removing the medium and cells. Trypsin was not used because it had been shown (Schuster, personal communication) to effect the tumor cells ability to reattach to the glass bottles. Mouse L-cells were harvested with 0.25% trypsin in trypsinizing citrate at pH 7.3. Both cell preparations were counted and then centrifuged, and the medium or trypsin removed. The cells were resuspended in saline to give a final concentration of 2.5 x 10^6 cells/ml. The saline suspension of cells was then sonicated on a Sonifier Cell Disruptor until no intact cells

could be detected by phase contrast microscopy. This preparation was termed the cell homogenate. If a cell extract was to be prepared, the homogenate was centrifuged at 100,000 x g for 60 minutes at 4°C. The supernatant was termed the cell extract.

Originally the homogenates of the tumor cells and L-cells were prepared by suspending an appropriate number of cells in saline and then using a Polytron homogenizer for the homogenization. This procedure was abandoned when it was observed that the cells were not disrupted by homogenization with the Polytron homogenizer.

D. Construction of the perifusion system

In order to study the interaction of the tumor cells with adipose tissue in vitro, it was desirable to have a system which meets the following requirements: 1) provides a stable environment which can be regulated as required; 2) allows constant volume samples to be taken regularly without disrupting the system; 3) permits the adipose tissue to be maintained in a viable state and to interact with the tumor cells, their extracts or other stimuli as desired; 4) allows for the recovery of the tissue at the end of the experiment; and 5) is easy to construct. These requirements were met by the apparatus described below and shown in figure 1.

The system illustrated in figure 1 was constructed from a glass bottle (1), a sterile plastic culture dish top (2), 16 gage stainless steel tubing (3), a disposable syringe (4), clamps (5), a peristaltic pump (6), a test tube rack (12), and a fraction

Figure 1

Schematic of perifusion system

1. glass bottle
2. sterile plastic culture dish top
3. 16 gage stainless steel tubing
4. disposable syringe
5. clamps
6. peristaltic pump
7. fraction collector
8. perifusion chamber
9. rubber stoppers with 16 gage stainless steel tubing
10. 1/8" Nalgene T-connector
11. Wedco incubator
12. test tube rack

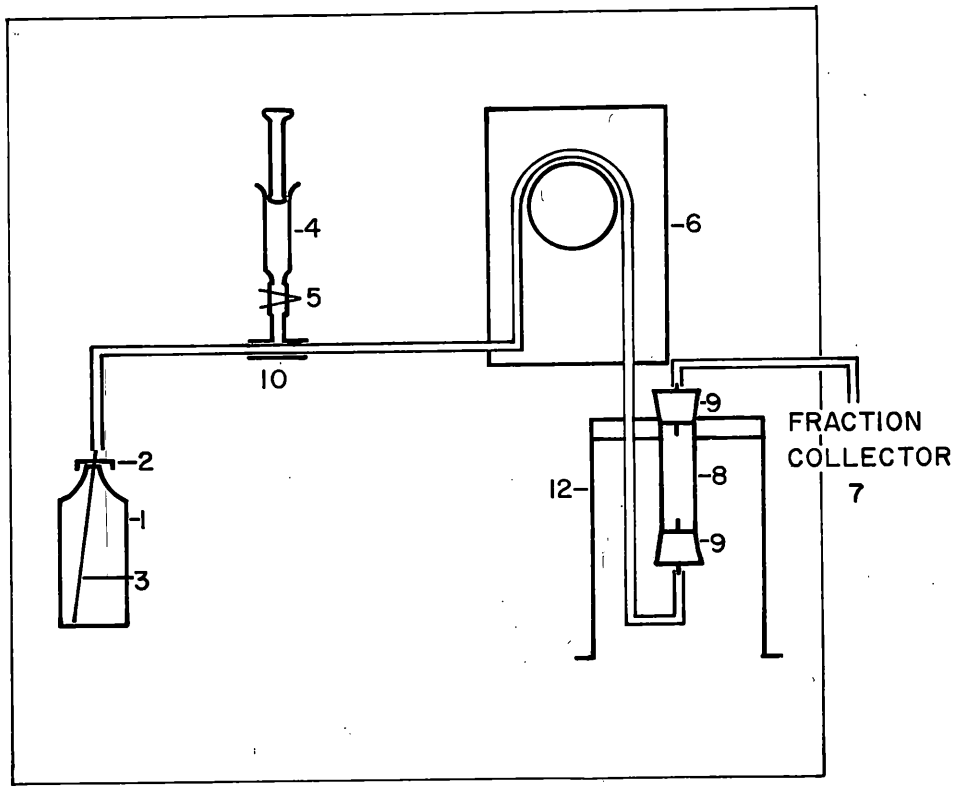


FIGURE 1

collector (7). The perifusion chamber (8) was constructed from a 3 ml. disposable syringe with the plunger removed and the barrel cut off at the 0.5 ml. mark. It was sealed with two rubber stoppers containing 16 gage stainless tubing (9) that protruded approximately 2 mm. into the chamber. The tubing between the bottle (1) and the 1/8" Nalgene T-connector (10) and the T-connector (10) and the chamber (8) is 1/16" ID., 1/8" OD. Tygon tubing. The tubing between the chamber (8) and the fraction collector (7) is .047 " ID., .067 " OD. Intramedic PHF. tubing. The tubing between the syringe (4) and the T-connector (10) is 1/8 " ID., 3/16 " OD. Tygon tubing. All components of the system except the fraction collector (7) will fit into a Wedco model 2-17 B incubator.

Medium is drawn by the pump from the bottle (1) and through the connecting tubing and T-connector (10) and enters the chamber (8) at the bottom. The chamber fills with medium bathing the tissue, then passes out the top of the chamber and through the tubing to the fraction collector (7). By having the medium enter at the bottom and exit at the top, we were able to eliminate the pulsing of the medium which was encountered when the flow was in the reverse direction. The flow rate was normally set at 0.5 ml. per minute for our experiments. However, the flow rate is limited only by the type of pump used. Tissue to be placed in the chamber is sliced or cut into appropriate size pieces and then placed on a thin piece of glass wool which is rolled into a cylinder that will fit inside the chamber (8). The chamber (8), is then sealed with the stoppers (9), placed in a test tube rack (12), and the tubing connected. At the end of the experiment

the cylinder of glass wool containing the tissue may be removed from the chamber (8), unrolled and the tissue recovered. At the start of perifusion, a clamp (5) is placed between the T-connector (10) and syringe connection preventing the flow of the test substance through the system. At the appropriate time this clamp (5) is moved from its original position and clamped on the tubing on the bottle (1) side of the T-connector (10). This blocks the flow of the medium from the bottle (1) and allows the test substance in the syringe (4) to be drawn into the system at the same rate as the original medium. During early experiments a small amount of tritiated water was included in the test substance to facilitate the standardization of the system. It was omitted in later experiments. At the end of the desired time period the clamp (5) is moved back to its original position, allowing the medium from the bottle to flow over the tissue again.

The system can be sterilized in the following manner: the medium bottle (1) and stainless steel tubing (3) can be autoclaved. The plastic plate (2) can be exposed to ultraviolet light. The tubing and chamber (8) can be disinfected by flushing the system for thirty minutes with a sterile saline solution containing potassium penicillin-G (100 units/ ml.), streptomycin sulfate (100 µg/ ml.) and amphotericin B (5 µg/ ml.). A sterile disposable syringe can be used to hold the test substance.

