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## Fat-Mobilizing Substance (FMS)

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FAT-MOBILIZING SUBSTANCE (FMS)

by

Dorothy A. Krupa

A Thesis Submitted to the Faculty of the Graduate School  
of Loyola University of Chicago in Partial Fulfillment  
of the Requirements for the Degree of  
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## VITA

The author, Dorothy A. Krupa, is the daughter of Walter R. Kulpinski and Mildred (Bowbin) Kulpinski. She was born in Chicago, Illinois.

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## CHAPTER I

### INTRODUCTION

An agent of biological origin endowed with the specific capacity of accelerating the mobilization of depot fat was described by Best and Campbell (1936) as occurring in the anterior pituitary gland. This adipokinin (adipokinetic substance) concentrated from aqueous extracts of beef anterior pituitary glands by precipitation, at pH 5.2, with 2 volumes of alcohol, upon injection into the fasted mouse produced an increase in the quantity of liver fat which was, within limits, linearly related to the dose injected; moreover, it accelerated the mobilization of depot fat and caused ketonemia. Weil and Stetten (1947), entertaining that variations might occur in the quantity of adipokinetic substance released under conditions of starvation or diabetes that might, in turn, be reflected by variations in an analogous activity in the urine, used a similar extraction procedure and were the first to show that lipid mobilizing activity could be demonstrated in the urine during fasting. These authors found that an alkaline extract of urine from fasting rabbits when injected into mice caused an increase in liver fat. Similarly prepared extracts of urine from fed rabbits had no such effect. Concurrently, it is still given to speculation, whether the

adipokinetic activity demonstrated to occur in the urine of the fasted rabbit is identical with that shown by Best and Campbell (1936) to occur in the normal anterior pituitary gland. However, during fasting and under conditions in which an increase in the mobilization of depot fat might be anticipated, there appears in the urine a material capable of provoking such an increase, which at least superficially resembles pituitary adipokinin (Weil and Stetten, 1947).

Chalmers et al. (1958) extended these observations to humans, based on studies on obese individuals. It appeared that a mechanism existed for controlling the mobilization of depot fat which must be sensitive to changes in the composition as well as the caloric content of the diet. Such a mechanism should be operating at its maximum during fasting, and if any hormonal agent were involved it should be detectable in the urine (Chalmers et al., 1958).

Therefore, Chalmers et al. (1958) studied the effect on fat metabolism of urine collected from healthy people during periods of fasting. Subjects were fasted for thirty-six hours. From the urine collected during the last twenty-four hours of this period, a fat-mobilizing material could be precipitated at pH 5.3 with two volumes of ethanol. Subsequent separation, and extraction of the material with dilute sodium carbonate solution resulted in an alkaline extract which, upon injection into mice, produced an increase

in liver fat. Animals receiving the urine extracts from the same subjects on a normal diet, without fasting, had liver fat values very similar to those found in saline-injected animals.

When the dose of fasting urine extract was varied by Chalmers (1960a) the increase in liver fat was found to be linearly related to the log dose. For convenience, the active material was referred to as Fat Mobilizing Substance (FMS) (Chalmers, 1965).

Further details of the characteristics of FMS were reported by the same authors and they postulated that FMS mobilizes fatty acids from adipose tissue by directly augmenting the lipolytic reaction. FMS activity did not appear in the urine of fasted hypophysectomized man or goat and Chalmers et al. (1960a) concluded that the pituitary (or adjacent hypothalamus) was necessary for its production. Comparison of FMS to known lipolytic substances of pituitary origin reveals important differences and FMS does not appear to be any of those lipolytic substances (Chalmers, 1965).

Following the extraction of FMS from the urine of fasting man, Stevenson et al. (1964) reported the extraction of a similar material (FMS I) by essentially the same procedure from the urine of fasting rats that also showed fat mobilizing and hypoglycemic properties in vitro and in vivo. FMS extracted from the urine of fasting rats caused an increase in release of free fatty acids from rat epididymal

fat pads in vitro and upon injection into intact rats produced a weight loss, transient hypoglycemia, hyperlipemia, and an increase in fatty acid content of the liver. FMS I differed from the material isolated by Chalmers et al. in that a single subcutaneous injection in the rat caused a transient but pronounced decrease in food intake. Beaton et al. (1964) fractionated FMS I into FMS IA and FMS IB on the basis of solubility in alkaline and water solutions. These two fractions were chemically distinct, the anorexigenic property of FMS I was attributable to the IA component and the fat mobilizing property to the IB component. FMS activity was also detected in the urine of fasting mice, dogs, sheep, goats and horses (Kekwick et al., 1967) but little information has been reported on the nature of these fat-mobilizing materials.

FMS has also been extracted from the urine of fasting chickens (Nir et al., 1969). The method used to extract FMS was essentially that of Chalmers et al. (1958) as modified by Stevenson et al. (1964) and Beaton et al. (1964).

#### Dietary Aspects and Application of Stress in Conditions in which FMS Activity is Found

FMS activity can be detected in human urine after about 12 hours of fasting (Chalmers, 1965). Low-calorie diets also elicit activity provided they contain little carbohydrate (Chalmers et al., 1960a). It disappears when caloric intake is adequate for maintenance, but reappears

during caloric deprivation (Gordon, 1970). On normal unrestricted diets, or low-calorie high-carbohydrate diets, activity is absent (Chalmers et al., 1960a). It seems to be most closely related to carbohydrate intake since dietary reduction of this specific nutrient will cause FMS to appear in the urine. In the human, a critical intake of 80 grams of carbohydrate daily is needed to prevent its appearance (Gordon, 1970).

However, as noted by Kekwick and Pawan (1967), patients who have had a pituitary ablation for carcinomatosis and who have been fasted or deprived of carbohydrates are unable to produce detectable amounts of FMS in the urine (Chalmers et al., 1960a). Chalmers et al. (1960a) observed no detectable fat mobilizing or ketogenic activity in the urine in patients with well-established pituitary deficiency receiving cortisone and thyroid in replacement dosage or in conditions of severe carbohydrate restriction or fasting up to 36 hours. Kekwick and Pawan (1967) reported that no activity could be detected in hypophysectomized fasting goats, as in human subjects with abnormal or absent pituitaries. An intact pituitary gland appears necessary for the production of FMS.

The effect of progressive restriction of dietary carbohydrate was studied in an obese patient (Chalmers et al., 1960a). Urine was collected on the last two-days of each four-day dietary period. Diets provided on thousand calories of which one hundred were in the form of protein.

At first, when carbohydrate provided ninety percent of the remaining calories and fat only ten percent, no ketogenic activity could be detected in the urine. Activity began to appear when the proportion of calories provided by carbohydrate was reduced to half, and it increased progressively with further carbohydrate restriction. This relationship holds true when the diets are given in the reverse order (Chalmers, 1965).

In very obese patients fasting and low carbohydrate diets appear to elicit a smaller output of FMS than in non-obese subjects (Chalmers, 1965), presenting the possibility that a primary defect of its availability plays a part in the development of obesity. However, the fasted hypothalamic obese rat excretes a greater amount of FMS of increased specific and total activities than does the control animal, regardless of whether laboratory chow or a high-fat diet is fed and with increased excretion when a high-fat diet is fed prior to fasting as opposed to a diet composed of high carbohydrate (Beaton et al., 1966). Beaton et al. (1966) stated that since these animals have increased lipogenesis, a net increase in body fat, and excrete approximately twice as much FMS during fasting as do controls, their ability to produce and excrete a substance for mobilization of fat appears not to be impaired. However, adipose tissue taken from hypothalamic obese rats has a reduced response to FMS (Beaton et al., 1966a). Beaton et al. (1966a) noted the gain

of body fat in these animals may be due to an increased food intake and lipogenesis, enhanced by a reduced response of their adipose tissue to one or more lipolytic agents.

In the absence of dietary restriction no activity has been detected in late pregnancy, nor after short periods of exercise (Chalmers et al., 1960a). A totally adrenalectomized patient found to have a corticotrophin secreting pituitary tumor did not excrete FMS while on an unrestricted diet but responded normally to carbohydrate deprivation (Chalmers, 1965). Chalmers (1965) cited that this observation supports the view that FMS is not corticotrophin-dependent and shows that functioning adrenal glands are not required for the production of activity in response to restriction of carbohydrate intake.

In a number of clinical situations FMS has been detected in the urine when carbohydrate intake was not severely restricted. Activity has been found in diabetic ketosis, in widespread malignant disease with wasting, and in the first few days after surgical operations (Chalmers, 1965), but in all cases the caloric intake was low (Chalmers et al., 1960a). Goth et al. (1965) confirmed the presence of FMS in the urine of patients with diabetes. In contrast, alloxan-diabetic rats excrete less total activity of FMS than do control animals (Beaton et al., 1966). No abnormal activity was found in a patient with lipodystrophy (Chalmers et al., 1960a), but in patients with diffuse lipotrophic diabetes

consumption of an unrestricted diet failed to suppress urinary activity (Chalmers 1960a; Louis et al., 1960, 1962). Chalmers (1965) noted that in one of two thyrotoxic patients activity was present which disappeared during treatment with carbimazole and therefore could not be attributed to TSH. Chalmers (1965) detected activity in three out of seven patients with acromegaly on normal unrestricted diets which ceased shortly after pituitary implantation in two patients.

FMS levels produced by altering the qualitative composition of isocaloric 1000 kcal diets in obese persons are in agreement with earlier suggestions by Kekwick and Pawan (1956 and 1957), that low carbohydrate diets are more effective in producing mobilization and loss of body fat than isocaloric diets high in carbohydrate (Pawan, 1971). Moreover, a high-fat low calorie diet produced higher FMS levels in obese subjects than an isocaloric diet high in protein.

Chalmers et al. (1960) detected no FMS in the urine of humans subsisting on a calorically restricted diet composed largely of carbohydrate. Beaton et al. (1965) investigated the effects of previous diet on the excretion of FMS in the urine of fasting rats. Of four diets (high carbohydrate, high protein, high fat, and high protein-high fat) the greatest excretion of FMS was observed when the high protein-high fat diet was fed ad libitum prior to fasting. Dietary protein had no significant effect upon FMS excretion. Excretion of FMS was increased by prior feeding of a high fat

diet and decreased by feeding of a high-carbohydrate diet. The greatest total activity excreted per twenty-four hours was obtained with the diet highest in noncarbohydrate calories. Kwok (1974) observed the effects of four diets (high carbohydrate, high fat, noncarbohydrate-high fat, and high protein) on the excretion of FMS in the urine of rats fasted for 24 hours at 15°C. The rats were fed ad libitum 7 days prior to their fast under cold stress. Kowk (1974) indicated that a positive correlation exists between the lipolytic potency of crude FMS and the proportion of calories from fat in the diet.

Previous dietary treatment can significantly affect the response of adipose tissue to FMS (Beaton et al., 1966a). Previous feeding of a high protein diet or noncarbohydrate-high fat diet inhibits the lipolytic response in vitro of adipose tissue to FMS. The release of free fatty acids was greatest with the high-carbohydrate and high fat diets. The release of free fatty acids in the absence of FMS was non-significantly different with the tissue obtained from rats fed the high carbohydrate, high fat, and noncarbohydrate-high fat diets. Kwok (1974) showed that rats fed a high carbohydrate diet prior to 72 hours starvation at 0° to 5°C before sacrifice would provide epididymal fat tissue with maximum response to FMS.

Karninen et al. (1962) reported results that suggest less activity may be excreted by humans in the second day of

fasting than on the first day. Chalmers (1965) stated that this report is in conflict with unpublished data from his laboratory showing a progressive increase in urinary activity in two lean subjects given a low-calorie, low-carbohydrate diet for 5 days. Pawan's (1971) observations compare favorably with that of Chalmers (1965). During a 36-hour fast in normal subjects FMS levels progressively increased with time, but upon resumption of normal eating, FMS output decreased and was absent by the eighteenth hour after re-feeding. Prolonging the period of fasting of obese persons produced an increase in FMS output from day 1 to day 5 of the fast, but by day 10 of the fast the FMS levels had again decreased (Pawan, 1971). Kekwick and Pawan (1967) observed in five female obese subjects that upon prolonged starvation FMS levels were virtually negligible by the fourteenth day. Crisp et al. (1968) have observed in subjects with primary anorexia nervosa of some duration who exhibited considerable fat loss, that FMS was undetectable in some cases, but on refeeding these persons, FMS reappeared transiently, for a few days, and then again disappeared as re-feeding continued (Pawan, 1971). Crisp et al. (1968) and Pawan (1971) stated that starvation, a powerful stimulus for FMS production, if continued without nutrient intake, might cause temporary exhaustion of the secretory cells.

