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# Lipolysis Induction in Adipocytes by a Protein From Tumor Cells

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Extracts of thymic lymphoma that are obtained from AKR mice and are kept in the cold for at least several days can induce lipolytic activity in rat adipocyte suspensions. Freshly prepared extracts have low activity but contain a low molecular weight material of less than 10,000 daltons that aggregates on standing in the cold and becomes active. Treatment of aged extracts with trypsin causes a loss in activity indicating that the active material is a protein. It has been obtained in partially purified form, is relatively heat stable, and is not a lipase.

Activity was also demonstrated in AKRXDBA/2 lymphoma (induced by AKR SL3-3 virus) and in transplanted lymphomas from a Friend-virus-induced erythroleukemia cell line in DBA/2 mice, but was not detected in normal thymus, spleen, liver, or other tissues. The partially purified material produced a massive fat mobilization when injected into normal mice.

**Key words: in vitro lipolysis, protein aggregation**

It has been common experience that cancer-bearing human patients and experimental animals are depleted of their adipose tissues, but the mechanism of the reaction has not been satisfactorily explained [1]. In previous studies using AKR mice with implanted adipose tissue labeled with [1-$^{14}$C]-linoleic acid, we have shown that in the fed mouse bearing a thymic lymphoma, fat is mobilized rapidly and appears largely in the membrane phospholipids of the tumor [2,3]. In the non-tumor-bearing AKR mice with the radioactive adipose graft, little mobilization occurs unless the mice are fasted, which causes an increase in respiratory $^{14}CO_2$. However, injection of serum from tumor-bearing mice into healthy mice carrying the labeled adipose tissue graft causes an immediate massive fat mobilization indicated by a dramatic increase in the expired $^{14}CO_2$ despite adequate food intake. Similar responses were produced by extracts from lymphoma tissue and by culture medium from an in vitro AKR lymphoma cell line (AKR SL3). In contrast, serum from normal mice and extracts of normal thymus have no such effect [3]. From these experiments, it seemed reasonable

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to conclude that the lymphoma cells produce a lipid mobilizing factor or factors that could play a part in adipose tissue depletion.

As a first step toward the goal of identifying and characterizing such a factor or factors, we have examined the effect of tumor extracts on adipose tissue. We report here that lipolysis is indeed induced by a protein obtained from thymic lymphoma. A preliminary characterization of this material is presented.

## MATERIALS AND METHODS

### Experimental Animals

Both male and female AKR mice were used. This strain has an almost 100% mortality from thymic lymphoma between the ages of 6 and 14 months with a peak incidence at nine to ten months [4]. If injected with Gross murine leukemia virus at three days, the onset of the disease is accelerated and peak incidence occurs at 2.7 months [5]. Animals with both spontaneous and virus-accelerated lymphomas were used. ADF1 mice (hybrid of AKR and DBA/2 mice) with lymphomas induced by neonatal inoculation of SL3-3 virus and animals bearing transplanted lymphoma from a Friend-virus-induced erythroleukemia (clone 745) were also studied. Two-month-old DBA/2 mice inoculated subcutaneously with $10^6$ cells develop local tumors at the site of inoculation within 21–28 days.

### Extraction of Tissues

In animals with lymphoma, fresh tissues from thymus, lymph nodes, and spleen that were almost completely replaced by malignant cells were used. In the DBA/2 mice bearing the clone 745 tumor, the subcutaneous tumor was removed, frozen at −70°C for several months and thawed just prior to use. The tissues were homogenized in five volumes of 0.01 M $KPO_4$ buffer (pH 7.2) per gram wet weight. A pH 5 extract was prepared by dropwise addition of 1 N HCl to the homogenate followed by centrifugation at 12,000g for 10 min. The supernatant solution was removed, and the solid material was extracted twice more with five volumes of the same buffer by HCl addition to pH 5 and centrifugation. The three supernatant solutions were combined. All operations were conducted at 4°C.

### Protein Determination

Protein was estimated by the method of Lowry et al [6].

### Digestion With Trypsin

A sample of the pH 5 extract containing 0.5 mg protein in 0.5 ml was incubated with 0.025 mg trypsin at 37°C for 3 hr. At the end of this time, 0.05 mg soybean trypsin inhibitor was added and incubation at 37°C was continued for 1 hr with gentle shaking.