E. Effect of cell homogenates and cell extracts on in vivo lipolysis.

Male mice of the CBA⁻ strain between twelve and sixteen weeks old were injected with cell homogenates or extracts prepared according to the procedure in section C. The animals were in-

jected at 9 A.M. and 4 P.M. on four consecutive days with 0.2 ml. of either the cell homogenate or extract. A control group received 0.2 ml. saline injections on the same schedule. At 9 A.M. on the fifth day the animals were sacrificed, and the weight of the inguinal and gonadal fat depots determined and used as an index of lipid mobilization (Liebelt, 1963).

F. Effect of conditioned media, cell homogenates and cell extracts on lipolysis in adipose tissue as measured by free fatty acid release.

The effect of these test substances was determined by incubating slices of adipose tissue in Krebs Ringer bicarbonate buffer pH 7.25, containing 2.5% (w/v) bovine serum albumin for thirty minutes at 37°C, in polyethylene vials and then adding varying amounts of conditioned medium, cell homogenates or cell extracts. The incubation was then continued for two additional hours. At the end of the incubation period the level of free fatty acids was determined by the procedure of Ko and Royer (1974). This involves extraction of the free fatty acids from the media or tissue with a mixture of isopropyl alcohol, heptane and sulfuric acid. To accomplish this a 4 ml. mixture of isopropyl alcohol: heptane (7:3) and 2 ml. of 0.033N sulfuric acid are added to 0.5 ml. of medium or 300 milligrams of tissue and mixed for ten seconds. If tissue is being extracted, it is homogenized in a hand homogenizer before mixing. After mechanical mixing, the mixture is allowed to separate into two layers. One ml. of the upper layer is removed and placed in a 15 ml. conical test tube to which 0.1 ml. of indicator (thymolphthalein 0.01 % in heptane: acetone 10:1) is added. The free fatty acids are then titrated with a tetrabutylammonium hydroxide,

(25% in methanol) standard diluted 1:100) in isopropyl alcohol. The end point is a change of the indicator from clear to blue. The titration is performed with a Gilmont ultrapipet. Mixing of the standard with the solution being titrated is accomplished by bubbling with nitrogen.

An increase in the amount of free fatty acid in the medium of the tissues stimulated with the test substances over that of the control was interpreted as an indication of a direct action of the substance on the tissue, causing the release of free fatty acids.

G. Changes in tissue adenosine 3'5' cyclic monophosphate (cAMP) levels in response to conditioned medium, cell homogenates and cell extracts.

Cyclic AMP levels were determined by incubating adipose tissue slices in Krebs Ringer bicarbonate (KRB) buffer pH 7.25 for thirty minutes and then adding the test substance. The incubation was continued for ten minutes. All incubations were performed in polyethylene vials placed in a 37°C, Dubnoff metabolic shaking incubator. At the end of the second incubation the tissue from the vials was quickly transferred to 15 milliliter Corex tubes. The reaction was stopped by adding 2 ml. of 5% trichloroacetic acid (TCA) in saline to the tubes and homogenizing the contents on a Brinkmann polytron homogenizer. Tritiated cAMP (15,000 to 20,000 cpm) was added to the homogenate, and the homogenate was centrifuged at 10,000 x g for thirty minutes. The supernatant was removed and washed five times with two volumes of ether. The pellet was saved for protein determination by the method of Lowry (1951) as modified by Oyama and

Eagle (1956). The washed supernatant was evaporated to dryness, resuspended in 100 microliters of water and spotted on Whatman 3MM chromatography paper. The chromatograms were developed in a solvent system containing ammonium hydroxide: N-propanol: water (15: 70: 15) by volume. At the end of the seventeen hour development time, the chromatograms were removed and air dried. The cAMP spots were detected using an ultraviolet light (254 nm) and cAMP standards as a reference. These areas were cut from the chromatogram and eluted with 50% ethanol. This eluate was evaporated to dryness, and the residue resuspended in distilled water for determination of cAMP levels by the competitive binding assay of Gilman (1970) as modified by Brown et. al. (1971).

An increase in the levels of cAMP in tissue treated with the tested substance over that in controls was interpreted as an indication that the test substance acts directly on the tissue to stimulate cAMP production.

H. Release of radioactively labeled free fatty acids (FFA) into perifusion fluid.

Adipose tissue was prelabeled as previously stated and the tissue was perifused in a controlled environment of 95% air and 5% CO_2 at 37°C, with a Krebs Ringer bicarbonate (KRB) buffer solution, pH 7.25, containing 2.5% (w/v) bovine serum albumin (BSA) fraction V until baseline levels of radioactivity were obtained in the perifusate. At this time the substance to be tested, to which 2.5% (w/v) BSA fraction V had been added, was perifused over the tissue. Fractions of the perifusate were assayed for radioactivity and free fatty acids. A release of

free fatty acids, as measured by these two parameters, was interpreted as evidence for a direct acting lipid mobilizer.

I. Statistical tests.

The statistical tests performed on the data were in accordance with the procedures outlined by Sokal and Rohlf.

RESULTS

In Vivo Lipid Mobilization

A. Lipid mobilization by mouse #2663 stomach carcinoma cells and mouse L-929 fibroblasts.

Animals of comparable age, weight, strain and sex were injected subcutaneously with either 0.2 ml. of Hanks balanced buffered salts or 2.5×10^6 #2663 tumor cells in a similar volume of the buffered salts. After injection the animals were allowed standard lab chow and water ad libitum for twenty-six days. Cachexia was not normally detectable until forty days after the initial injection of tumor cells. The animals were killed on the twenty-sixth day to insure the presence of adipose tissue in the fat depots. The weight of the inguinal and gonadal fat depots was determined at this time. The weight change, when compared to sham injected controls, was used as an index of lipid mobilization (Liebelt, 1963). The data in table 1 are from a single experiment and are representative of data obtained from ten similar experiments. The differences in the lipid index between experimental and control animals is significant at the .001 level.

Experiments were performed to determine if the mobilization of lipid was a common response of mice injected with cultured mouse cells or was limited to the injection of specific tumor cells. A procedure identical to that emplyed with the mouse #2663 tumor cells was used but cultured mouse L-929 fibroblasts were substituted for the tumor cells. Table 2 is a composite of the

Table 1

In vivo lipid mobilization by mouse #2663 tumor cells.

Animals 1 through 6 received a single subcutaneous injection of 2.9×10^6 tumor cells in 0.2 ml. of Hanks balanced buffered salts (BBS). Animals 7 through 11 received a single subcutaneous injection of 0.2 ml. of Hanks BSS. Both groups were permitted food and water ad libitum until the time of sacrifice, 26 days after the injection of cells or Hanks BSS. The data in this experiment are representative of the data obtained in 10 similar experiments. The P value was determined using a t-test for a population with equal variance. The lipid index is the amount of adipose tissue remaining in the fat depots per 50 gram animal at the time of autopsy. All values are reported $\pm$ SEM.

a. $P < .001$ for the comparison between the lipid index of tumor-bearing animals and control animals.

b. $P < .001$ for the comparison between the final body weight of tumor-bearing animals and control animals.