Beaton et al. (1966) reported observations on the duration of fasting on excretion of lipid-mobilizing acti-

vity in the urine of rats. During fasting at 24°C the greatest excretion of specific and total activities occurred in the first twenty-four hours and diminished thereafter up to seventy-two hours. Beaton (1966) noted that the sudden increase of excreted activity may be related to the coincident mobilization of fat in these animals during fasting and that decreased activity during prolonged fasting could possibly be due to decreased production or secretion of FMS. The observations by Kwok (1974) compare favorably with those of Beaton et al. (1966). With fasting, there was appreciable excretion of active FMS, the excreted specific activity being greatest in the first 24 hour fasting period and diminishing thereafter. Kwok (1974) in agreement with Beaton et al. (1966) stated that the sudden increase of excreted activity may be related to the coincident mobilization of fat in the rat during fasting, noting the frequent observation that in situations in which the mobilization of fat from depots is known to occur, there is an increased excretion of FMS.

Mobilization of fat during acute exposure to cold stress is known to occur, presumably in response to the increased demand for energy. The urinary excretion of a fat-mobilizing substance by rabbits during exposure to cold has been reported by Braun and Mosinger (1961). Observations by Beaton et al. (1964a) showed that FMS is not excreted by rats during feeding at an ambient temperature but is excreted by fed rats during acute exposure to cold of an

environmental temperature of 5°C. Stevenson et al. (1965) observed a progressively increasing excretion of FMS in the urine of fasting rats with decreasing environmental temperature from 30°C to 5°C. Kwok (1974), upon decreasing the environmental temperature from 30°C to 5°C observed a progressive increase in the excretion of crude FMS to a maximum at 15°C and expressed the opinion that it may well be that increased spontaneous activity (exercise) or shivering is the causative factor in increased excretion of FMS at about 15°C. Exercise has been reported by Basu et al. (1960) to increase serum concentration of free fatty acids, an index of fat mobilization.

#### Sex, Age, Metabolic Obesity and Weight Reduction

Pawan (1971) studied the effect of age on the production of FMS in normal persons fasted for 36 hours. Adult human subjects, forty years of age produced less FMS than younger persons. Levels of FMS decreased with increasing age above forty years. Stevenson et al. (1965) observed that infant and young children excreted more fat-mobilizing activity than did young male adults during an 18 or 24 hour fast.

Beaton (1966) reported observations on the effect of age and sex on the excretion of FMS. Young male rats excrete a greater amount of FMS than do adult rats, the greatest excreted total activity per 100 gram body weight being at 37 days of age (102 grams). The amount of FMS excreted did

not vary appreciably between 37 (102 grams) and 62 (197 grams) days of age but was decreased between 62 (197 grams) and 78 (297 grams) days. It is generally accepted that puberty in the rat is reached between 50 and 70 days. At the age of 27 days, i.e., prepuberty, male rats fed a high fat diet excreted more FMS with a higher activity than did female rats. However, at 90 days of age (post-puberty), there was no apparent sex difference in the amount of total activity of FMS excreted per rat but when expressed per 100 gram body weight, females excrete more FMS than do males.

Beaton et al. (1966a) investigated the lipolytic response of adipose tissue to rat FMS as affected by sex, age, and location of tissue. There was no sex difference in the response of perinephric and omentum adipose tissue. The response of adipose tissue of a young male rat was considerably less than that of the adult male rat.

Nobody knows the relationship, if any, between FMS and the problem of human obesity. It is apparent, however, from even a brief perusal of its biological effects, that this lipolytic factor probably arising in, or controlled by, the pituitary gland, may be part of a physiological homeostatic control of body mass (Gordon, 1970). The sensor or detector and the entire afferent portion of the mechanism have not been identified, either anatomically or physiologically, but its potential importance to the problem of weight control cannot be doubted (Gordon, 1970).

From observations on very obese patients, fasting and low carbohydrate diets appear to elicit a smaller output of FMS than in non-obese subjects (Chalmers, 1965). He considered the possibility that a primary defect of this mechanism plays a part in the development of obesity. Assuming that the urinary FMS is a reflection of the production of this substance in the body and its excretion by the kidneys, the higher mean urinary FMS levels in the group of non-obese as compared with the mean values in the obese subjects, together with the observations of Chalmers et al. (1960) and Kekwick and Pawan (1968) that injected FMS causes a mobilization and reduction of the body fat content, suggest that a relative deficiency of FMS in obese persons may be associated with development of their obesity (Pawan, 1971). Pawan (1971) noted that it is of some interest that levels of FMS decrease in individuals with increasing age above forty years as there is evidence from actuarial studies of a positive correlation between incidence of obesity and age above forty years, particularly in women. The high FMS values in subjects with diffuse lipomatrophy observed by Pawan (1971), together with the elevated levels in physiological and pathological conditions of increased fat catabolism, support the hypothesis that FMS may be involved in the regulation of body fat stores (Pawan, 1971).

Sustained use of FMS induces a fall in body weight in rats and in humans. Kekwick and Pawan (1968) placed six

obese patients on a 1500 calorie diet containing 120 grams of carbohydrate and gave injections every second day for varying periods of time, either with saline or with 25-100 milligrams of FMS. After investigation for a total of 158 patient-days, 80 with saline and 78 with FMS, 61 days of weight loss and 17 days without weight loss was reported during the 78 days of FMS injections in contrast to 21 days of weight loss and 59 days without weight loss during the 80 days of saline injections. Kekwick and Pawan (1963) observed significant weight losses in mice kept in a metabolic chamber as a result of administering FMS, with loss of weight being produced in animals even when food intake remained constant upon presentation of an excess of food to the animals throughout the experiment.

Repeated administration of FMS into animals for a period of up to three weeks causes weight loss; control animals receiving saline or non-fasting urine extract do not lose weight (Chalmers et al., 1960a). Loss of weight is not due to suppression of appetite; the treated animals remain in good conditions and their food intake does not differ significantly from that of the controls (Chalmers et al., 1958, 1960a). Carcass analysis showed that loss of fat accounts for more than half the weight loss; the remainder, water and body protein, is unchanged (Chalmers, 1960a, 1965).

## FMS and Pituitary Hormones

The ability to produce FMS in response to fasting, carbohydrate deprivation, and other conditions associated with increased catabolism of fat depends on the integrity of the adenohypophysis (or adjacent hypothalamus). FMS does not appear during fasting of hypophysectomized animals or of human subjects with abnormal or absent pituitaries. Therefore, an intact pituitary gland appears to be necessary for the production of FMS.

While the urine of fasting, normal, and sham operated control animals contained fat-mobilizing activity, no such activity could be detected in the urine of hypophysectomized goats fasting for up to five days (Kekwick et al., 1967). Beaton et al., (1964a) demonstrated pituitary-dependence in rats. Urine extracts from fasting hypophysectomized rats contained no fat-mobilizing activity in vitro or in vivo.

Chalmers et al. (1960a) reported seven studies in six patients with anterior pituitary deficiency. These included a patient whose pituitary had been removed for breast carcinoma three weeks before, three cases of chromophobe adenoma, a case of Sheehan's syndrome and a woman in whom the cause of hypopituitarism was unknown. All patients were receiving cortisone and thyroid in replacement dosage. When these patients were subjected to severe carbohydrate restriction or fasting for up to thirty-six hours, no fat-mobilizing or ketogenic activity was detected in the urine

by in vitro or in vivo techniques.

Interestingly, FMS has been detected in the urine of hypophysectomized chickens (Nir et al., 1969).

The relationship of the pituitary gland and its hormones to the fat-mobilizing substance of urine is unclear. It is not known, whether the pituitary is the origin of FMS in mammals or if it simply controls the formation and release of FMS elsewhere. However, comparisons of FMS with lipolytic substances of known pituitary origin reveal important differences. Although preparations of FMS show weak melanophore-stimulating and in vitro corticotrophic activity (Chalmers, 1965) the evidence against the fat-mobilizing property being due to corticotrophin or MSH appears to be quite conclusive. Comparison of a preparation of FMS with corticotrophin A<sub>1</sub> peptide gave widely differing potency ratios in three assay systems (Chalmers et al., 1960a). These were (FMS/corticotrophin): (1) release of corticoids by rat adrenal in vitro-5/1000; (2) release of FFA by rat epididymal adipose tissue in vitro-25/1000; (3) increase in blood ketones in intact, unanesthetized mice-10-100/1. The corticotrophin peptide could be inactivated with hydrogen peroxide and partially reactivated with cysteine; FMS could be inactivated with hydrogen peroxide but could not be reactivated with cysteine (Chalmers et al., 1960a). Both calcium and magnesium ions are required for the lipolytic effect of corticotrophin in vitro: FMS requires calcium

but not magnesium (Chalmers, 1964; Beaton et al., 1965, 1966). Finally, both corticotrophin and melanocyte-stimulating hormone are lipolytic in the rabbit as well as in the rat (Raben et al., 1961) but FMS is inactive in leporine adipose tissue (Chalmers, 1965). Species differences in the responsiveness of adipose tissue also differentiate FMS from the lipolytic peptides isolated from anterior pituitary glands by Rudman et al. (1961) and by Friesen et al. (1962), since these substances are active in rabbits and guinea pigs but not in rats or mice. Administration of commercial corticotrophin to normal subjects does not lead to the appearance of fat-mobilizing activity in the urine and the urine of a totally adrenalectomized patient with a corticotrophic-secreting pituitary tumor contained no FMS while on an unrestricted diet but responded normally to carbohydrate deprivation with excretion of FMS (Chalmers, 1965). This observation supports the view that FMS is not corticotrophin-dependent and shows that functioning adrenal glands are not required for the production of activity in response to restriction of carbohydrate intake.

The relationship between FMS and growth hormone remains an unresolved problem. Chalmers et al. (1960a) could find no fat-mobilizing activity in the urine of two hypophysectomized patients after injection of human growth hormone (5 mg intramuscularly) although plasma free fatty acids rose two to three fold in each case. The rise in plasma free fatty

acids produced by growth hormone is slower than that due to FMS and in vitro, growth hormone shows little or no lipolytic activity (Vaughan and Steinberg, 1963), suggesting that the adipokinetic effect may belong not to growth hormone itself but to an agent produced in response to growth hormone or possibly as a metabolite (Chalmers, 1965). Evidence that FMS is such an agent has not yet been obtained. Preparations of FMS do not show growth-promoting activity and do not cross-react with antisera to human growth hormone (Chalmers et al., 1960). Using an assay technique capable of detecting more than 1 microgram of growth hormone, Professor F. C. Greenwood could find no evidence for growth hormone in FMS (1 to 100  $\mu\text{g}$ ): It seems safe on these, and other grounds presented by Chalmers et al. (1960) and Chalmers (1965) to exclude growth hormone as the fat mobilizing factor in FMS (Pawan, 1971).

In 1964, in the course of improving the method for the isolation of ACTH from ovine pituitaries, a new peptide was obtained which differed from all other then known pituitary hormones and had distinctive properties quite different from those of ACTH (Li, 1964; Birk and Li, 1964). Its biological behavior was tested after it had been characterized chemically. Upon isolation in pure form and establishment of its primary structure (Li, 1965), it was designated  $\beta$ -LPH (beta-lipotropin) by Li because of its lipolytic activity. Li suggested the use of the term lipotropin "LPH" to define

specific fat mobilizing pituitary hormones.  $\beta$ -LPH has since been isolated from bovine (Lohmar and Li, 1967), porcine (Graf and Cseh, 1968; Gilardeau and Chretien, 1970) and human (Chretien, 1976; Cseh, 1968; and Scott and Lowry, 1974) pituitaries. Li (1965) isolated  $\beta$ -LPH from sheep blood.

The complete amino acid sequences of porcine (Graf, 1971) and human (Li, 1976)  $\beta$ -lipoprotein have been proposed. Comparison of the amino acid sequence of human  $\beta$ -lipotropin with that of the ovine and porcine  $\beta$ -LPH reveals the sequence of the COOH terminal 56 residues to be homologous, whereas the amino acid sequences at the  $\text{NH}_2$  terminus exhibit considerable variability.