### Preparation of Adipocytes

Adipocytes from epididymal fat pads from male rats were prepared by Rodbell's procedure [7]. The animal was decapitated and epididymal fat pads were removed. To each gram of tissue (wet weight) in a plastic vial, 2 ml Krebs-Ringer-phosphate buffer containing 1% bovine serum albumin (fatty acid-free, Sigma) and 3.3 mg collagenase (Sigma) were added. The vials were shaken vigorously in a water bath at 37°C for 1 hr. Further operations were carried out at room temperature. The liquid

was then filtered through three layers of gauze into a plastic tube fitted with a stopcock at the bottom and was centrifuged briefly at low speed. The cell debris and supernate were removed through the stopcock and the adipocytes were suspended in two volumes of the Krebs-Ringer-phosphate-albumin buffer by gentle mixing with a plastic stirrer. The cells were washed three times more by resuspension and centrifugation and finally suspended in 5 ml of the same solution per gram original tussue.

### Assay for Lipolysis

Lipolytic activity was measured by analysis of glycerol liberated from adipocytes after incubation with tumor tissue extracts. Routinely, a suspension of 0.5 ml of freshly prepared adipocytes was mixed with the material to be tested in a final volume of 1.0 ml and the suspension was incubated at 37°C with gentle shaking for 2 hr. The mixture was then centrifuged briefly at low speed, and up to 0.4 ml of the clear solution was removed from below the top layer of adipocytes. Plastic vessels and pipettes were used to this point. Glycerol assay was carried out by the procedure of Galletti [8]. This is based on periodate oxidation of glycerol and reaction of the formaldehyde released with phenylhydrazine and potassium ferricyanide. The optical density was measured at 520 nm. Controls were carried out in the absence of cells and in the absence of sample, and control values were subtracted from readings obtained with the complete system. The release of 1 $\mu$g glycerol was defined as one unit of lipolytic activity.

## RESULTS

### Induction of Lipolysis by Lymphoma Extracts

The first experiments were carried out with 50–100 mg of washed and minced rat epididymal adipose tissue, but background levels were found to be too variable for routine use. A satisfactory assay was then developed with adipocytes prepared with collagenase and washed thoroughly before use. A freshly prepared cell suspension released only trace levels of glycerol during a 2 hr incubation.

Samples of thymus tumor tissue were extracted by several methods. Tissue was homogenized in 2 N acetic acid, the extract was dried, dissolved in phosphate buffer, and found to cause release of glycerol from adipocytes. However, the total activity recovered was low. Extraction was incomplete even when it was repeated several times. Specific activity levels were in the range of 1–3 units/mg of protein.

When tissues were homogenized in 0.01 M potassium phosphate buffer and the whole homogenate was incubated with adipocytes, some activity could also be detected. An extract prepared by addition of HCl to pH 5 and removal of insoluble material resulted in a partial purification of the active factor. However, the results were highly variable. Similar tumor tissue sometimes had little activity and at other times gave relatively active preparations. The variability was eventually found to be a function of the time at which the preparation was allowed to stand before examination for lipolytic activity. Freshly prepared homogenates or pH 5 extracts had little activity. Samples allowed to age at pH 5 in the cold for at least several days became active. This is illustrated in Table I. It may be seen that significant lipolytic activity began to appear in the whole homogenate after four or five days and in the pH 5 extract after about two days. The maximum level, a specific activity of 200 units/mg protein, was reached after about one week. This was the most active preparation obtained so far. In other experiments, specific activities of 60 to 100 were seen. There was some difference in adipocyte response from day to day, and cells from young rats

gave a better response than did those from older rats.

Purifications of between 10- and 20-fold were obtained by the pH 5 treatment with yields of about 60%. When fresh pH 5 extracts were adjusted to pH 7 and allowed to stand in the cold, activation also occurred and similar levels of activity were reached.

For most of the experiments described below, pH 5 extracts held at 4°C for at least five days were used and were kept frozen after aging.

In all cases, experiments were performed several times and typical results are presented.

## Properties of the pH 5 Extract

The effect of the concentration of a pH 5 extract on the release of glycerol from adipocytes is shown in Figure 1. It may be seen that the response is linear to about 36 μg protein. Incubation of adipocytes with a pH 5 extract was carried out for various times as shown in Figure 2. Lipolysis was complete in about 2 hr.

To determine the nature of the active material, a sample of pH 5 extract was digested with trypsin, the reaction was stopped with soybean trypsin inhibitor, and the solution was assayed. Trypsin treatment was found to completely destroy lipolytic activity.

The heat stability of the active factor was tested by heating a sample of the pH 5 extract at 80°C for 5 min. After heating, 75% of the lipolytic activity was retained.

The presence of a lipase in the pH 5 extract was examined by incubation of a sample of the active material with 250 μg of triolein under the standard conditions of the assay. No glycerol was released, indicating the absence of a triglyceride lipase in the pH 5 extract.