Table 1

Animal	Body weight Initial	Final	Tumor weight	Lipid index
-	(g)		(mg)	(mg)
1	28.3	27.0	424	0
2	28.4	25.0	1000	0
3	29.4	32.0	662	859
4	27.4	24.8	270	105
5	26.4	31.0	226	1280
6	27.0	30.0	338	543
Mean + SEM	27.8 +0.4	28.3 + 1.3b	495 + 119	465 + 215a
7	29.0	31.6	0	1477
8	30.1	34.0	0	1722
9	30.1	34.8	0	3152
10	27.3	33.0	0	2989
11	32.6	36.1	0	2427
Mean ± SEM	29.8 ± 0.8	33.9 ± 0.8	0	2353 ± 333

Table 2

In vivo lipid mobilization by mouse #2663 tumor cells and and mouse L-929 fibroblasts: a composite

Animals receiving the #2663 tumor cells received approximately 2.5×10^6 cells in 0.2 ml. of Hanks balanced buffered salts in a single subcutaneous injection. Animals receiving the L-929 fibroblasts received a single injection of 2.7×10^6 cells in 0.2 ml. of Hanks BBS. The control groups of animals received 0.2 ml. of Hanks BBS in a single injection. All groups were permitted food and water ad libitum until the time of sacrifice, 26 days after the initial injection. The mean lipid index is the average amount of adipose tissue remaining in the animal's fat depots when corrected to a 50 gram animal. All values are reported $\pm$ SEM. The P values were determined using a t-test for a population with equal varience.

a. $P < .001$ for a comparison of the lipid index of animals receiving tumor cells and L-cells
b. $P < .001$ for a comparison of the lipid index of animals receiving tumor cells and Hanks BBS.
c. $P < .001$ for a comparison of the final body weight of animals receiving tumor cells and L-cells
d. $P < .001$ for a comparison of the final body weight of animals receiving tumor cells and Hanks BBS

Table 2

no. animals	Preparation	Body wt. Initial	Body wt. Final	Tumor wt.	Lipid index
-	-	(g)	(g)	(mg)	(mg)
43	Tumor cells	28.7 ± 0.4	29.5 ± 0.4c,d	393 ± 44	1252 ± 93a,b
12	L-cells	29.0 ± 0.5	32.2 ± 0.6	0	1971 ± 136
48	Hanks BBS	29.0 ± 0.4	32.2 ± 0.4	0	2010 ± 69

data from all *in vivo* experiments performed using the mouse #2663 tumor cells, mouse L-929 fibroblasts and Hanks BBS. The differences in the lipid index of animals bearing the mouse #2663 tumor from both control groups is significant at the .001 level. The data presented in tables 1 and 2 indicate that the mobilization of lipid observed in the CBA⁻ mice was a result of the presence of the mouse #2663 tumor cells and not a common response of mice injected with cultured mouse cells or Hanks BBS.

B. *Effect of cell homogenates and cell extracts from mouse #2663 tumor cells and mouse L-929 fibroblasts on in vivo lipolysis.*

Male CBA⁻ mice twelve to sixteen weeks old were injected subcutaneously with 0.2 ml. of cell homogenate, cell extract or saline at 9 A.M. and 4 P.M. on four consecutive days. At 9 A.M. of the fifth day the animals were sacrificed, and the weight of the inguinal and gonadal fat depots determined and used as an index of lipid mobilization (Liebelt, 1963). The data in tables 3 and 4 indicate that neither cell homogenates nor cell extracts from mouse #2663 tumor cells possess greater lipid mobilizing ability than do similar preparations of mouse L-929 fibroblasts. In fact, the amount of lipid mobilized by the preparations from the tumor cells and L-929 cells was not significantly different from that of the saline controls.

Male CBA⁻ mice, twelve to sixteen weeks old, injected and sacrificed on the same time schedule as above with cell homogenates from #2663 mouse tumor cells prepared by Polytron homogenization showed a loss of fat depot weight over that of control animals receiving a similar preparation of L-cells. Those animals re-

Table 3

Effect of cell homogenates from mouse #2663 tumor cells and mouse L-929 fibroblasts on in vivo lipolysis.

Cell homogenates for both groups were made from the same number of cells suspended in saline and sonicated until no intact cells could be detected by phase contrast microscopy. The animals received twice daily subcutaneous injections for 4 days according to the schedule described in the test, of amount equivalent to 400,000 cells/injection of either the tumor cells or the L-cells. Another group received 0.2 ml. saline injections on the same schedule. On the fifth day the animals were sacrificed and the weight of the fat depots determined. The lipid index is the average of 4 animals and is the amount of adipose tissue remaining in the fat depots per 50 gram animal at the time of autopsy. The P value was determined using a t-test and the values are reported ± SEM.

a. $P > .9$ for the comparison between the lipid index of mice receiving tumor cell homogenates and L-cell homogenates
B. $P > .2$ for the comparison between the lipid index of mice receiving tumor cell homogenates and saline.

Table 3

no. of animals	Preparation	Body wt. Initial	Body wt. Final	Tumor weight	Lipid index
-	-	(g)		(mg)	(mg)
4	L-cell homogenate	27.6 ± 2.4	27.5 ± 2.0	-	1661 ± 261
4	Tumor cell homogenate	27.6 ± 1.2	27.0 ± 0.9	-	1667 ± 65a,b
4	Saline	27.6 ± 0.8	27.4 ± 0.8	-	1579 ± 91

Table 4

Effect of cell extracts from mouse #2663 tumor cells and mouse L-929 fibroblasts on in vivo lipolysis.

Cell extracts for both groups are the 100,000 x g. supernatant and were prepared by suspending an equal number tumor cells and L-cells in saline and sonicating them until no intact cells could be detected by phase contrast microscopy and then centrifuging them for 1 hr. at 100,000 x g. The animals received twice daily subcutaneous injections for 4 days of an amount of cell equivalent to 400,000 cells per injection of either the tumor cells or the L-cells. Another group received 0.2 ml. injections of saline on the same schedule. On the fifth day the animals were sacrificed and the weight of the fat depots determined. The lipid index is the average of 4 animals and is the amount of adipose tissue remaining in the fat depots per 50 gram animal at the time of autopsy. The P value was determined using a t-test for populations with equal varience. All values are reported ± SEM.

a. $P > .5$ for a comparison between the lipid index of animals receiving tumor cells extracts and L-cells extracts.
b. $P > .9$ for a comparison between the lipid index of animals receiving tumor cells extracts and saline.

Table 4

no. of animals	Preparation	Body wt. Initial	Body wt. Final	Tumor weight	Lipid index
-	-	(g)		(mg)	(mg)
4	L-cell extract	27.8 ± 0.8	27.9 ± 0.6	-	1625 ± 100
4	Tumor cell extract	27.8 ± 0.9	27.2 ± 0.8	-	1562 ± 129 a,b
4	Saline	27.6 ± 0.8	27.4 ± 0.7	-	1579 ± 46

ceiving this tumor cell homogenate also had small nodules at the site of injection that average seventy-three milligrams. Histopathological examination of these nodules revealed that they contained intact tumor cells. Upon reexamination of the Polytron homogenization procedure it was observed that the procedure did not adequately disrupt the cells strongly suggesting that injections of homogenates prepared using this instrument contained intact cells. Table 5 presents the data from the injection of Polytron homogenates and suggests that lipid mobilization may be detected within four days. The homogenate data obtained with the Polytron homogenization procedure are consistent with that reported by Liebelt et. al. (1974), and suggests that the lipid mobilization they observed could have been caused by the presence of intact cells in their homogenates rather than by a factor produced and/or secreted by the #2663 mouse tumor cells.