Chretien and Li (1967) isolated in a homogeneous form another peptide with lipolytic activity calling it  $\gamma$ -LPH. Sequence of the 58 amino acids forming the  $\gamma$ -LPH molecule proved to be identical with the 1-58 N-terminal sequence of  $\beta$ -LPH. Both  $\gamma$ -LPH and  $\beta$ -LPH contain the entire sequence of  $\beta$ -MSH representing positions 41-58 of both the LPHs. Alpha-MSH has the same amino acid sequence as the 1-13 residues of ACTH. ACTH, MSH, and LPH all contain a heptapeptide common core occurring at positions 4-10 in ACTH and  $\alpha$ -MSH and at 46-52 in the  $\beta$ -MSH related peptides  $\beta$ -LPH and  $\gamma$ -LPH. All pituitary peptides which contain the heptapeptide core cause melanophore dispersion in frog skin, stimulate adrenal steroidogenesis and mobilize lipids from adipose tissue. It

has been shown by Sceh and Graf (1971) that the in vitro activity of the lipotropins, the most specific lipolytic pituitary peptides, has not surpassed that of ACTH which exerts other, e.g. steroidogenic potency too. In equimolar concentrations, ACTH was more potent than  $\beta$ -LPH, while  $\gamma$ -LPH was ineffective in inducing lipolysis in rat adipose tissue. Human  $\beta$ -LPH, in contrast to human ACTH, is effective on human adipose tissue (Goth, 1971).

$\beta$ -LPH is the prohormone for morphine like peptides (met-enkephalin and endorphins). In 1975, Hughes reported the isolation of potent opiate-agonist activity of two pentapeptides called met-enkephalin and leu-enkephalin, corresponding to residues 61-65 in  $\beta$ -lipotropin. The C-terminal fragment of  $\beta$ -LPH (61-91) containing this 61-65 sequence of  $\beta$ -LPH was found to be 20 times more potent as an analgesic than the enkephalins (Bradbury, 1976). In 1976, [ $\beta$ -LPH-(61-91)] was named  $\beta$ -endorphin (Li, 1976). Besides  $\beta$ -endorphin, Lazarus (1976) described the isolation and properties of  $\alpha$ -endorphin [ $\beta$ -LPH-(61-76)] and  $\alpha$ -endorphin [( $\beta$ -LPH-(61-77))].

Pelletier (1977) has shown that adrenocorticotrophic hormone (ACTH) and  $\beta$ -LPH are found in the same storage granules in corticotrophs and cells of the intermediate lobe of the pituitary. Mains (1977) using a mouse pituitary tumor cell line has shown that ACTH and  $\beta$ -LPH have a common protein precursor and that ACTH,  $\beta$ -LPH, and  $\beta$ -endorphin are

stored and then released together. Yalow and Berson (1971) believe 'big ACTH' synthesized by the pituitary gland to be the precursor molecule of both ACTH and LPH.

Anselmino and Hoffman (1931) extracted from the anterior lobe of the pituitary a substance inducing fat mobilization and ketosis, and called it "Fettstoffwechselhormon". Etzrodt (1978) in studies on the pituitary "Fettstoffwechselhormon" isolated two peptides P-LF II C and P-LF II D from porcine pituitary glands which display a higher lipolytic activity than ACTH (Schleyer, Voight and Pfeiffer, 1976). Complete cross reaction was observed between porcine  $1-39$ ACTH and P-LF II D. Etzrodt (1978) concluded that P-LF II D has the same immunodeterminants as  $1-39$ ACTH.

Lelek (1963), Burns (1967), Trygstad (1967, 1968), and others have isolated fractions with lipolytic activity from the pituitary and blood. The characterization of their chemical and biological activities has not yet been done.

Currently, it is not at all clear whether there exists a relationship between fat mobilizing substance (FMS) isolated from the urine and many of these lipolytic substances isolated from the pituitary and blood.

#### Mode of Lipolytic Action of FMS

On the isolated rat fat pad, FMS produces an increased rate of release of free fatty acids into the incubation medium. This parameter has been utilized as the basis for the in vitro measurement of FMS lipolytic activity. Chalmers

et al. (1960a) demonstrated that a quantitative dose response curve could be obtained. When the dose of fasting urine extract was varied the increase in liver fat was found to be linearly related to the log dose. FMS is active in vitro in releasing free fatty acids from rat adipose tissue at a concentration of less than 1 microgram per milliliter (Chalmers et al., 1960a), and some preparations of FMS are active at less than 0.1  $\mu\text{g}$  per ml (Chalmers, 1965). These authors showed that the rate of release is about half that produced by adrenaline and 40 times that produced by corticotrophin on a weight for weight basis.

In the intact mouse, subcutaneous injection caused a prompt fall in blood sugar, a rise in blood ketones as well as an increase in liver fat (Chalmers et al., 1960). The transient hypoglycemia reported by Chalmers et al. (1960a), lasting for about 2 to 4 hours, which diminishes on repeated injection presumably corresponds with the increased glucose metabolism noted on isolated fat pads (Kekwick et al., 1967). The effects on liver fat and blood ketones were maximal at six hours and subsided within twelve hours (Chalmers et al., 1960a). Blood lipids, including total lipids, phospholipids, cholesterol and free fatty acids also increased six hours after FMS injection (Chalmers, 1965). The increase in ketones and free fatty acids in the blood may last for over 48 hours after a single large injection (Kekwick et al., 1967). The effects on liver fat and on the

circulating levels of lipids and ketone bodies can be considered as the result of an increased rate of entry into the liver of free fatty acids released from adipose tissue. The following evidence support this view: (1) both rats and mice injected with FMS show a prompt rise in plasma free fatty acid concentration (Chalmers, 1965); (2) epididymal adipose tissue removed from rats previously injected with FMS release free fatty acids faster than tissue from saline-injected animals (Chalmers, 1965); (3) addition of FMS to rat adipose tissue in vitro causes a marked increase in the rate of release of free fatty acids (Chalmers, 1965).

Kekwick and Pawan (1963) studied the effects of administering FMS to mice kept in a metabolic chamber. Respiratory gas exchange was measured as well as carbon excretion in stools and urine. Daily injection caused only a small rise in oxygen consumption; carbon dioxide output did not increase and sometimes actually diminished, which was reflected in a drop in the 24 hour R.Q. (respiratory quotient). Carbon balance, however, became markedly negative due to increased loss of carbon in the urine and, to a lesser extent, in the stools. Similar changes were found after daily injections of FMS for three weeks. The form in which the additional carbon is excreted is not completely clear but it is probably mainly in the form of ketone bodies representing the disposal of free fatty acids mobilized in excess of energy requirements. These results

appear to exclude the possibility that FMS, besides releasing free fatty acids into the circulation, may directly augment their oxidation in peripheral tissues (Chalmers, 1965).

Moreover, FMS has been demonstrated to have no effect on the utilization of short-chain fatty acids by the isolated perfused rat heart (Chalmers, 1965).

Upon extraction of FMS from the urine of fasting chickens, injection into chickens caused transient decrease in food intake and transient increase in plasma free fatty acids (Nir et al., 1969). These effects were accompanied by persisting decrease in plasma triglyceride, cholesterol and lipid phosphorus. Slight elevation of blood glucose occurred but it was not statistically significant. These results, so widely different from those obtained with mammals, can only be possible if the process for the metabolism of carbohydrate and fats differ widely in chickens and mammals. Some data indicate such a possibility. Protamine zinc insulin, for example, causes hyperphagia in mammals but, conversely, it causes anorexia in chickens (Lepkovsky et al., 1965). Moreover, the FMS from the urine of fasting chickens has both anorexigenic and fat-mobilizing properties that cannot be separated into two fractions by the procedure of Beaton et al. (1964).

Carbohydrate metabolism in rat adipose tissue is altered in a pattern similar to that of other lipolytic agents such as epinephrine and corticotrophin (Cahill et al., 1961). Cahill et al. (1961) studied the in vitro

effect of FMS on glucose metabolism and observed that FMS increases the incorporation of glucose carbon into glyceride-glycerol in the same manner as does ACTH, epinephrine and growth hormone (Cahill et al., 1960; Lynn et al., 1960; and Leboeuf et al., 1961). These workers suggested that FMS primarily accelerates lipolysis and does not exert its action via inhibition of esterification. The rise in intracellular fatty acids resulting from accelerated lipolysis induces the following: (1) increased glucose uptake; (2) increased glyceride-glycerol synthesis; (3) increased metabolism of glucose via the Embden-Meyerhof (glycolytic) pathway; and (4) increased oxidation by the tricarboxylic pathway. Therefore, the action of FMS appears to be at the cellular level.

It would appear that these in vitro observations support the in vivo situation. Louis et al. (1962, 1963) reported that FMS has strong anti-insulin activity in the dog and suggested that the anti-insulin effect may be due to the increased fatty acid production. Randle et al. (1963) have shown that glucose uptake by muscle is inhibited by increased availability of free fatty acids for oxidation.

The in vitro relationship between FMS and glucose was further studied by Beaton et al. (1966a) who observed that the in vitro lipolytic response of FMS can be abolished by addition of glucose to the incubation medium. It is possible that glucose aids triglyceride synthesis resulting in a net decrease in lipolysis.

In vivo, the hypoglycemic but not the ketonemic effect of FMS can be abolished in mice by intraperitoneal injection of glucose at the time of FMS injection (Chalmers). It has been shown that the hypoglycemic and fat-mobilizing properties of FMS are not mutually dependent and can be dissociated by ultrafiltration (Beaton et al., 1966b).

FMS produces no marked effect on protein metabolism. Nitrogenous equilibrium is undisturbed by injections of FMS as compared with periods on identical food intakes and saline injections (Kekwick et al., 1963). Repeated injections of FMS produce a significant diminution of carcass fat, but little alteration in carcass protein (Kekwick et al., 1963, 1967).

#### Mechanism of Action

In opposition to the hypothesis that all lipolytic agents act by releasing noradrenaline from tissue stores (Paoletti et al., 1961), observations of Chalmers and Goldiner (unpublished) do not support noradrenaline as an essential mediator of the lipolytic response to FMS: (1) Most adrenaline blocking agents when added, in vitro, depress the lipolytic response to FMS, namely; methalide, dibenzyline, tolazoline, and dihydroergotamine. (2) Some inhibition of the lipolytic effect of FMS by dichloroisoproterenol is seen but is far from complete even at higher concentration. At higher concentrations the effects of adrenaline and noradrenaline are completely blocked. (3) Rat adipose tissue depleted of

catecholamines by reserpine is fully responsive to FMS.

Now, it is generally accepted that cyclic AMP plays a key role in hormonal regulation of lipid mobilization (Vaughan, 1961; Butcher et al., 1965; Robison et al., 1971a and; Kwok, 1974). Investigation of FMS with labeled isolated fat cells indicates strongly that cyclic AMP plays an important role in lipolysis promoted by FMS (Kwok, 1973). Incubation of isolated fat cells for a brief period of time with increasing concentration of FMS resulted in increased cyclic AMP levels without a significant increase in lipolysis. Longer incubation times demonstrated that lipolysis occurred and that it occurred after the maximal stimulation of adenylate cyclase. A synergistic effect of 1,2 dimethylxanthine and FMS on lipolysis was demonstrated; while neither 0.25 mM theophylline nor 25  $\mu$ g of FMS strongly stimulated lipolysis, the combination of the two produced a striking response. Moreover, FMS is capable of increasing the intracellular cyclic AMP levels in intact isolated fat cells while inactive FMS (heat-treated FMS, proven to be inactive) did not increase cyclic AMP levels (Kwok, 1974).

That cyclic AMP levels are elevated before lipolysis is detected in cells treated with FMS (Kwok, 1974), is consistent with the suggestion of previous investigators (Butcher and Sutherland, 1962) that cyclic AMP is the cellular mediator of hormone actions and may be related to the lipolytic cascade proposed by Steinberg (1971). After FMS

stimulation of the adenylate cyclase system, cyclic AMP activates protein kinase which in turn phosphorylates the hormone sensitive lipase, converting it from an inactive to a more active form. In this active form the enzyme controls the lipolysis of triglyceride to free fatty acids, FFA.

#### Isolation and Purification of FMS

FMS has been isolated from the urine by a four stage extraction procedure involving: benzoic acid precipitation, sodium carbonate solution or water extraction, alcoholic precipitation, and lyophilization (Beaton et al., 1964; Kekwick et al., 1967; Kwok, 1974). Chalmers et al., (1960a) reported that the use of oxycellulose increased the potency of the isolated product twenty to thirty times while Beaton et al. (1966b) reported that Millipore filtration and carboxymethylcellulose treatment are without any significant effect on the activity of the isolated product. Both Beaton et al. (1966b) and Kekwick and Pawan (1967) achieved some purification of the material by ultrafiltration through Visking dialysis membranes. The greater portion of the activity was found in the ultrafiltrate. Kwok (1974) achieved some purification of the material by passage through a Diaflo ultrafiltration apparatus fitted with a PM-10 membrane. Upon further purification of the active PM-10 ultrafiltrate by ultrafiltration with a UM-2 Diaflo membrane, the activity was found to be associated with the UM-2 residue (retentate).