## Aggregation of the Factor

The requirement for aging of lymphoma extracts indicated that some kind of modification is associated with increased adipokinetic activity. To examine this phenomenon further, thymic lymphoma was homogenized, pH 5 extract was prepared, and a portion of the freshly prepared solution was adjusted to pH 7.2 and passed

**TABLE I. Lipolytic Induction as a Function of Time at 4°C***

| | Whole homogenate | | pH 5 extract | |
|---|---|---|---|---|
| Day | Units/ml | Units/mg protein | Units/ml | Units/mg protein |
| 0 | trace | trace | trace | trace |
| 1 | trace | trace | trace | trace |
| 2 | trace | trace | 0.83 | 1.89 |
| 3 | trace | trace | 2.5 | 5.7 |
| 4 | 25.0 | 1.28 | 20.0 | 45.5 |
| 6 | 100.0 | 5.14 | 30.0 | 100.0 |
| 8 | 313.0 | 16.1 | 60.0 | 200.0 |
| 10 | 175.0 | 9.0 | 40.0 | 133.0 |

*Whole homogenate and pH 5 extract were allowed to stand at 4°C for the indicated length of time. Samples were then incubated with rat adipocyte suspensions in Krebs-Ringer-phosphate-albumin solutions for 2 hr at 37°C and analyzed for lipolytic induction.

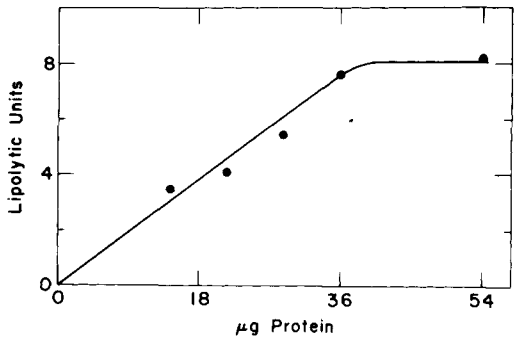


Fig. 1. Relationship of amount of protein to lipolytic activity. Cell suspensions were incubated at 37°C for 2 hr with increasing amounts of pH 5 extract and lipolytic activity was measured as described in the text. Concentration of protein in the pH 5 extract was 0.72 mg per ml.

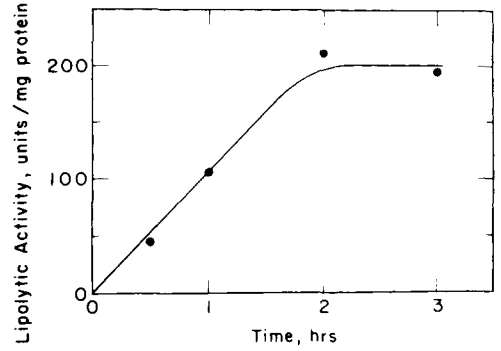


Fig 2. Effect of incubation time. The pH 5 extract, 50 μl (36μg protein), was incubated with cell suspensions at 37°C for various time periods and lipolytic activity was measured.

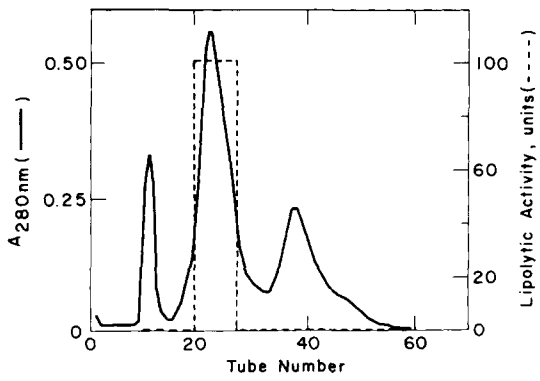


Fig. 3. Gel filtration of pH 5 extract on a Sephadex G-25 column. Fresh pH 5 extract, 2.0 ml, was applied to a Sephadex G-25 column (1.5 × 22.5 cm) equilibrated with 0.01 M potassium phosphate buffer (pH 7.2). Elution was carried out with the same buffer. Fractions of 1.6 ml were collected and analyzed for protein by absorbance at 280 nm. Fractions were pooled as indicated and assayed for lipolytic induction.

through a Sephadex G-25 column. The elution pattern is shown in Figure 3. When fractions were pooled, as indicated, and assayed, no activity was found. However, after standing for one week in the cold, the retarded fraction (tubes 19–27) was found to induce lipolytic activity. This active fraction was concentrated by lyophilization and passed through the same column. Now, all the activity appeared in the void volume.

A second sample of aged pH 5 extract was filtered through a Sephadex G-50 column. In this case, most of the activity was found in the void volume, containing fractions of large size.

### Adipokinetic Activity in Normal Tissue and Other Tumor Tissue

Cells from two other tumors, ADFl-virus-induced lymphoma and transplanted erythroleukemia in DBA/2 mice and from normal thymus, spleen, liver, kidney, lung, and muscle tissue of healthy AKR mice and of healthy rats were homogenized in dilute phosphate buffer, brought to pH 5, and the supernatant solutions were allowed to stand in the cold for about a week in the same manner as for the AKR thymic lymphoma. Lipolytic activity in the pH 5 extracts was then assayed (Table II). It may be seen that both tumor extracts were active, but normal tissue had no detectable activity.