The data from the two homogenization procedures and the lipid mobilization observed in vivo with viable cells suggest that mouse #2663 tumor cells do not contain a lipid mobilizing factor that can be released by cell disruption.

Table 5

Effect of Polytron cell homogenates from mouse #2663 tumor cells and mouse L-929 fibroblasts on *in vivo* lipolysis.

Cell homogenates for both groups were made from the same number of cells suspended in saline and homogenized on a Polytron homogenizer. The animals received twice daily subcutaneous injections for 4 days of an amount equivalent to 400,000 cells/injection of either tumor cells or L-cells. Another group received 0.2 ml. saline injections on the same schedule. On the fifth day the animals were sacrificed and the weight of the fat depots determined. The lipid index is the average from 4 animals and is the amount of adipose tissue remaining in the fat depots per 50 gram animal at the time of autopsy. The P value was determined using a t-test for a population with unequal varience. Values are reported $\pm$ SEM.

a. $P < .01$ for a comparison between the lipid index of animals receiving tumor cell homogenates and L-cell homogenates.
b. $P > .3$ for a comparison between the final body weight of animals receiving tumor cell homogenates and L-cell homogenates.
c. $P > .2$ for a comparison between the initial body weight of animals receiving tumor cell homogenates and L-cell homogenates.

Table 5

no. of animals	Preparation	Body wt. Initial	Body wt. Final	Tumor weight	Lipid index
-	-	(g)		(mg)	(mg)
4	Tumor cell homogenate	30.8 ± 0.7c	29.2 ± 0.6b	73.8 ± 11.4	1712 ± 17 a
4	L-cell homogenate	29.2 ± 1.4	28.4 ± 1.6	0	2416 ± 117

In Vitro Lipid Mobilization

A. Effect of conditioned medium, cell homogenates and cell extracts on in vitro lipolysis in adipose tissue as measured by free fatty acid release.

The effects of conditioned medium, cell homogenates, cell extracts and fresh medium on in vitro lipolysis were determined by incubating slices of adipose tissue in Krebs Ringer bicarbonate buffer pH 7.25 containing 2.5% bovine serum albumin (w/v) for thirty minutes at 37°C and then adding varying amounts of the substances to be tested. The incubation was continued for two additional hours. At the end of the incubation period the level of free fatty acids in the medium was determined. Preliminary studies had shown that most of the free fatty acids released from triglycerides in adipose tissue were in the incubation medium and not in the adipose tissue. Table 6 shows the effect of increasing concentrations of L-epinephrine, a known lipolytic agent, on the release of free fatty acids from adipose tissue, and demonstrates the ability of the assay to detect them in the incubation medium. Table 7 shows that the amount of free fatty acids in fresh medium is greater than in medium which has been incubated with cells for forty-eight hours. The data also indicate that tumor cells and L-cells remove a comparable amount of free fatty acids from the medium. Table 8 indicates that fresh medium possesses a slightly greater lipolytic ability than does forty-eight hour conditioned medium from either tumor cells or L-cells. The possibility that more free fatty acids might have been present in the fresh medium initially than were present in either of the conditioned media after forty-eight hours may not be entirely ruled out. The important point to note in table 8 is that the levels of free fatty acids in the

Table 6

Effect of L-epinephrine on in vitro lipolysis in adipose tissue.

250 mg of adipose tissue was incubated in 1.95 ml. Krebs Ringer bicarbonate buffer (KRB) containing 2.5% (w/v) albumin for 30 min. Powdered L-epinephrine dissolved in 0.1N HCl was then added to give the indicated concentration in a volume of 2 ml. The incubation was continued for 2 hrs. At that time the amount of free fatty acids in the incubation medium was determined. The values are the average of duplicate readings for two samples at each concentration.

Table 6

Concentration of L-epinephrine (M)	nmoles FFA/L mg. adipose tissue
0	3.4
1×10^{-6}	3.4
1×10^{-5}	18.2
1×10^{-4}	20.9

Table 7

Free fatty acid concentration in fresh and 48 hour conditioned medium.

Medium was removed from bottles containing a similar number of tumor cells and L-cells and these, with the fresh medium, were centrifuged at 900 x g for 10 min. The volume of medium indicated was added to Krebs Ringer bicarbonate buffer containing 2.5% (w/v) albumin to give a final volume of 2 ml. and incubated at 37° C for 2 hrs at which time the amount of free fatty acids in the incubation medium was determined. The values are the means of duplicate samples from 4 bottles. The results are expressed ± SEM.

Table 7

volume of medium added	nmoles FFA/L		
	Tumor cell medium	L-cell medium	Fresh medium
(ml)			
0	0.685 ± .139	0.685 ± .139	0.685 ± .139
0.1	0.976 ± .030	0.917 ± 0.074	0.976 ± .045
0.5	1.156 ± .046	1.151 ± .076	1.211 ± .049
1.0	1.635 ± .020	1.640 ± .013	1.624 ± .015

Table 8

Effect of fresh medium and 48 hour conditioned medium on in vitro lipolysis in adipose tissue.

Medium was removed from bottles containing a similar number of tumor cells and L-cells and these, along with fresh medium, were centrifuged at 900 x g for 10 min. Approximately 250 mg of mouse adipose tissue was preincubated in Krebs Ringer bicarbonate buffer containing 2.5% (w/v) albumin, for 30 min. At the end of this time the volume of medium indicated was added to give a final volume of 2 ml. The incubation was continued for 2 hrs and at the end of this time the amounts of free fatty acid in the incubation media were determined. The values are the composite of 6 experiments and are the means of 12 samples. The results are expressed $\pm$ SEM.

Table 8

volume of medium added	nmoles FFA/L mg. adipose tissue		
	Tumor cell medium	L-cell medium	Fresh medium
(ml)			
0	2.8 ± 0.6	2.8 ± 0.6	2.8 ± 0.6
0.1	4.2 ± 0.7	3.8 ± 0.2	4.2 ± 0.4
0.5	5.5 ± 0.9	5.3 ± 0.1	6.6 ± 0.3
1.0	8.2 ± 0.7	7.5 ± 0.3	10.2 ± 0.9

incubation media are similar for both tumor cells and L-cells.

The data from tables 7 and 8 thus show that tumor cells and L-cells use a comparable amount of free fatty acids from fresh medium and that neither possess a significant amount of a releaseable lipolytic factor.

Tables 9 and 10 show the effects of cell homogenates and cell extracts from both mouse #2663 tumor cells and mouse L-929 fibroblasts on in vitro lipolysis. As can be seen in the tables, the addition of mouse #2663 tumor cell homogenates and extracts to the incubation medium containing adipose tissue does not cause significantly more lipolysis then do homogenates and extracts of mouse L-929 cells.

The cell homogenate and extract data and the data from the fresh and forty-eight hour conditioned medium experiments suggest that mouse #2663 tumor cells either do not secrete a lipid mobilizing substance or, that not enough is secreted or remains active during the incubation period. It also indicates that tumor cells and L-cells remove a comparable amount of free fatty acids from the fresh medium suggesting both use free fatty acids at a similar rate.