When Beaton et al. (1966b) further fractionated the active ultrafiltrate, extracted from urine of fasting rats, through Sephadex G-25 chromatographic column with elution with distilled water, three active fractions were obtained. These activities did not appear to be necessarily associated with protein. The first active fraction coincided with the presence of carbohydrate although in small amount. The second active fraction coincided with the presence of carbohydrate and some protein. The third active fraction coincided with some protein. Consideration of these observations immediately raised the question whether rat FMS contains three separate fat-mobilizing substances or whether there is but one compound attached to or absorbed by different carrier substances. The answer to this question has not yet been secured. In contrast, when the active UM-2 residue (retentate) ultrafiltration product of Kwok (1974) was fractionated by passage through a Sephadex G-25 column with elution with distilled water, the activity was found to be associated only with the first ten 5 ml fractions.

When Kekwick and Pawan (1967) passed their active ultrafiltrate extracted from the urine of fasting humans through a column of Sephadex G-50 and then through a column of Bio-Gel P-10, a reasonably homogeneous extract was obtained which migrated as a single band when subjected to electrophoresis on polyacrylamide gel and had one major component peak in the ultracentrifuge with a small subsidiary

peak. Kwok (1974) achieved further purification of his active UM-2 residue ultrafiltration product by column chromatography with a column of Sephadex G-50 and Bio-Gel P-10 set in tandem. When the active eluant peak fraction was subjected to electrophoresis on cellulose acetate and polyacrylamide gel, the preparation migrated as a single protein spot.

#### Chemical Nature of FMS

Chalmers et al. (1960a) described the fat-mobilizing material they had obtained from the urine of fasting humans as a polypeptide with molecular weight less than 18,000. It is not ultrafiltrable and its behavior in dextran gel suspensions suggests a molecular weight below 5,000 (Chalmers, 1965). It contained about 8% nitrogen and after acid hydrolysis, it yielded the following amino acids: histidine, phenylalanine, leucine, serine, cystine, aspartic acid and a trace of alanine (Chalmers et al., 1960a). It gave a weak Molisch test for carbohydrate material. The biological activity was thermostable up to 80°C in 0.1 N sodium hydroxide. It was destroyed by boiling for two minutes. Activity was completely destroyed by digestion with trypsin and chymotrypsin but some activity remained after peptic digestion.

Preliminary analysis of rat FMS extract by Stevenson et al. (1964) by paper chromatography after acid hydrolysis indicate that it is a polypeptide composed of histidine,

phenylalanine, leucine, serine, cystine, aspartic acid and alanine, with traces of 2 other amino acids. The extract obtained from fasted rats contained about 10% nitrogen and 6% carbohydrate. Unpurified fat-mobilizing substance from rat urine was reported to contain several amino acids in addition to those listed by Chalmers et al.: The nitrogen contents of human and rat FMS are essentially the same (Beaton et al., 1964). Carbohydrate is also present in crude FMS obtained from rat urine (Beaton et al., 1964). FMS fractionation on Sephadex G-25 dextran gel by Beaton et al. (1966b) suggests a polypeptide molecular weight between 1,000 and 5,000. Kwok (1974) fractionated crude rat FMS into substances greater than 1,000 and less than 10,000 in molecular weight by Diaflo ultrafiltration utilizing respectively, UM-2 and PM-10 Diaflo membranes; he showed that the active material, as isolated from rat urine, had a molecular weight between 1,000 and 10,000. Results from passage of UM-2 retentate through Sephadex G-25 columns suggested that the active material may have a molecular weight between 5,000 and 10,000 daltons.

Seifter et al. (1968), after studying the lipid mobilizing peptides from various sources, suggested that all lipid mobilizing peptides reported since 1956 are closely related chemically. Many questions concerning FMS cannot be answered until it can be isolated in pure form.

### Statement of the Problem

Production of FMS has been measured in vivo by using the ability of the urinary extracts to (1) raise blood ketones, (2) raise free fatty acids levels in the blood of intact mice, and (3) diminish mouse carcass fat. Production has been measured in vitro by the ability of FMS to release free fatty acids from the in vitro fat pad. During lipolysis both free fatty acids and glycerol are released. The fatty acids can be activated and reesterified. Glycerol, however, is not metabolized to any significant extent. There exists the inherent danger of relying on only one criterion for measuring in vitro FMS lipolytic activity. To date, no in vitro bioassay utilizing glycerol has been employed in a quantitative comparison to free fatty acid release as an index of in vitro FMS lipolytic activity. It has become increasingly evident that differences in adipose tissue due to the number of adipose tissue cells and the size of these adipocytes can have an effect upon the metabolic activity of the adipose tissue fat pad. Accordingly, attention need be directed to modification of assay procedures to express the lipolytic activity of FMS as a function of cell size and number.

The precise chemical nature of FMS remains elusive, and none of its physical characteristics have been reported. Progress has been hampered by the lack of highly purified preparations in sufficient quantity for basic studies. Application of the technique of iso-electric focusing to

this biologically active material may prove to be a powerful tool for future analytical and/or preparative separation of FMS. Physicochemical information can be obtained from isoelectric data to contribute towards characterization of FMS in terms of its physical properties.

Obesity and age have been shown to affect the urinary excretion of FMS. Hence body weight probably has an effect on the quantity and quality of its excretion and should be studied in more detail.

The purpose of this thesis is to obtain further information relevant to the characterization of this lipolytic factor. Attention will be directed to the following parameters: (1) to improve the efficiency of purification of the active principle; (2) to investigate the influence of weight and age factors on its urinary excretion; and (3) to establish an assay system more specific for target cell-FMS interaction than has been used previously, and based upon the release of glycerol and of fatty acid from fat cells of known size and lipid content.

## CHAPTER II

### MATERIALS AND METHODS

#### General Experimental Procedures

The first objective at the start of this work was to obtain a sufficient quantity of FMS for basic studies. Male albino rats of the Sprague-Dawley strain were placed on a high fat diet for a 7 day period. On the 7th day, the animals were placed in a cold room at 15°C and their urine was collected during a 24 hr fast.

Crude FMS was extracted from the urine by a four stage process: (1) benzoic acid precipitation of FMS from freshly passed urine at pH 5.3; (2) extraction of FMS from the precipitate with distilled water; (3) alcoholic precipitation at pH 5.3; and (4) lyophilization of the precipitate after redissolving in distilled water. Crude, FMS was then bioassayed in vitro for lipolytic activity.

The influence of weight, and hence age factors, on FMS bulk urinary excretion and activity were studied. Eleven groups of rats, 30 in each group and varying in mean group weight, were subjected to the above stated routine. The urine collected from the rats within a group was pooled, and the FMS extracted, weighed and bioassayed in vitro for lipolytic activity.

Three different assay procedures were used to test the lipolytic activity of FMS. Being a relatively rapid and

accurate method, the lipolytic activity of FMS was assayed in vitro, at each stage of purification, by its ability to increase the release of free fatty acids (FFA) from epididymal fat pads of the rat in a medium of Krebs-Ringer phosphate buffer (pH 7.4) containing 4% bovine serum albumin. The quantitative release of FFA into the medium was determined by the method of Duncombe (1964) as modified and used by Kwok (1974). In this assay, chloroform-soluble copper soaps of the FFA are formed and the chloroform-soluble copper is converted to a colored complex by reaction with 1,5 diphenylcarbohydrazide.

To improve the sensitivity of this biological assay an experimental procedure was set up to assay the increased release of free fatty acids via direct FMS-fat cell interaction. Fat cell suspensions were used for this purpose and were prepared according to the method of Rodbell (1964).

To remove the inherent problem of relying on only one criterion for measuring in vitro FMS lipolytic activity, a third assay, was established. In this case, increased glycerol release from direct FMS-target cell interaction was quantitatively measured as an index of in vitro FMS lipolytic activity.

Glycerol release was determined by modification of the sensitive enzymatic method of Davidson and Karjala (1970). An Aminco-Bowman spectrophotofluorometer was used with excitation set at 350 nm and emission at 455 nm to measure

$\text{NADH}_2$  which is formed when glycerol is oxidized by glycerol dehydrogenase.

Data obtained in FMS-target cell interaction assays were expressed in two ways. In the first approach, total lipid was extracted from the fat cells used in the assay system, by the biphasic system established by Folch et al. (1957) mixing chloroform, methanol and a known aliquot of cell suspension; the glycerol and/or free fatty acid release was expressed on a per unit cell lipid basis.

The second mode of data expression provided an opportunity to study FMS lipolytic activity as a function of cell size and number. Increasing evidence shows that cellularity of the adipose tissue can affect the metabolic activity of this tissue, and can provide an inaccurate amount of reported activity per target cell if expressed on a per unit wet weight of tissue basis. FFA release was expressed per  $10^6$  target cells, using rats weighing 130-150 grams.

Adipose tissue cellularity was determined on every cell suspension prepared for bioassay by use of the Coulter Electronic Counter, which gave cell number, as well as the means of obtaining the diameter and volume of the target cells used.

The general approach and techniques of subjecting crude FMS to partial purification by ultrafiltration with PM-10 and UM-2 Diaflo membranes, and then further fraction-

ation by passage through Sephadex G-50 and Bio-Gel P-10 chromatographic columns, were similar to those which have been used previously by Kwok (1974) in our laboratory but with some modifications.

In the ultrafiltration studies the usefulness of the substitution of a DM-5 Diaflo membrane, for the UM-2 membrane or of its incorporation into the existing purification protocol along with the PM-10 and UM-2 membranes was investigated.

Column chromatography was applied to the active fractions obtained from each of these modes of ultrafiltration. Both Sephadex G-50 and Bio-Gel P-10 columns in tandem and Bio-Gel P-10 columns alone were used in the further purification of FMS.

Highly purified FMS preparations were subjected to isoelectric focusing. The effluent was monitored with an LKB Absorptionmeter at 280 nm and fractions representing elution peaks were pooled, dialyzed, lyophilized, and bioassayed in vitro for lipolytic activity.

The active variants of FMS were examined for homogeneity, amide gel electrophoresis and densitometry of the destained gel columns.

Chemicals

- Acrylamide, Eastman Organic Chemicals, No. 5521
- Albumin, Bovine, Fraction V Powder, Sigma Chemical Company,  
No. A4378
- Albumin, Dried Human Protein Standard, Dade (Div. of American Hospital Supply Corp., No. PR5-411
- Alcohol, 95%, Commercial Solvents Corporation
- Alcohol, absolute, Commercial Solvents Corporation
- Ammonium Persulfate, Mallinckrodt Chemical Works, AR, No. 3460
- Ampholine Carrier Ampholytes, pH range 3-10, LKB Instruments Inc., Rockville, Maryland
- Benzoic Acid, Aldrich Chemical Company, No. 10,947-9
- Bio-Gel P-10, 50-100 mesh, Bio-Rad Laboratories, No. 81102
- BIS (N,N'-Methylene-bisacrylamide), Eastman Organic Chemicals, No. P 8383
- Bromphenol Blue (Water Soluble), Hartman-Leddon Company,  
No. 859
- Calcium Chloride, Anhydrous, Mallinckrodt Chemical Works,  
AR, No. 4132
- Casein, Nutritional Biochemicals Corporation
- Chloroform, Mallinckrodt Chemical Works, AR, No. 4440
- Collagenase, Type III, Fraction A, Sigma Chemical Company,  
No. C-0255
- Coomassie Brilliant Blue R-250, Colab Laboratories, Inc.,  
No. 11-152B

Copper Sulfate·5H<sub>2</sub>O, Mallinckrodt Chemical Works, AR, No.

4844

Crisco, Proctor and Gamble Company

Cupric Nitrate·3H<sub>2</sub>O, Fisher Scientific Company, No. 771342

1,5 Diphenylcarbohydrazide, Eastman Kodak Company, No. 618

Fiber, Non-nutritive, Nutritional Biochemicals Corp.

Glycerol, Reagent Grade, Fisher Scientific Co., No. G33

Glycerol Dehydrogenase, Grade I, Sigma Chemical Co., No.

50C-6860-1

Glycine (Ammonia Free), Eastman Organic Chemicals, No. 445

Hydrochloric Acid, J. T. Baker Chemical Co., No. 9535

Isopropyl Alcohol, Mallinckrodt Chemical Works, AR, No.

3032

Kodak Photo-Flo

Liver Powder, Nutritional Biochemicals Corporation

Magnesium Sulfate·7H<sub>2</sub>O, Mallinckrodt Chemical Works, AR,

No. 6056

Methanol, Anhydrous, Mallinckrodt Chemical Works, AR,

No. 3004

β-Nicotinamide-adenine dinucleotide, Grade III, Sigma

Chemical Co., No. D 5755

Palmitic Acid, Sigma Chemical Company, No. P-0500

Perchloric Acid, Mallinckrodt Chemical Works, AR, No. 2766

Phenol Reagent, Folin and Ciocalteu, Harleco Co.