### In Vivo Activity of pH 5 Extract

To test whether the aged pH 5 extract of thymic lymphoma had lipid mobilization activity in vivo, [$^{14}$C]-adipose tissue was grafted into a healthy AKR mouse as described earlier [3]. Injection of the tumor extract caused a rapid release of $^{14}CO_2$, while an extract of normal thymus had no effect (Fig. 4).

## DISCUSSION

The stimulus for the experiments described here was the observation, reported previously [3], that extracts of a murine thymic lymphoma cause a rapid lipid mobilization when injected into healthy mice. It is significant that lipid mobilization was produced by serum from tumor-bearing mice and cancer-bearing human patients and particularly that it was produced by medium from the culture of cancer cells. Extracts of normal tissues and serum from healthy subjects had no such effects. These results indicate that the factor is produced by the tumor cells themselves and is not the product of normal cells. These in vivo experiments, however, could not distinguish between a direct action of the factor on adipose tissue or an indirect one mediated through other organs or tissues resulting in lipid mobilization.

The present experiments demonstrate that the active material is a protein and that it acts directly on adipocytes causing them to release glycerol (and fatty acids) from triglycerides. In the latter respect, it is similar to other adipokinetic substances,

**TABLE II. Lipolytic Induction by Various Tissues**

| pH 5 extract[a] | Units/mg protein |
|---|---|
| AKR lymphoma | 78.4 |
| ADF1 lymphoma | 90.0 |
| Erythroleukemia | 30.2 |
| Normal thymus | 0 |
| Normal spleen | 0 |
| Normal liver | 0 |
| Normal kidney | 0 |
| Normal lung | 0 |
| Normal muscle | 0 |

[a]pH 5 extracts were prepared, allowed to stand at 4°C for a week, incubated with rat adipocyte suspensions, and analysed for lipolytic induction.

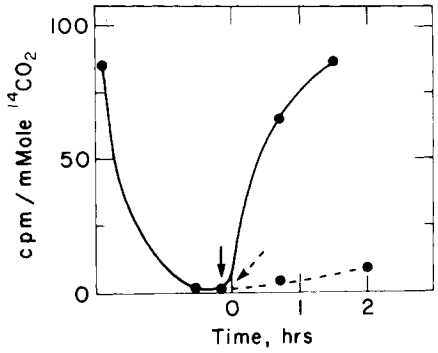


Fig. 4. Effect on respiratory $^{14}CO_2$ of injection of pH 5 extract of control or lymphoma-bearing mice. Solid arrow shows injection of lymphoma thymus pH 5 extract (50 $\mu$l) through the tail vein. Dotted arrow shows injection of control thymus pH 5 extract (50 $\mu$l) through the tail vein. See reference 3 for details of the procedure.

such as the catecholamines, which have no lipolytic activity in themselves but cause lipolysis by their reaction with receptor sites on the cell surface of the adipocytes. The pituitary peptide hormone, $\beta$-lipotropin [9], may have a similar action.

It is particularly interesting that only small amounts of activity are present in freshly prepared extracts. Full activity, usually more than 100-fold greater, appears only after standing. In additional experiments (to be reported later), activation has been induced very rapidly under more physiological conditions (temperature, metal ion concentration, presence of sulfhydryl groups). Activation occurs not only in whole homogenates and in pH 5 extracts but in a fraction of low molecular weight separated on Sephadex. Activation is associated with an increase in molecular weight; this argues for activation as an aggregation phenomenon and not as a covalent modification. It is important to note that activity was not found in any extract of nonneoplastic tissue treated in exactly the same manner.

Why the lymphoma should produce and secrete an inactive small protein is not obvious. Possibly a peptide of this size is more readily released from the cell, or it is possible that a strong lipolytic action within the tumor cell itself is avoided in this manner. Answers to these questions must await the results of further research. It might be recalled, however, that possibly for similar reasons, the important enzyme, acetyl coenzyme A carboxylase, is also produced by the cells as an inactive protomer, that is aggregated and activated on incubation in the presence of tricarboxylic acids [10,11] or on standing in their absence [12].

The existence of a lipid mobilization factor has been suspected by a number of workers in this area [13–16]. Recently, a lipolytic factor called Toxohormone-L has been isolated from cell-free fluid of ascites Sarcoma 180 [17]. Toxohormone-L is a protein of 75,000 daltons. On treatment with trypsin much of the activity remains. On the basis of these observations Toxohormone-L and the factor described here are not the same substance. Further characterization of the substance identified in this paper is in progress.

## ACKNOWLEDGMENTS

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