B. Changes in tissue cyclic AMP levels in response to conditioned medium, cell homogenates and cell extracts.

Adenosine 3'5' cyclic monophosphate levels in adipose tissue slices were determined by incubating them in Krebs Ringer bicarbonate buffer at pH 7.25 for thirty minutes and then adding the test substances. The incubation was continued for ten minutes. At the end of the second incubation the tissue was quickly transferred to a Corex tube and the reaction stopped by

Table 9

Effect of cell homogenates on in vitro lipolysis in adipose tissue.

Tumor cell and L-cell homogenates were made by suspending the same number of cells in saline and disrupting them by sonication until no intact cells could be detected by phase contrast microscopy. Approximately 250 mg of mouse adipose tissue was preincubated in Krebs Ringer bicarbonate buffer (KRB) containing 2.5% albumin (w/v) for 30 min. At the end of this time the volume of homogenate indicated was added to give a final volume of 2 ml. The incubation was continued for 2 hrs. At the end of this time the amount of fre fatty acid in the incubation medium was determined. The values are the means of duplicate reading for 6 samples at each volume. The results are expressed ± SEM.

Table 9

volume of homogenate added (ml.)	nmoles FFA/L mg. adipose tissue	
	Tumor cell homogenate	L-cell homogenat
0	1.8 ± 0.1	1.8 ± 0.1
0.1	2.5 ± 0.3	2.4 ± 0.1
0.5	3.6 ± 0.2	3.5 ± 0.2
1.0	4.2 ± 0.3	4.5 ± 0.1

Table 10

Effect of cell extracts on in vitro lipolysis in adipose tissue.

Cell extracts for both groups are the 100,000 x g supernatant and were prepared by suspending an equal number of tumor cells and L-cells in saline and sonicating them until no intact cells could be detected by phase contrast microscopy and then centrifuging them for 1 hr. at 100,000 x g. Approximately 250 mg of mouse adipose tissue was preincubated in Krebs Ringer bicarbonate buffer containing 2.5% albumin (w/v) for 30 min. At the end of this time the volume of extract indicated was added, to give a final volume of 2 ml. The incubation was continued for 2 hrs. At the end of this time the amount of free fatty acid in the incubation medium was determined. The values are the means of duplicate readings for 6 samples at each volume. The results are expressed $\pm$ SEM.

Table 10

volume of extract added (ml.)	nmoles FFA/L mg. adipose tissue	
	Tumor cell extract	L-cell extract
0	1.8 ± 0.1	1.8 ± 0.1
0.1	2.5 ± 0.1	3.1 ± 0.1
0.5	3.6 ± 0.2	4.5 ± 0.1
1.0	4.6 ± 0.3	5.6 ± 0.3

adding trichloroacetic acid and homogenizing the contents of the tube. Cyclic AMP was extracted and determined by the competitive binding assay of Gilman (1970) as modified by Brown et. al. (1971). Initial studies in which both incubating medium cyclic AMP levels and tissue cyclic AMP levels were determined together resulted in recovery values for cyclic AMP of less than 10%. When tissue and medium levels of cyclic AMP were determined separately the levels of recovery in tissue was approximately 30% while that for medium was less than 10%. Because of the interference with recovery of cyclic AMP by medium, cyclic AMP levels were determined in tissue only. Table 11 shows that the amount of cyclic AMP in the adipose tissue increased slightly with increasing amounts of fresh medium and forty-eight hour conditioned medium from L-cells and tumor cells. Although cyclic AMP levels in the adipose tissue were slightly higher for the tissue incubated with conditioned medium from the tumor cells, the range of the increase of cyclic AMP was comparable for the three media tested. These data would seem to indicate that the increases in cyclic AMP were due to an increase in the amount of serum containing fresh or forty-eight hour conditioned medium added rather than to a substance secreted into the medium by the cells. Tables 12 and 13 show the effect of cell homogenates and cell extracts on cyclic AMP levels in adipose tissue. The data indicate that increasing amounts of cell homogenates or cell extracts prepared from mouse #2663 tumor cells or mouse L-929 fibroblasts and added to the incubation medium cause cyclic AMP levels to increase in adipose tissue. Although the cyclic AMP levels in adipose tissue

Table 11

Effect of fresh medium and 48 hour conditioned medium on cAMP levels in adipose tissue.

Medium was removed from bottles containing a similar number of tumor cells and L-cells and these, along with fresh medium, were centrifuged at 900 x g for 10 min. Approximately 250 mg of mouse adipose tissue was preincubated in Krebs Ringer bicarbonate buffer for 30 min. At the end of this time the volume of medium indicated was added to give a final volume of 2 ml. The incubation was continued for 10 min. At the end of this time the amount of cAMP in the adipose tissue was determined. The values are the means of duplicate readings of samples at each time period.

Table 11

volume of medium added	picomoles cAMP / mg. adipose tissue protein		
	Tumor cell medium	L-cell medium	Fresh medium
(ml)			
0	43.0	43.0	43.0
0.1	64.0	60.0	53.0
0.5	74.0	66.0	64.0
1.0	81.0	78.0	72.0

Table 12

Effect of cell homogenates on cAMP levels in adipose tissue.

Tumor cell and L-cell homogenates were made by suspending the same number of cells in saline and disrupting them by sonication until no intact cells could be detected by phase contrast microscopy. Approximately 250 mg of mouse adipose tissue was preincubated in Krebs Ringer bicarbonate buffer for 30 min. At the end of this time the volume of medium indicated was added to give a final volume of 2 ml. The incubation was continued for 10 min. and at the end of this time the amount of cAMP in the adipose tissue was determined. The values are the means of duplicate readings of 6 samples at each volume. The results are expressed $\pm$ SEM.

Table 12

volume of homogenate added (ml.)	picomoles cAMP / mg. adipose tissue protein: Tumor cell homogenate	L-cell homogenate
0	64 ± 7	64 ± 7
0.1	166 ± 7	98 ± 3
0.5	237 ± 1	274 ± 4
1.0	432 ± 20	433 ± 17

Table 13

Effect of cell extracts on cAMP levels in adipose tissue.

Cell extracts for both groups are the 100,000 x g supernatant and were prepared by suspending an equal number of tumor cells and L-cells in saline and sonicating them until no intact cells could be detected by phase contrast microscopy, then centrifuging them for 1 hr at 100,000 x g. Approximately 250 mg of mouse adipose tissue was preincubated in Krebs Ringer bicarbonate buffer for 30 min. At the end of this time the volume of extract indicate was added, to give a final volume of 2 ml. The incubation was continued for 10 min. At the end of this time the amount of cAMP in the adipose tissue was determined. The values are the means of duplicate readings of 6 samples at each volume. The results are expressed $\pm$ SEM.

Table 13

volume of extract added (ml.)	picomoles cAMP / mg adipose tissue protein: Tumor cell extract	L-cell extract
0	64 ± 7	64 ± 7
0.1	156 ± 18	190 ± 5
0.5	185 ± 3	223 ± 13
1.0	205 ± 14	236 ± 9

stimulated with preparations from the L-929 cells were slightly higher than the levels obtained with the tumor cell preparations it appears that the increases in tissue cyclic AMP levels in both homogenates and extracts, prepared from both cell lines were due to the release from the disrupted cells of substances that stimulate tissue cyclic AMP production or protect against degradation of cyclic AMP.

C. Radioactive labeling of adipose tissue, extraction of lipid and the release of labeled free fatty acids into perifusion medium.