Phosphoric Acid, Mallinckrodt Chemical Works, AR, No. 2796

Potassium Chloride, Granular, Mallinckrodt Chemical Works,

AR, No. 6858

Potassium Ferricyanide, Sargent-Welch Scientific Co., AR,  
No. 14180

Potassium Hydroxide, Matheson, Coleman and Bell, No. CB  
1158

Riboflavin, Eastman Organic Chemicals, No. 5181

Salt Mixture, Wesson Modified Osborne-Mendel, General Bio-  
chemicals, No. 170900

Sephadex G-50, Medium, Pharmacia Fine Chemicals Inc.

Siliclad, Clay Adams Company

Sodium Carbonate, Anhydrous, Mallinckrodt Chemical Works,  
AR, No. 7521

Sodium Chloride, Mallinckrodt Chemical Works, AR, No. 7581

Sodium Dodecyl Sulfate (SDS), Bio-Rad Laboratories, No. C-  
161-0300

Sodium Hydroxide, Pellets, Mallinckrodt Chemical Works, AR,  
No. 7708

Sodium Phosphate Dibasic, Anhydrous, Mallinckrodt Chemical  
Works, AR, No. 7917

Sodium Tartrate, Mallinckrodt Chemical Works, AR, No. 2386

Sucrose, Nutritional Biochemicals Corporation

Sulfuric Acid, Mallinckrodt Chemical Works, AR, No. 2876

TEMED (N,N,N',N'-Tetramethylethylenediamine), Eastman  
Organic Chemicals, No. 8178

Thymol, Aldrich Chemical Company, No. 11,209-7

Toluene, Mallinckrodt Chemical Works, AR, No. 8608

Trichloroacetic Acid, Mallinckrodt Chemical Works, AR,

No. 2928

Triethanolamine, Eastman Organic Chemicals, No. 1599

TRIZMA-BASE, Sigma Chemical Company, No. T 1503

TRIZMA-HCl, Sigma Chemical Company, No. T 3253

Trypsin, Type III, from Bovine Pancreas, Sigma Chemical  
Company, No. T-8253

Trypsin Inhibitor, Type II-O, from Ovomuroid (Egg White),  
Sigma Chemical Company, No. T-9253

Vitamin Diet Fortification Mixture, Nutritional Biochemi-  
cals Corporation

#### Urine Collection and Extraction of Crude FMS

In all experiments, both for the collection of urinary FMS and for its bioassay in vitro, white male, albino rats of the Sprague-Dawley strain purchased from Locke-Erickson Laboratories, Maywood, Illinois were used. For the collection experiments, groups of 30 rats were immediately housed upon arrival into individual screen-bottomed cages at an environmental temperature of  $25 \pm 1^{\circ}\text{C}$  and fed laboratory chow ad libitum for the next 7 days.

Following this period they were placed on a high fat diet for the following 7 days. The composition of the high fat diet is given in Table 1. The diet provided 84.7% of of the Calories as fat, 9.4% as protein and 5.9% as carbohydrate with an average of 5.22 Calories per gram. The high fat diet was prepared every two days and was provided

TABLE 1  
CONSTITUENTS AND CALORIC CONTENT OF THE  
HIGH FAT DIET\*

Constituent	Percent by Weight	Calories/ Gram
Casein	13	0.49
Sucrose	8	0.31
Crisco	50	4.42
Non-Nutritive Fiber	24	
Salt Mixture	3	
Liver Powder	2	
Total	100	5.22

\* Plus 2% vitamins.

daily to the animals. Fresh drinking water was available ad libitum.

On the seventh day of this regimen, the animals were weighed and transferred to individual stainless steel metabolism cages, fitted with urine-feces separators, in the cold room at 15°C. The animals were then fasted for the next 24 hours, during which time urine was collected and pooled. The urine was collected in 125 ml Erlenmeyer flasks containing 5.0 mls of 10% thymol in isopropanol as a preservative. The preservative was not used however if the urine was to be processed immediately after its collection and this was generally the case.

Following their 24 hour fast under cold stress, the rats were refed laboratory chow ad libitum at room temperature for the next 7 days before being placed back on the high fat diet and subsequently, the 24 hour fasting period and the cycle repeated. The experimental cycle was repeated for purposes of urine collection until the rats had reached weights over a group mean of 450 grams.

Crude FMS was extracted from the urine according to the method of Beaton et al. (1964). The pH of the pooled 24 hour urine collection from groups of 30 rats was pH adjusted to 5.3 with 1.0 N HCl. One volume of saturated benzoic acid solution in 100% ethanol was added for each 10 volumes of urine with constant stirring. The slurry was mixed thoroughly for at least 15 minutes. Precipita-

tion was allowed to proceed overnight at 4°C in a stoppered container. The bulky precipitate which formed served to adsorb and coprecipitate FMS from the urine. The contents were transferred to 100 ml Corning centrifuge tubes. The precipitate was centrifuged at 5,000 x g at 0° to 5°C, in an International Centrifuge, Model PR-2 (840a head), for 30 minutes. The supernatant was discarded.

In order to remove the benzoic acid, the precipitate was washed in the large centrifuge tubes with cold 95% ethanol at pH 5.3. A glass rod was used to break up any lumps and to mix the contents thoroughly. The aqueous solution was then centrifuged at 5,000 x g at 0° to 5°C for 30 minutes. The ethanolic washings were discarded on each occasion after centrifugation. The precipitate was washed twice.

FMS was then eluted from the precipitate by adding 100 ml of distilled water and mixing thoroughly over a period of about 15 minutes using a glass rod to break up any lumps. The aqueous solution was then centrifuged as before and the supernatant retained.

Reprecipitation of FMS was carried out by adjusting the pH of the aqueous solution to pH 5.3 with 1.0 N HCl or 1.0 N NaOH as required, and slowly adding 2 volumes of cold absolute alcohol while stirring constantly with a magnetic stirrer.

The suspension was put in the cold room at 4°C for the

next 24 hours in a stoppered container for precipitation to be completed. The suspension was then transferred to 100 ml Corning centrifuge tubes, and centrifuged at 5000 x g at 0° to 5°C in the International Centrifuge, Model PR-2, for 30 minutes. The supernatant was discarded.

To the residue 50 ml of distilled water was added and the slurry mixed thoroughly and left for 15 minutes to dissolve the FMS. After centrifugation for 30 minutes at 0° to 5°C at 5000 x g, the supernatant was collected and lyophilized. The lyophilized product, crude FMS, was stored in the refrigerator in a tightly stoppered container.

#### Free Fatty Acid Determination

The lipolytic activity of FMS was assayed in vitro by incubation with epididymal fat pads from male rats in a medium of Krebs-Ringer phosphate buffer (pH 7.4) containing 4% bovine serum albumin. The colorimetric method developed by Duncombe (1964), as modified by Kwok (1974), was used for the quantitative determination of free fatty acids (FFA) released into the incubation medium. In this assay, chloroform soluble copper soaps of the FFA are formed and the chloroform soluble copper is converted to a colored complex by treatment with 1,5 diphenylcarbohydrazide.

#### Reagents

1 M Triethanolamine: 14.9 gms of triethanolamine was dis-

solved in deionized water and diluted to 100.0 ml with deionized water.

6.45%  $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ : 6.45 gms of cupric nitrate trihydrate was dissolved in deionized water and diluted to 100.0 ml with deionized water.

Copper reagent: A mixture of equal volumes of 1 M triethanolamine and 6.45% cupric nitrate trihydrate solution was prepared when needed.

1,5 Diphenylcarbohydrazide Solution: 0.5 gm of 1,5 diphenylcarbohydrazide was dissolved in absolute methyl alcohol and diluted to 100.0 ml with absolute methyl alcohol. This solution was prepared just prior to use, as aging of this reagent results in increased blank values.

Standard Fatty Acid Solutions: Six standard solutions of palmitic acid ranging from 20  $\mu\text{M}$  to 120  $\mu\text{M}$  in chloroform were prepared namely: 20  $\mu\text{M}$ , 40  $\mu\text{M}$ , 60  $\mu\text{M}$ , 80  $\mu\text{M}$ , 100  $\mu\text{M}$ , and 120  $\mu\text{M}$ .

Krebs-Ringer Phosphate Buffer (pH 7.4) Containing 4% Albumin: Krebs-Ringer phosphate buffer containing the following components was prepared:  $\text{NaCl}$ , 128  $\text{mM}$ ;  $\text{MgSO}_4$ , 1.4  $\text{mM}$ ;  $\text{CaCl}_2$ , 1.4  $\text{mM}$ ;  $\text{KCl}$ , 5.2  $\text{mM}$ ; and  $\text{Na}_2\text{HPO}_4$ , 10  $\text{mM}$ . The phosphate buffer was made up fresh daily and the pH adjusted to 7.4 after addition of sufficient bovine serum albumin to yield a 4% (w/v) solution.

### Preparation of Standard Curve

5.0 ml of each of the standard palmitic acid solutions were placed in glass-stoppered 17 x 135 mm (12 ml) Pyrex centrifuge tubes. In addition, 5.0 ml of chloroform was pipetted into an identical glass-stoppered centrifuge tube to serve as the blank. 2.5 ml of copper reagent was then added to each tube and the solutions were mixed thoroughly with a Vortex mixer set at top speed for 30 seconds.

The resulting two layers were allowed to separate and approximately 4 ml of the chloroform layer was removed with a 6", 18 gauge needle and syringe, and transferred to another 17 x 135 mm glass-stoppered Pyrex centrifuge tube. Extreme care was taken not to transfer any of the copper solution. About 2 ml of deionized water was added to the top of the chloroform layer in each of the tubes without any mixing of the two layers. The tubes were centrifuged in an IEC Clinical Centrifuge (809 head) set at speed #6 for 10 minutes.

The aqueous layer was completely removed along with a small amount of chloroform layer with a glass disposable pipet therefore, insuring that any small amount of the copper reagent remaining in the chloroform layer was removed.

Aliquots of 1.0 ml of the chloroform solution of the soap were pipetted into 5.0 ml volumetric flasks. 1.0 ml of the 1,5 diphenylcarbohydrazide solution was added to

each flask, and the mixture was diluted to volume with chloroform.

The absorbance of each solution at 540 nm was read in a Beckman DU Spectrophotometer exactly 5 minutes after the addition of the complexing agent. The data for this standard curve appear in Table 2. A linear relationship between the absorbance at 540 nm and the concentration of palmitic acid over the range of 4  $\mu\text{M}$  to 24  $\mu\text{M}$  is shown in Figure 1.

#### Determination of Biological Activity of FMS Based on FFA Release from Epididymal Fat Pads

An epididymal fat pad weighing 250 mgs, removed from 130-150 gm male rats was placed in a 25 ml Erlenmeyer flask containing 4 ml of Krebs-Ringer phosphate buffer containing 4% albumin. After the addition of 100 ug of FMS (or test material) in 1.0 ml of the Krebs-Ringer phosphate buffer, the flask contents were incubated for 3 hours at 37°C in a Dubnoff Metabolic Shaker using air as the gas phase.

At the end of the incubation, 1.0 ml of the incubation mixture was diluted with 1.0 ml of distilled water and extracted in a 18 x 150 mm glass-stoppered centrifuge tube with 5.0 ml of chloroform by mixing for 1 minute on a Vortex mixer set at top speed. The mixture was centrifuged in an IEC Clinical Centrifuge (setting 6: 809 head) for 20 minutes. The aqueous and chloroform layers were separated by a disc of precipitated protein. 3.0 ml of the chloro-

TABLE 2  
Standard Curve Data for Determination of  
Free Fatty Acids

Palmitic Acid uM	No. of Determinations	Absorbance* 540 nm
4	12	0.061 $\pm$ 0.021
8	12	0.161 $\pm$ 0.018
12	12	0.258 $\pm$ 0.021
16	12	0.382 $\pm$ 0.016
20	12	0.493 $\pm$ 0.021
24	12	0.614 $\pm$ 0.013

\* Mean  $\pm$  standard deviation

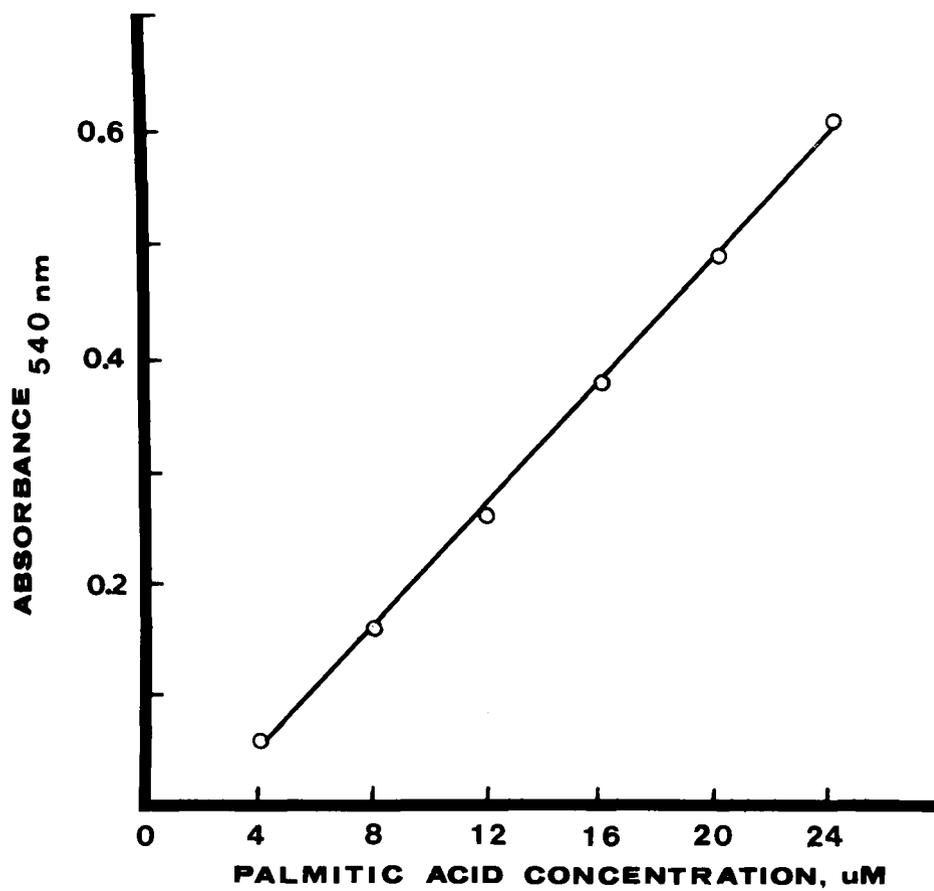


Figure 1. Standard Curve for the Determination of Free Fatty Acids.

form solution were pipetted into a clean dry tube and analyzed for free fatty acids. The concentration of the FFA in the circulation medium was calculated from the regression line equation of absorbance versus concentration determined by the method of least squares, namely  $F = 36.01A + 2.18$ , where  $F$  represents the  $\mu\text{M}$  concentration of FFA and  $A$ , the absorbance value. The total FFA released to the medium as umoles was calculated by multiplying the value of  $F$  by the dilution factor.

#### Preparation of Isolated Fat Cells

Isolated fat cells were used in place of intact epididymal fat pads in an attempt to improve the existing assay used for free fatty acid release by the removal of the heterogeneous cell population that exists in fat pads (Butcher and Baird, 1968), and to increase the sensitivity of the fat cells to FMS through the increased sensitivity attributed to fat cells when assayed in their isolated form to lipolytic agents (Fain et al., 1972).

Fat cells were isolated according to the procedure of Rodbell (1964), as modified by Moskowitz and Fain (1970), and applied by Kwok (1974) in his investigation of FMS elevation of cAMP levels with labeled adipocytes.

White male albino rats weighing not more than 100 gms were purchased from Locke Erickson Laboratories in Maywood, Illinois. The rats were maintained on laboratory chow ad libitum until they weighed 130-150 gms, upon which, they

were killed by decapitation. It has become increasingly evident that differences in adipose tissue mass, due to the number of adipose cells in the tissue and their respective size, can have an effect on the metabolic activity of this tissue. Therefore, all preparations were made from rats weighing between 130-150 grams. The mean diameter of fat cells obtained from rats in this weight range is approximately 45  $\mu\text{m}$ , in contrast to a mean diameter of approximately 78  $\mu\text{m}$  in rats weighing 400 to 450 grams. Small cells were used rather than the large cells since it has been demonstrated that large adipocytes from adult rats were less sensitive to glucagon stimulation of lipolysis than small cells from young animals (Manganiello and Vaughan, 1972; Livingston et al., 1974).

Immediately after decapitation, the epididymal fat pads were removed quickly and rinsed in 0.9% NaCl. Polyethylene centrifuge tubes and siliconized glass vessels were used during the preparation and incubation of the fat cells. Up to 10 grams of pooled rat epididymal fat pads were added to a siliconized 25 ml flask containing 10 ml of Krebs-Ringer phosphate buffer, pH 7.4, with 4% (w/v) bovine serum albumin. 10 mgs of collagenase and 4.0 mgs of crystalline trypsin were added to each 25 ml siliconized Erlenmeyer flask. Cells were isolated by digestion with trypsin in addition to collagenase in order to diminish the effects of insulin and insulin-like substances on the re-

sponse to lipolytic agents. No glucose was present during isolation or the incubation of the cells. When the in vitro relationship between FMS and glucose was studied by Beaton et al. (1966a), it was observed that the in vitro lipolytic response of FMS was abolished by addition of glucose to the incubation medium.

The mixture was incubated at 37°C for 1 hour in a Dubnoff Metabolic Shaker. The whole digest was then transferred to a polyethylene centrifuge tube containing 20 ml of Krebs-Ringer buffer warmed to 37°C.

Fat cells were liberated from the tissue fragments by gentle stirring with a siliconized glass rod. Liberation of the fat cells was manifested by an increased turbidity of the medium. Fragments of tissue still remaining after this treatment were removed with forceps.

The suspended cells were then centrifuged in polyethylene centrifuge tubes for 1 minute at 400 x g. The fat cells floated to the surface and the stromal vascular cells sedimented. The stromal-vascular cells were removed by aspiration. After gentle stirring of the cell suspension, fat droplets which formed from the breakage of some of the fat cells, floated more rapidly to the surface than the fat cells. These fat droplets were aspirated off from the surface. The adipocytes were then washed by suspending them in 10 ml of warm Krebs-Ringer phosphate buffer and centrifuging for 1 minute at 400 x g; any remaining stromal vas-

cular cells were aspirated off after centrifugation. Any remaining fat droplets were aspirated off the surface after gentle stirring. The washing procedure was repeated three times in order to insure complete removal of the stromal vascular cells. The adipocytes were finally suspended in 20 to 25 ml of Krebs-Ringer phosphate buffer containing 0.1 mg per ml of trypsin inhibitor. The cell suspension was swirled to ensure delivery of uniform suspension of cells immediately prior to use.

#### Determination of the Biological Activity of FMS Based on FFA Release from Epididymal Fat Cells

The lipolytic activity of FMS was measured in vitro on the basis of the increased release of free fatty acids from isolated fat cells in a medium of Krebs-Ringer phosphate buffer (pH 7.4) containing 4% bovine serum albumin. The colorimetric method developed by Duncombe (1964) as modified by Kwok (1974) was used, as it was applied to the in vitro bioassay using epididymal fat pads, for the quantitative determination of free fatty acids released into the medium.

2.0 ml of fat cell suspension was placed in a 25 ml Erlenmeyer flask containing 2.0 ml of Krebs-Ringer phosphate buffer with 4% albumin. After the addition to the flask of 100 ug of FMS (or test material) contained in 1.0 ml of the Krebs-Ringer phosphate buffer, incubation was allowed to proceed for 3 hours at 37°C in a Dubnoff Metabolic Shaker

using air as the gas phase.

At the end of incubation, the incubation mixture was very carefully transferred to polyethylene centrifuge tubes. Fat cells were separated from the aqueous incubation mixture by rapid centrifugation for 1 minute in an IEC Clinical Centrifuge (speed setting 6: 809 head) for 20 minutes. The adipocyte layer was removed by aspiration.

1.0 ml of the remaining incubation mixture was placed into a 17 x 135 mm glass-stoppered Corning centrifuge tube containing 1.0 ml of distilled water and extracted with 5.0 ml of chloroform by mixing for 1 minute on a Vortex mixer set at top speed. The mixture was then centrifuged as before in the clinical centrifuge for 20 minutes. 3.0 ml of the chloroform solution were pipetted into a clean dry tube and analyzed for free fatty acids as described previously. The total FFA released to the medium as umoles was calculated by multiplying the value of F by the dilution factor and then dividing by 1000.

The total FFA released to the medium was expressed as umoles of free fatty acids released by 100 ug of FMS (or test material) acting on rat epididymal adipose tissue fat cell suspension equivalent to 1 gram of lipid for 3 hours at 37°C, and as umoles of free fatty acids released per  $10^6$  epididymal fat cells being acted upon by 100 ug of FMS (or test material) for 3 hours at 37°C.

## Extraction of Total Lipid from Fat Cell Suspensions

The lipid concentration of the adipose tissue cell suspension was measured by the method developed by Folch et al. (1957).

### Reagents

Chloroform-Methanol Mixture: 2:1 (w/v).

0.05% (w/v) Aqueous CaCl<sub>2</sub>: 50 mg of CaCl<sub>2</sub> was dissolved into distilled water and diluted to 100.0 ml with distilled water.

Pure Solvent Upper Phase: Prepared by mixing at 3.0 ml aliquot of chloroform with 48.0 ml of methanol and 47.0 ml of 0.05% CaCl<sub>2</sub>.

### Procedure

8.0 ml of chloroform and 4.0 ml of methanol were placed in a separatory funnel. 2.0 ml of cell suspension in Krebs-Ringer phosphate buffer, pH 7.4, with 4% bovine albumin was added. The contents of the separatory funnel were shaken vigorously for approximately 30 minutes. Upon standing, a biphasic system was obtained, which contained no interfacial fluff. The volumes of the upper and lower phase were respectively, 40 and 60 per cent of the total volume of the system. The upper phase containing the non-lipid substances. The lower phase containing essentially all the lipids.

This biphasic system was allowed to stand for 4 hours.

Afterwards, as much of the upper phase as possible was removed by siphoning, and removal of its solutes was completed by rinsing the interphase three times with pure solvent upper phase mixture that contained 0.05% (w/v)  $\text{CaCl}_2$  in place of water. In rinsing, 2.0 ml of pure solvent upper phase mixture was allowed to flow gently from a pipette onto the walls of the separatory funnel. The washing fluid collected on top of the lower phase without any mixing of the two phases. After the separatory funnel was rotated gently, the upper phase mixture was removed by siphoning.

The lower phase was emptied from the separatory funnel into a tared beaker. Following overnight evaporation the difference in weight represented the total lipid per 2.0 ml cell suspension.

#### Determination of Adipose Cell Size and Number

In exploring the direct lipolytic effect of FMS on epididymal fat tissue consideration should be given to the cellularity of the chosen fat pads in terms of their cell size and number. It has become increasingly evident that differences in adipose tissue mass due to the number of adipose cells in the tissue and the size of these adipocytes can have an effect on the metabolic activity of this tissue.

It has been demonstrated that large adipocytes exhibit a marked resistance to the lipolytic action of glucagon when compared to small cells (Manganiello et al., 1972; Living-

ston et al., 1974). In vitro studies utilizing rat adipose tissue or isolated fat cells have indicated that fat tissue from older animals is less sensitive to catecholamines than fat tissue from younger animals. These observations connected with the knowledge that fat cell size in the rat increases throughout growth, has led to the concept that fat cells may lose their sensitivity to the lipolytic effects of catecholamines as they enlarge. Extending this concept to the obese individual who has fat cells of increased size projects the expectation of finding the same apparent metabolic deficit (Hartman et al., 1971).

Hartman et al. (1971) demonstrated that when lipolysis is based on the amount of triglyceride in the incubation medium or on tissue weight, as is the usual custom, or on the cell surface area, lipolysis inversely relates to cell size. But when norepinephrine stimulated lipolysis was expressed per  $10^6$  cells lipolysis was independent of cell size.

Experimental findings such as those noted above indicate that the lipolytic action of FMS on adipose tissue may vary when using the tissue from rats differing in body weight and/or subjected to different experimental treatment if tissue cellularity is not taken into consideration. Therefore, a method based on cellularity may prevent misleading information about the metabolic potential of FMS on its target tissue.

Various methods for determining adipose tissue cellularity include the following: (1) microscopic examination of isolated fat cells in the unfixed state with the aid of an ocular micrometer, (2) DNA content which produces a nuclear, and hence cellular tally, and (3) electronic counting. The first method is tedious since it involves the measurement and counting of large numbers of cells which have the appearance of irregular polygons in various orientations to the cut plane. The measurement of DNA in the intact tissue is an inexact estimate of adipose cellularity, since blood vessels, fibrous tissue, mast cells, etc., contribute unknown amounts of DNA to the total. A determination of the DNA content of adipose cell suspensions may not be a valid estimate due to the cell breakage caused by collagenase treatment. As a result, the Coulter Electronic Counter, Model B (Coulter Electronics, Inc., Hialeah, Florida) was used to count and size the epididymal fat cells, because of its simplicity, accuracy and general applicability.