Adipose tissue was labeled by incubating pieces of tissue in minimal essential medium supplemented with sodium bicarbonate, potassium penicillin-G, streptomycin sulfate, fetal bovine serum and 1-C^{14} acetate (2.5 μCi/ml.). The amounts of radioactivity incorporated into triglycerides, diglycerides and phospholipids were determined by extracting the adipose tissue with chloroform and methanol (Folch et. al. 1957), chromatographing the extract on silica gel loaded paper, exposing the dried chromatogram to X-ray film, and then cutting these areas from the original chromatogram and measuring the radioactivity. Figure 2 is an illustration of the separation of the lipid classes obtained by chromatography. Table 14 demonstrates the increased radioactive labeling of the various classes with increasing incubation time. Radioactive labeling of the lipids was maximal at four hours and only slightly decreased for the two major lipid classes for the two hour incubation. Since experiments using the labeled adipose tissue would take approximately two hours, it was decided to use a two hour incubation period for the labeling in order to insure tissue viability throughout the experiment.

Figure 2

Separation of phospholipid, diglyceride and triglyceride by paper chromotography.

250 mg of mouse adipose tissue cut into small pieces was incubated in minimal essential medium supplemented with sodium bicarbonate, penicillin-G, streptomycin sulfate, 5% fetal calf serum and 1-C^{14} acetate (2.5 μCi/ml.) for the indicated times. The tissue was extracted with chloroform and methanol and chromatographed on silica gel loaded paper. The lipid classes were visualized by radic autography and the spots cut from the silica gel loaded paper and measured for radioactivity. This data is representative of 5 similar experiments.

Abbreviations

PL= phospholipid
DG= diglyceride
TG= triglyceride

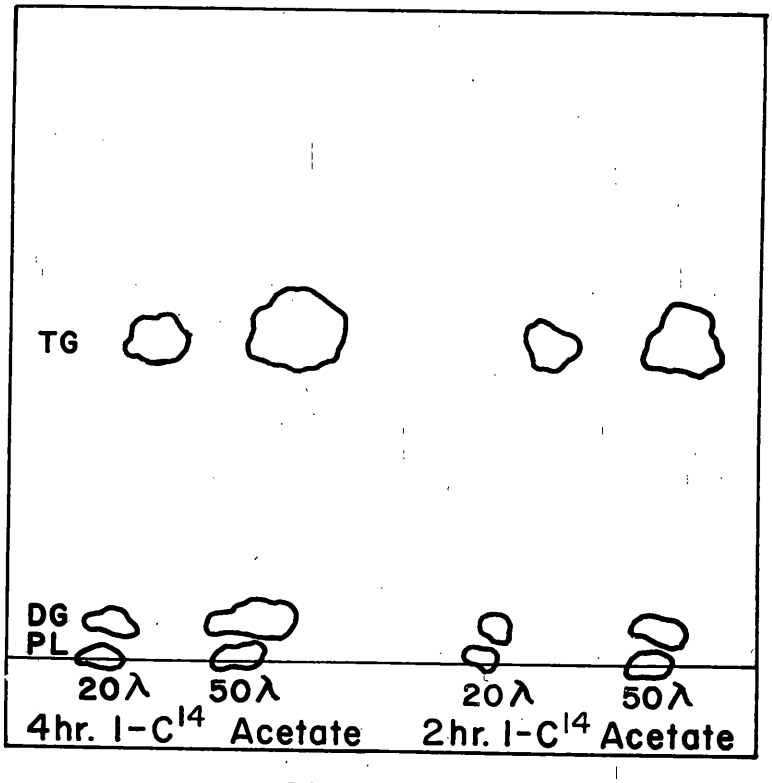


FIGURE 2

Table 14

The incorporation of C^{14} acetate into phospholipids, diglycerides, and triglycerides over a 4 hour period.

250 mg of mouse adipose tissue cut into small pieces was incubated in minimal essential medium supplemented with sodium bicarbonate, penicillin-G, streptomycin sulfate, 5% fetal calf serum and 1-C^{14} acetate (2.5 μCi/ml.) for the indicated times. The tissue was extracted with chloroform and methanol and chromatographed on silica gel loaded paper and measured for radioactivity. This data is representative of 5 similar experiments.

Abbreviations

PL= phospholipid
DG= diglyceride
TG= triglyceride

Table 14

Incubation Time	Lipid Class	CPM/100 mg tissue
.5 hr.	PL	2384
	DG	2634
	TG	4677
1 hr.	PL	2301
	DG	9285
	TG	11,285
2 hr.	PL	5262
	DG	28,494
	TG	35,840
4 hr.	PL	12,088
	DG	31,059
	TG	45,742

Prelabeled adipose tissue was perifused with a Krebs Ringer bicarbonate (KRB) buffer solution, pH 7.25, containing 2.5% (w/v) bovine serum albumin (BSA) fraction V until baseline levels of radioactivity were obtained in the perifusate. At this time the substance to be tested, to which 2.5% (w/v) BSA fraction V had been added, was perifused over the tissue and fractions of the perifusate were assayed for radioactivity. As seen in Figure 3a, L-epinephrine, a known lipolytic agent, releases approximately 3.5 times as much radioactively labeled free fatty acids from prelabeled adipose tissue as does KRB buffer. Figure 3b is a graph of the release of radioactively labeled free fatty acids in response to either fresh medium or medium in which #2663 tumor cells had grown for forty-eight hours. As can be seen from the graph fresh medium causes a slightly greater release of free fatty acids than does forty-eight hour conditioned medium from #2663 cells, suggesting that fresh medium contains slightly more active lipolytic factors than does conditioned medium.

Figure 3a, 3b

Release of radioactively labeled free fatty acids into perifusion medium.

Approximately 250 mg of mouse adipose tissue was prelabeled by incubation in minimal essential medium containing 1-C^{14} (2.5 µCi/ml. was perifused for 40 min. with Krebs Ringer bicarbonate buffer. At the end of this time the tissue was perifused with L-epinephrine (1×10^{-5}M), fresh medium adjusted to the pH of 48 hr. conditioned medium, or 48 hr. conditioned medium from #2663 tumor cells for 20 min. At the end of this time period the tissue was perifused an additional 40 min. with Krebs Ringer bicarbonate buffer. Fractions containing 2 ml. were collected every 4 min. and 0.5 ml. of these were assayed for radioactivity.