#### Coulter Counter Principle

Particles or cells, suspended in an electrolyte can be sized and counted by passing them through an aperture with a specific path of current flow for a given length of time.

As particles or cells pass through the aperture and displace an equal volume of electrolyte, the resistance in

the path of current changes. This results in corresponding current and voltage changes. The quantity (magnitude) of this change is directly proportional to the volumetric size of the particle or cell. The number of changes within a specific length of time is proportional to the number of particles or cells within the suspension.

#### Preparation of Adipocytes

Albino male rats of the Sprague Dawley strain (body weight range, 130-150 gms) were stunned by a sharp blow to the head and decapitated and the fat cells were isolated from epididymal fat pads after exposure to collagenase according to the method of Rodbell (1964). Cell preparations were made only from rats weighing between 130 gms and 150 gms in the testing of FMS lipolytic activity on adipocytes (FMS target cells).

While the cell suspension was gently stirred, a 1.0 ml aliquot was withdrawn into a siliconized glass pipette, added to isotonic saline, and the volume brought up to 10.0 ml. A 1.0 ml aliquot of this stirred suspension was withdrawn into a siliconized glass pipette, added to isotonic saline, and the volume brought up to 10.0 ml; obtaining an overall dilution of 1:100. This same double step dilution method was repeated when using Krebs-Ringer phosphate buffer as the diluent. The dilution was such that the cell concen-

tration in the 0.5 ml sample to be monitored would fall between 1,000 and 80,000 cells.

Agitation is often required to maintain uniformity in suspensions containing particles over 5 microns. Maintenance of an uniform cell suspension during counting was achieved via continued gentle agitation during the process. The desired control settings were chosen and the count cycle initiated.

Sixteen counts per threshold level (272 counts in total) were taken for each and every cell suspension preparation used for an in vitro bioassay of FMS. For one-half of these counts isotonic saline was used as diluting fluid (electrolyte) and for the other half, Krebs-Ringer phosphate buffer was used.

On every cell suspension preparation the cell number per unit volume of cell suspension was calculated, and the average diameter of the cells in the cell suspension calculated. A frequency distribution of cell diameters was provided graphically for each cell suspension preparation.

#### Glycerol Determination

The biological activity of FMS was also measured, in vitro, by its ability to increase the release of glycerol from epididymal fat cell suspensions of the rat in a medium of Krebs-Ringer phosphate buffer (pH 7.4) containing 4% bovine serum albumin. The amount of glycerol released into the medium was determined by the method of Davidson and

Karjala (1970), with modifications. The assay utilizes the fluorometric measurement of the reduced adenine dinucleotide,  $\text{NADH}_2$ , formed when glycerol is oxidized to dihydroxyacetone by glycerol dehydrogenase.

### Reagents

1 N Perchloric Acid: 44.8 ml of the 70% (11.14 N), perchloric acid was dissolved into distilled water and diluted to 500.0 ml with distilled water.

1.8 N KOH: 249.3 gms of 45% (w/v) KOH was mixed with distilled water and diluted to 1.0 liter with distilled water.

Glycerol, Stock solution: 3.68 mg of glycerol was dissolved into distilled water and diluted to 100.0 ml with distilled water. Stock solution; 0.4 umole/ml.

Glycerol Standards: Standard solutions of glycerol were prepared ranging from 0.04 to 0.40 umole per tube.

0.1 M Glycine buffer, pH 9.5: 751 mgs of glycine, ammonia-free, was dissolved into distilled water and diluted to 100 ml with distilled water. The pH was adjusted to 9.5 with 0.1 M NaOH.

NAD: 3.0 mg of coenzyme NAD per ml of 0.1 M glycine buffer, pH 9.5 was prepared just prior to incubation.

Glycerol dehydrogenase: 0.15 Unit glycerol dehydrogenase per ml distilled water. 1 Unit oxidizes 1 umole of glycerol per minutes to dihydroxyacetone at pH 10.0 at 25°C.

An Aminco-Bowman Spectrophotofluorometer and Ratio

Photometer were used to measure the relative fluorescence intensity of  $\text{NADH}_2$  with excitation set at 350 nm and an emission setting of 455 nm. The "Sensitivity" control on the ratio photometer was set at 3. The "Sensitivity Vernier" control was set at its counterclockwise limit.

#### Preparation of Standard Curve

Standard samples for the preparation of a standard curve were prepared by diluting various aliquots of the stock glycerol standard solution to 1.0 ml with distilled water in glass stoppered 17 x 135 mm Corning centrifuge tubes. Standard glycerol solutions were prepared ranging from 0.04 to 0.40 umole of glycerol per tube. One ml of distilled water was used as the enzyme-coenzyme blank. This 1.0 ml of distilled water, because it is carried through the deproteinization procedure along with the glycerol standards, is referred to as deproteinized water in the table on "Blank and Sample Protocol for the In Vitro Bioassay of FMS". The glycerol standards were treated in the same manner as the unknown samples to compensate for any loss of substrate which may occur to the unknown samples during their deproteinization. When glycerol dehydrogenase (enzyme) and NAD are kept separate the fluorescence of each remains constant, but when the two are incubated together a small gradual increase in fluorescence occurs. With addition of 1.0 ml of distilled water (carried through the deproteinization procedure) the resulting fluorescence is slightly greater. As a result an enzyme-coenzyme blank must be sub-

tracted from all standard and unknown sample readings. A sample blank must also be subtracted from the corresponding unknown sample reading.

Deproteinization was achieved by adding 2.0 ml of 1 N  $\text{HClO}_4$  to each standard and enzyme-coenzyme blank. The contents were mixed for 30 seconds with a vortex mixer and then centrifuged for 15 minutes in an IEC Clinical Centrifuge (setting 6: 809 head). The standard and blank solutions were then very carefully transferred to a new set of glass stoppered 17 x 135 mm Corning centrifuge tubes. 1.0 ml of 1.8 N KOH was added to each glycerol standard and the enzyme-coenzyme blank to neutralize the standards and blank to pH 7-8. The tubes were then cooled in ice for 10 minutes to ensure complete precipitation of potassium perchlorate and afterwards centrifuged for 10 minutes in an IEC Clinical Centrifuge. The resulting supernatant was decanted into a 4.0 ml volumetric flask and brought up to 4.0 ml in volume with distilled water. Final volume was 4.0 ml for all the standards and enzyme-coenzyme blank.

1.0 ml aliquots from each deproteinized standard and enzyme-coenzyme blank were placed in 17 x 135 mm graduated reaction tubes. 6.0 mg of NAD in 2.0 ml of 0.1 M glycine buffer, pH 9.5, was added to each reaction tube and to the enzyme-coenzyme blank. 0.15 Unit of glycerol dehydrogenase dissolved in 1.0 ml of distilled water was added to each tube and to the enzyme-coenzyme blank. After the above additions the standards and the enzyme-coenzyme blank were

incubated in a thermo-regulated water bath set at room temperature, 25°C, using air as the gas phase for 90 minutes. The assay was performed at room temperature to avoid the variable cooling of the reaction mixtures between incubation and reading. The completeness of the reaction and the stability of NADH<sub>2</sub> eliminate the necessity of exact timing. With the monochromator wavelength settings of excitation 350 nm and emission at 455 nm, the relative fluorescence intensity of each unknown sample was read quickly to avoid errors in measurement due to possible heating of the samples in the cell chamber.

The fluorescence of each solution was read with excitation at 350 nm and emission at 455 nm. The fluorescence of the enzyme-coenzyme blank was subtracted from each standard using the ratio photometer's "Blank Subtract" control. Figure 2 shows a linear relationship between relative fluorescence intensity and the concentration of glycerol within the range of 0.01 to 0.10 umole of glycerol per tube. The data for this standard curve appear in Table 3.

#### Determination of Biological Activity of FMS Based on Glycerol Release from Epididymal Fat Cells

1.0 ml of epididymal fat cell suspension was placed in a 25 ml Erlenmeyer flask containing 3.0 ml of Krebs-Ringer phosphate buffer with 4% albumin. After 100 ug of FMS (or test material) in 1.0 ml of Krebs-Ringer phosphate buffer was added to the flask, incubation was allowed to proceed

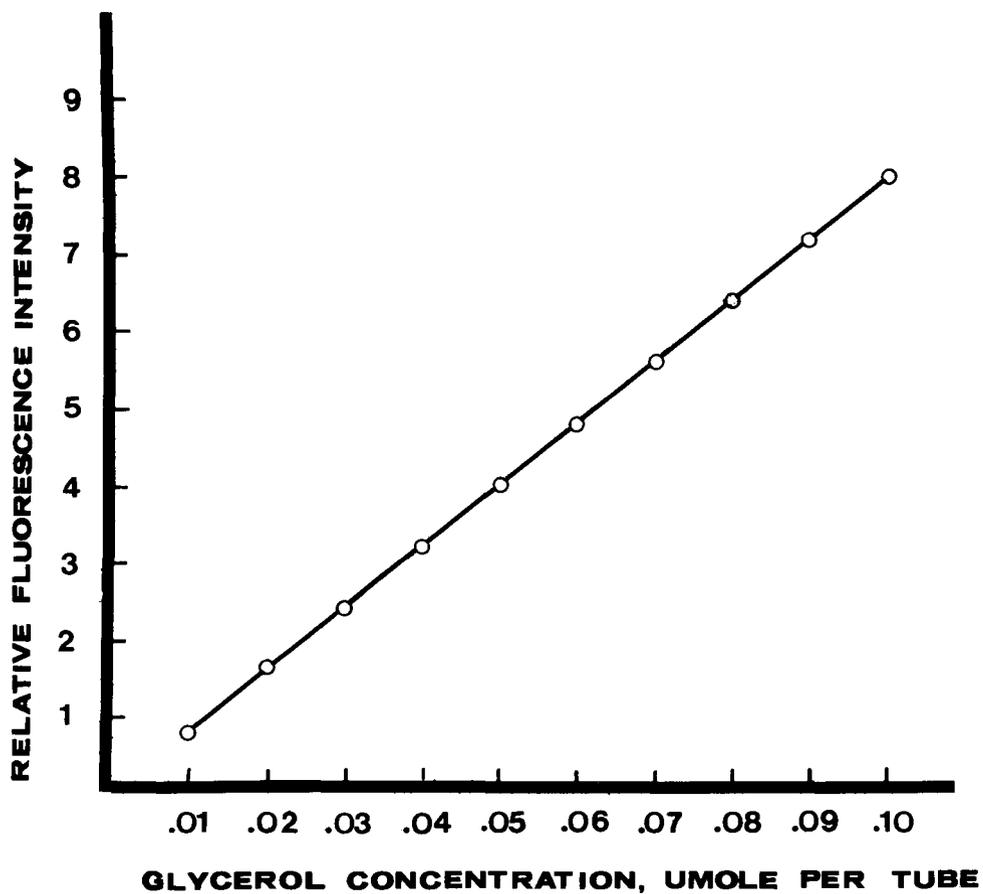


Figure 2. Standard Curve for Determination of Glycerol.

TABLE 3  
Standard Curve Data for  
Determination of Glycerol

Glycerol umole	No. of Determinations	Relative Fluorescence Intensity
0.01	20	0.8
0.02	20	1.6
0.03	20	2.4
0.04	20	3.2
0.05	20	4.0
0.06	20	4.8
0.07	20	5.6
0.08	20	6.4
0.09	20	7.2
0.10	20	8.1

Instrumental Parameters:

- (1) Aminco Ratio Photometer - photomultiplier tube detector (PMT):  
R 456 (unpotted) PM tube and Cable PM tube
- (2) Slit No:                    3   4   7  
Slit size (mm)            2   2   2
- (3) Excitation wavelength    350 nm  
Emission wavelength        450 nm  
Xenon Lamp Cat. No.        416-922
- (4) Sensitivity setting 3  
Sensitivity vernier setting - Counterclockwise limit

for 3 hours at 37°C in a Dubnoff Metabolic Shaker using air as the gas phase.

At the end of the incubation, a 1.0 ml aliquot was withdrawn from each of the incubation flasks and placed in glass-stoppered 17 x 135 mm Corning centrifuge tubes. The remaining contents from all the incubation flasks were pooled and 1.0 ml of the pooled incubation mixtures pipetted into a centrifuge tube to serve as the sample blank, which must be subtracted from each of the unknown sample readings. In addition, 1.0 ml of distilled water was pipetted into a Corning centrifuge tube to serve as the enzyme-coenzyme blank. (A minimum of four glycerol standards (two pair), one enzyme-coenzyme blank, and one sample blank were prepared per assay.)