o 1. L-epinephrine, 1×10^{-5} M

□ 2. Krebs Ringer bicarbonate buffer pH 7.25

⊗ 3. Fresh medium, pH adjusted to that of 48 hr. conditioned medium

x 4. 48 hour conditioned medium from #2663 tumor cells

FIGURE 3a

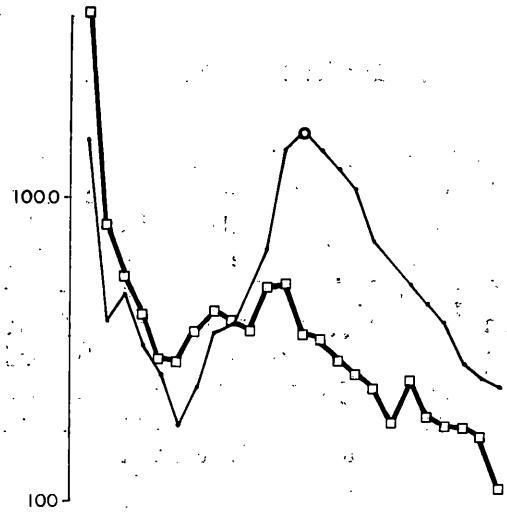
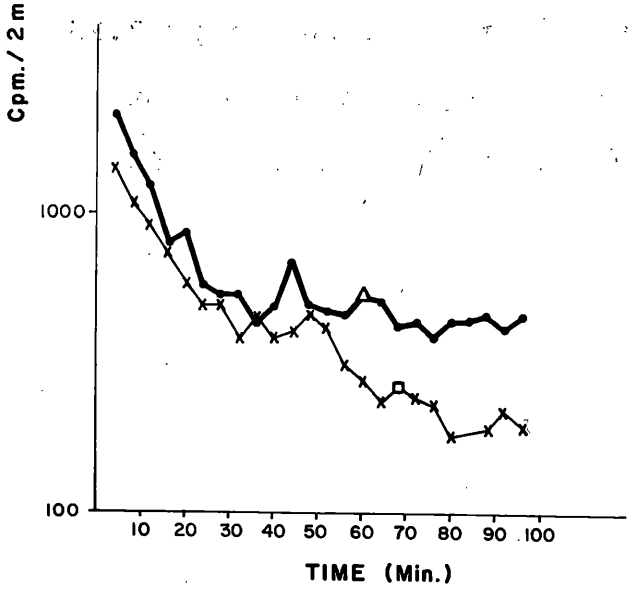


FIGURE 3b



DISCUSSION

Anorexia and cachexia in cancer patients can cause serious clinical problems and none of the current theories of how tumors exert their effects on the host's metabolism adequately explain these clinical responses. A possible explanation of anorexia and cachexia that has not been fully explored is the possible involvement of lipid mobilization seen with some cancers. It has been reported that in the latter stages of certain types of malignant diseases, of both human and laboratory animals, there is a greater loss of body weight, with an accompanying hyperlipemia, than can be explained by decreased appetite. If this hyperlipemia is produced by substances associated with the tumor and is accompanined by an impairment of the body's ability to utilize the lipids that have been made available, then anorexia and cachexia may, in part, be explained by postulating that the hyperlipemia interferes with the body's appetite control mechanism and this, coupled with impaired utilization, allows the body to starve. The present study examines whether the cachexia and lipid mobilization seen in animals bearing the mouse stomach carcinoma #2663 is a direct response to a lipid mobilizing factor produced by the tumor or is a stimulation of normal physiological lipolysis by the presence of this tumor.

The *in vivo* data from Tables 1, and 2, utilizing tumor cells and L-cells demonstrate the selective ability of the mouse #2663 tumor cell to cause massive lipid mobilization and indicates that

this mobilization is not a response of mice injected with cultured mouse cells.

It was reported by Liebelt and co-workers in 1974 that when CBA$^-$ mice were injected with homogenates made from #2663 tumor cells a decrease in their lipid index was observed in four days. This suggested that the tumor cells contained a lipid mobilizing factor that could be released by cell disruption. In an effort to verify these results, an experiment was performed using mice of the same strain, sex, and age, injected with homogenates made from a similar number of cells and sacrificed on the same time schedule. The results from this experiment are presented in Tables 3 and 4 and are opposite from those obtained by Liebelt in 1974. There are two possible explanations for the differing results: 1) the mouse #2663 tumor cells have undergone a change in later passages in culture or 2) the homogenates prepared by Liebelt contained intact tumor cells. Since the tumor cells used in this experiment were a later passage of the same cells used by Liebelt and have been maintained under identical conditions in the same laboratory and still demonstrate the <u>in vivo</u> lipid mobilizing ability and the same growth characteristics in culture, it seems unlikely that the cells have undergone a major change in character. The second possibility, that the homogenates prepared in the earlier study contained viable cells, was inadvertently tested when homogenates of the cells were prepared in saline using a Polytron homogenizer. This treatment was assumed to cause cell lysis and the Polytron homogenate thus formed was injected into the animals. The results of this experiment, reported in table 5, are in agreement with those of

Liebelt et. al., (1974). However, upon reexamination of the homogenization procedure it was found that the use of the Polytron caused very little lysis. The experiment was repeated using homogenates made by sonicating tumor cells suspended in saline on a Sonifier Cell Disruptor until no intact cells would be detected by phase contrast microscopy and injecting this homogenate. The results shown in Table 3 are from an experiment performed with sonicated cells and demonstrates that neither homogenates of mouse #2663 cells nor mouse L-929 cells cause in vivo lipid mobilization. Thus it seems most likely that the difference between the results of Liebelt's study and those described here are due to the presence of viable cells in the homogenate of the former study and that these were sufficient to cause a decrease in the fat depot weight without the tumor being detected.

It should be noted that tumors arose in all the animals that received the Polytron homogenates of tumor cells and that they were detectable after four days. These lesions were induced by twice daily injections for four days of approximately 4×10^5 cells for a total cell injection of 3.2×10^6 cells. Normally the tumors, induced in animals with a single injection of 2.5×10^6 cells, are detectable only after ten to fourteen days, and only appear with this dosage level in about seventy-five percent of the animals. This dosage level was originally decided upon by Dr. R. A. Liebelt because it was estimated that the piece of tumor tissue transplanted by the trochar method (Liebelt and Liebelt, 1967) and known to initiate lipid mobilization contained approximately this number of cells. A secondary reason was that while increasing the number of cells to approximately 3.2×10^6 cells per in-

jection increased the percentage of tumors appearing, it did not decrease the time at which they were detectable. A single injection of more than 3.2×10^6 cells did not increase the percentage of tumors appearing or decrease the time of their appearance. The results described here suggest that multiple injections of a small number of cells produce tumors more efficiently than a single injection of a large number of cells. The increased effectiveness of multiple injections may result from either the first few injections providing a protective covering for the following injections thus protecting them from non-specific attacks by macrophages or that they might stimulate the production of blocking or enhancing antibodies in the host that serve to protect the injected tumor cells from the lytic activities of immune lymphoid cells. This later possibility is time dependent and may or may not have time to occur. The net effect of the several injections seems to be that more viable cells remain present for establishment of the tumor than with a single injection, thus increasing the percentage of tumor production and also decreasing the time required for them to appear.

The in vivo data has enabled the quantification of the loss of depot fat for CBA⁻ lean male hosts: it is approximately thirty milligrams per day per animal and represents a thirty-eight percent total decrease in depot lipid weight at the end of twenty-six days. This value agrees well with the values reported by Liebelt et. al., (1971), which ranged from twenty-eight to thirty-six percent. These studies also provided evidence that suggests that the tumor cells do not contain a lipid mobilizing factor that can be released by cell disruption and thus suggests that the decrease

in the weight of the depot fat is not the result of a direct acting lipid mobilizing substance produced by the tumor cells.

In order to verify the in vivo results and test for a direct acting lipid mobilizing substance a series of experiments was performed in which attempts were made to stimulate mobilization from adipose tissue with fresh medium, forty-eight hour conditioned medium, cell homogenates and cell extracts, with the degree of stimulation being determined by the amount of free fatty acids released into the incubation medium as well as the amount of increase in tissue cyclic AMP levels. Tissue levels of cyclic AMP were determined for two reasons. First, because it has been reported by Butcher (1965), and confirmed by several other investigators (Weiss et. al., 1966; Humes et. al., 1968; Huttunen and Steinberg, 1971,1971; Khoo et. al, 1974), that an increase in cyclic AMP levels in adipose tissue occurs prior to an increase in lipolysis, and second because of the greater sensitivity of the assay, it seemed more likely to show smaller changes in lipolysis than did measurement of free fatty acid release.

In those experiments in which adipose tissue was stimulated with cell homogenates or cell extracts from mouse #2663 cells, the release of free fatty acids from the tissue was not significantly greater than the release caused by control cell homogenates or extracts. When cyclic AMP levels in adipose tissue stimulated with homogenates and extracts were determined, it was found that the cyclic AMP levels in the tissue increased with the addition of increasing amounts of cell homogenates or extracts. This was true for both #2663 tumor cells and L-929 cells. This suggested that the

ability of the homogenates and extracts to stimulate the increase in cyclic AMP levels was due to cyclic AMP modulators common to both cells and not specific for the tumor cells. The inability of the tumor cell homogenates or extracts to cause an increase in the levels of free fatty acids in the incubation medium or the levels of cyclic AMP in the adipose tissue over those levels observed with control L-929 cell homogenates and extracts *in vitro* is consistent with the *in vivo* data and is evidence against the presence of a direct acting lipid mobilizer in the tumor cells.

In experiments in which fresh medium and forty-eight hour conditioned medium were added to the incubation medium and incubated for two hours, the data indicate that as the amount of medium increased so did the detectable amounts of free fatty acids in the incubation medium. It is apparent from table 7 and 8 that tumor cells and L-cells remove a similar amount of free fatty acids from fresh medium and that neither possess a significant amount of a releaseable lipolytic factor.

The *in vivo* data indicate that viable mouse #2663 tumor cells injected into CBA⁻ mice do cause a significant loss of depot fat and that the weight is lost at approximately 30 mg per day. *In vitro* attempts to induce the release of free fatty acids from adipose tissue and to increase cyclic AMP levels in adipose tissue as well as *in vivo* attempts to duplicate the loss of depot fat with preparations other than viable cells were not successful. Therefore, the presence of a direct acting lipid mobilizer produced by the tumor cells was examined from several points of view and the failure of the *in vivo* experiments using cell homogenates

and extracts and of all the *in vitro* experiments to cause lipid mobilization is strong evidence against the lipid mobilization observed *in vivo* being caused by a direct acting substance produced by the growing tumor.

There are several possible ways in which the tumor cells could cause their effect *in vivo* that are consistent with both the *in vivo* and *in vitro* data reported. 1) The tumor cells themselves might be altered in such a way that oxidative phosphorylation is uncoupled. This would cause the tumor cells to use fatty acids at the normal rate, which is supported by the data in tables 7 and 8, while deriving little or no energy from their oxidation. A secondary effect of this would be that the tumor cells would depend more heavily on glycolysis for their energy needs, thus increasing their use of glucose. The increased use of glucose would result in lower blood glucose levels, thus initiating by normal physiological pathways the release of glycogen and free fatty acids. Since the tumor cells are continually growing, the need for glucose by them would continue to grow and cause depletion of the host's glycogen supplies. The host would then be similar to an animal under starvation conditons and would divert tricarboxylic acid cycle intermediates toward gluconeogenesis and increase the oxidation of fatty acids to help meet the energy requirements of the host. The result of these changes would be a persistent hyperlipemia, a loss of depot fat and the wasting of the animal's body. All of these are observed *in vivo*. 2) The energy demand placed on the host by this growing tumor could cause a decrease in the blood glucose levels thus initiating gluconeogenesis in the liver and the release of free fatty acids from adipose tissue. Since the tumor is

continuing to grow, the energy demand would continue to increase while the glucose supply decreases. This would be manifested the same as if it were due to the uncoupling of oxidative phosphorylation. Either of these mechanisms for an indirect effect are consistent with both the in vivo and in vitro data and are supported by preliminary studies (McChesney and Schuster, unpublished data) that indicate that the tumor cells use approximately three times as much glucose as L-cells. 3) The tumor cells could produce a substance that would suppress pancreatic lipase, thus altering the absorption of fatty acids from the small intestine. This would impede the host's ability to replace depot fat. This , coupled with an increased energy demand placed on the host by the tumor, would serve to lower blood glucose levels and lead to the release of free fatty acids from adipose tissue by normal physiological means. Coupled with an impaired ability to absorb fatty acids from the intestine, this would cause an accelerated loss of depot fat and a persistent hyperlipemia. Althoughthe in vivo cell homogenate and extract data seem to rule this out, it is possible that the continual presence of the substance that suppresses pancreatic lipase is required. 4) The tumor could synthesize a substance that could cause the release of a lipolytic substance from the anterior pituitary gland, or of epinephrine from the adrenal medulla. One could postulate that the continual presence of one or more of these lipolytic substances could accelerate the loss of depot fat and also produce the persistent hyperlipemia. The in vivo cell homogenate and extract data would seem to rule out this possibility. It is however possible that the continual presence of the substance that stimulates the release of the lipolytic hormones is required for

increase in lipid mobilization to be observed. Thus these last two possibilities would both require viable tumor cells to be present.

The present study has provided a variety of data against the *in vivo* lipid mobilization being a direct response to a substance produced by the tumor and by so doing has suggested that the actual lipid mobilization is probably due to the stimulation of the normal physiological activators of lipolysis. It is postulated that the tumor cells effect the host in such a way that one or more of the normal activators (catecholamines, glucagon or peptide hormones) of adipose tissue adenylate cyclase are released thus causing the subsequent activation of lipases which can initiate the release of free fatty acids from triglycerides. The release of these activators of cyclic AMP would be due to: 1) the uncoupling of oxidative phosphorylation in the tumor cells, 2) an increase energy demand placed on the host by the tumor's growth and its increased glucose requirement, 3) the presence of a substance, produced by the tumor that suppresses pancreatic lipase, 4) synthesis by the tumor of a substance that stimulates the release of a lipolytic factor from the anterior pituitary gland or the adrenal medulla.

SUMMARY

1. Animals bearing tumors induced by mouse #2663 tumor cells show a loss of weight from their fat depots and cachexia after carrying the tumor for forty days.
2. Viable #2663 tumor cells cause a significantly greater loss of depot fat than do sham injected controls or animals injected with mouse L-929 cells.
3. Cell homogenates and cell extracts from mouse #2663 tumor cells do not cause *in vivo* loss of depot fat.
4. Cell homogenates and cell extracts from #2663 tumor cells do not increase the *in vitro* release of free fatty acids from adipose tissue or the levels of cyclic AMP in adipose tissue any more than do similar L-929 cell preparations.
5. Forty-eight hour conditioned medium from mouse #2663 tumor cells does not stimulate the release of free fatty acids from adipose tissue into incubation medium as determined by direct measurements or measuring the release of radioactively labeled free fatty acids nor does it increase adipose tissue cyclic AMP levels any more than does conditioned medium from L-929 cells.
6. Mouse #2663 tumor cells and L-929 fibroblasts utilize a comparable amount of free fatty acids from fresh medium.
7. Mouse #2663 tumor cells apparently do not exert their effect on the host's fat depots by a direct effect. That is, they do not produce a substance which acts directly on the fat depots to mobilize lipids.

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