The protocol for the in vitro bioassay of FMS based on glycerol release is given in Table 4. The fluorescence of the sample blank was subtracted from the final reading of each unknown sample. The amount of glycerol present in the sample was calculated by reference to the standard curve of relative fluorescence intensity versus glycerol concentration. The total glycerol released into the medium as umoles was calculated by multiplying the concentration value obtained from the standard curve by the dilution factor. Glycerol release was expressed umoles of glycerol released by 100 ug of FMS (or test material) acting on rat epididymal adipose tissue fat cell suspension equivalent to 1 gram of lipid for 3 hours at 37°C.

TABLE 4  
 Protocol for the  
In Vitro Bioassay of FMS

	Enzyme-Coenzyme Blank	Sample Blank	Sample	Standard
Deproteinized Pooled sample	-	1.0 ml	-	-
Deproteinized Sample	-	-	1.0 ml	-
Deproteinized Standard	-	-	-	1.0 ml
Deproteinized H <sub>2</sub> O	1.0 ml	-	-	-
NAD	2.0 ml	-	2.0 ml	2.0 ml
Enzyme	1.0 ml	-	1.0 ml	1.0 ml
H <sub>2</sub> O	-	3.0 ml	-	-

Final reaction mixture contained 6.0 mg of NAD and 0.15 U glycerol dehydrogenase in a total volume of 4.0 ml of 0.05 M glycine buffer, pH 9.5 and incubated at 25°C for 90 minutes.

Purification of Crude FMS by Ultrafiltration and Column  
Chromatography

After assaying, on an individual basis, the crude FMS extracted from the urine of each group of rats, all the remaining crude FMS was pooled, dissolved in deionized water and the solution was lyophilized in a VirTis Uni-Trap Freeze Dryer (The VirTis Company, Inc., Gardiner, New York). The lyophilized pooled residue of crude FMS was then divided into three parts and each of the three parts was carried through an ultrafiltration procedure, separately, employing PM-10 and UM-2 Diaflo membranes.

PM-10 Diaflo Membrane

A 0.05% (w/v) to 0.5% (w/v) aqueous solution of crude FMS was transferred to an Amicon Stirred Standard Ultrafiltration Cell, Model 52 (Amicon Corporation, Scientific Systems Division, Lexington, Massachusetts), fitted with a PM-10 Diaflo Membrane, which has a molecular weight cut-off of 10,000. A pressure of 30 psi was applied and the cell stirred at a relatively high rate.

Ultrafiltration was allowed to proceed for about 1.5 hours at an environmental temperature of 0° to 5°C. The ultrafiltrate and the residue were freeze-dried in the VirTis Uni-Trap Freeze Dryer, and bioassayed in vitro for lipolytic activity.

### UM-2 Diaflo Membrane

The biologically active fraction, the PM-10 filtrate, was then subjected to ultrafiltration, in a like manner, using a UM-2 Diaflo Membrane, with a cut-off at 1,000 in molecular weight. The ultrafiltrate and the residue were freeze-dried in the Virtis Uni-Trap Freeze Dryer and bioassayed in vitro for lipolytic activity.

### DM-5 Diaflo Membrane

The biologically active fraction, the UM-2 residue, was further purified and the molecular weight of FMS further approximated by subjecting this fraction to ultrafiltration using the newly patented DM-5 Diaflo Membrane, with a cut-off at 5,000 in molecular weight. The DM-Series is similar to the UM series of membranes in flow rate and rejection characteristics, and exhibits resistance to detergents, salts and phosphates, which affect the UM structure. The UM-2 residue was subjected to ultrafiltration with the DM-5 membrane in the same manner as with the PM-10 and UM-2 membranes and the ultrafiltrate and residue bioassayed as before.

### Bio-Gel P-10

A 0.35% (w/v) aqueous solution of UM-2 residue, the biologically active fraction from the ultrafiltration with the UM-2 Membrane, was passed through a column (K 25/45, 2.5 x 45 cm, jacketed, Pharmacia Fine Chemicals Inc., Piscataway, N.J.) of Bio-Gel P-10 fitted with a Pharmacia

Flow Adaptor, A-25.

The temperature of the column was maintained at 0<sup>o</sup>-5<sup>o</sup>C. Elution with distilled water was carried out at a rate of 1.8 ml per minute and serial fractions 5 ml in volume were collected with an Unifrac Fraction Collector (Savant Instruments Inc., Hicksville, New York). The transmission of the effluent at 280 nm was monitored with a LKB Uvicord Absorptiometer and Recorder (LKB Instruments Inc., Rockville, Maryland). The total volume of eluate was 500 ml (100 tubes). The collected fractions corresponding to individual elution peaks were pooled and lyophilized in the Virtis Freeze Dryer. Each peak was bioassayed in vitro for lipolytic activity.

#### Sephadex G-50 and Bio-Gel P-10

A 0.01% (w/v) aqueous solution of UM-2 residue, the biologically active fraction from the ultrafiltration with the UM-2 Membrane, was passed through a prepared column (K 25/45, 2.5 x 45 cm, jacketed, Pharmacia Fine Chemicals Inc., Piscataway, N.J.) of Sephadex G-50 and then through a column (identical in type to the one just mentioned) of Bio-Gel P-10, in tandem, as per Kwok (1974).

The column procedure used was that of Kwok (1974). With the temperature of the column maintained at 0<sup>o</sup>-5<sup>o</sup>C elution with distilled water was carried out at a rate of 2.5 ml per minute and serial fractions 5 ml in volume were collected with the Unifrac Fraction Collector. The trans-

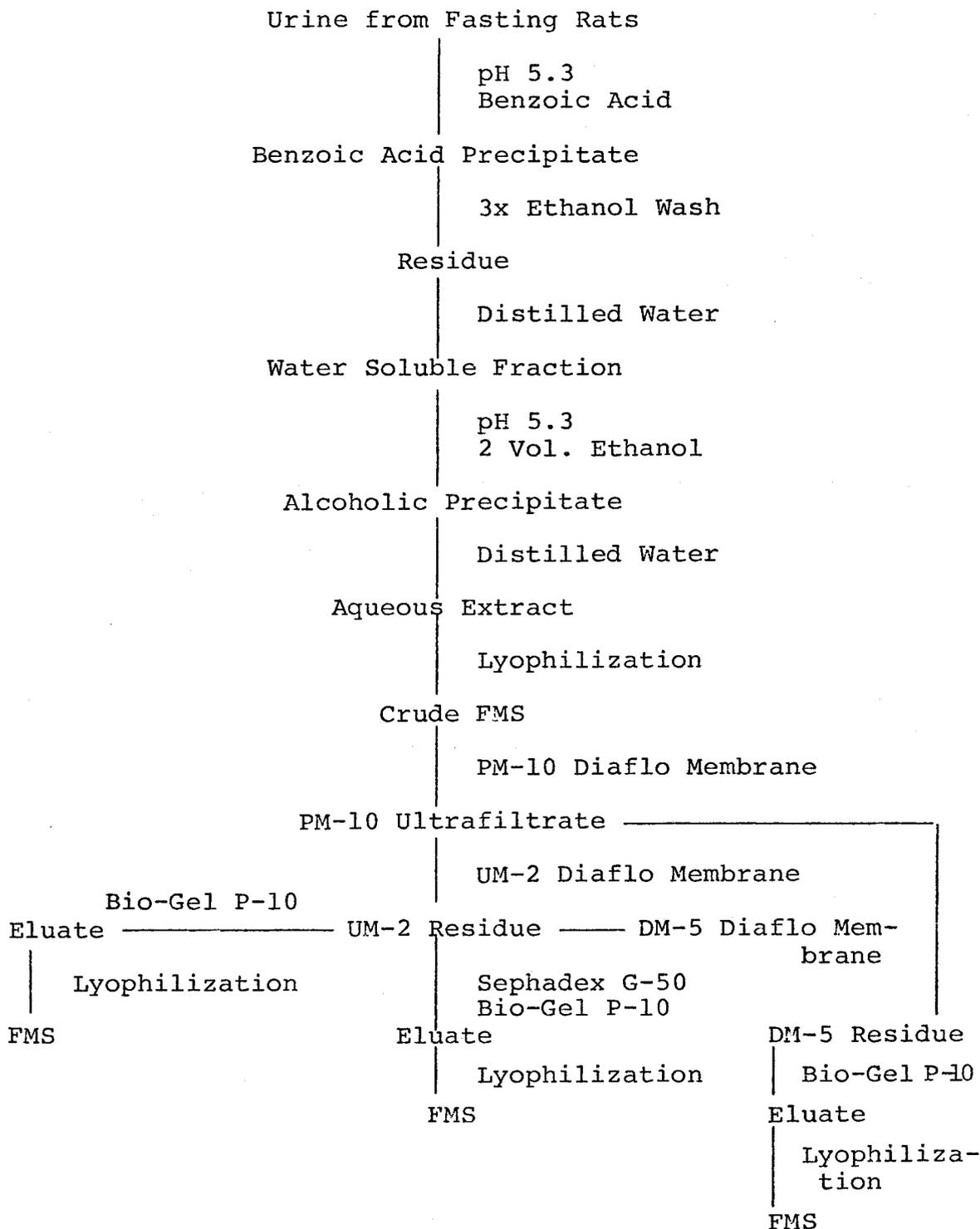
mission of the effluent was monitored with the Uvicord Absorptiometer and Recorder, set for monitoring at 280 nm. The total volume of the eluate was 500 ml (100 tubes). The fractions corresponding to elution peaks were pooled and lyophilized in the Virtis Uni-Trap Freeze Dryer, followed by in vitro bioassay for lipolytic activity. A diagram of the scheme for the extraction of crude FMS from rat urine and purification of FMS via ultrafiltration and column chromatography is shown in Figure 3.

### Isoelectric Focusing

Isoelectric focusing has become, in recent years, a powerful tool for the analytical or preparative separation of ampholytes, especially proteins, and for the characterization of the ampholyte by determining its isoelectric point (pI). The technique is characterized by a very high analytical resolution and by simplicity of apparatus and method. Isoelectric point determinations of resolved proteins can now be accomplished conveniently and with great reproducibility in a single experiment. Proteins with as small a difference as 0.02 pH unit separating their isoelectric points have been successfully resolved.

Isoelectric focusing is obtained by imposing a direct current potential on a system of electrolytes. The whole interval between the electrodes is occupied by a series of ampholytes with pI ascending from anode to cathode, thus creating a pH gradient defined in each point by carrier

FIGURE 3



Flow Diagram for Isolation and Purification of FMS

ampholytes which are in their isoelectric states. Each site along the gradient has its pH determined by the pH of the ampholyte at that point. When one adds a mixture of proteins with isoelectric points occurring within the pH range of the pH gradient, each protein molecule of the mixture migrates towards the pH value in the column where its net charge is zero. The pH gradient reaches a stable equilibrium if the system is stable for the duration of the experiment. Each protein of the mixture will be resolved at the point in the electrofocusing column where the pH is equal to the pI. Since this focusing, or resolution of proteins, is caused by an electric field, it has been named "Isoelectric Focusing".

#### Ampholine Carrier Ampholytes

Carrier ampholytes covering a pH range from pH 3 to pH 10, manufactured and marketed by LKB - Produkter AB, S-161 25 Bromma Sweden, were obtained from LKB Instruments, Inc., Rockville, Maryland, under the trade name of Ampholine Carrier Ampholytes, pH range 3-10. These synthetic ampholytes are aliphatic polyamino-polycarboxylic acids composed of a very large number of homologues and isomers of the same type of molecule which exhibit different, closely spaced pK and pI values.

The ready-for-use mixture came in a 25 ml bottle at a concentration of 40% (w/v) water solution, and was stored in the dark at a low temperature (below 6°C) to prevent decomposition, discolorization, and contamination by microorganisms.

The recommended concentration for electrofocusing proteins is 1 gm of Ampholine per 100 ml of solution. A 2% (w/v) ampholine solution was used when electrofocusing FMS in order to increase protein solubility. Ampholine chemicals contribute to the solubility of proteins.

### Electrode Solutions

Because the electrodes can communicate directly with the separation compartment, the carrier ampholytes adjacent to the electrodes must be protected from anodic oxidation or cathodic reduction. This is accomplished by surrounding the anode with a dilute solution of phosphoric acid and the cathode with a dilute solution of sodium hydroxide.

Electrolysis, during the electrofocusing experiment, draws the dilute acid and base solutions to their respective electrodes; the dilute phosphoric acid solution giving a net positive charge to any nearby carrier ampholytes, causing them to be repelled from the anode and hence protected from electrolytic decomposition. At the cathode, the dilute NaOH solution, gives a negative charge to the nearby carrier ampholytes which are then repelled from the cathode.

### Phosphoric Acid Solution: Anode Electrode Solution

The solution was composed of 0.2 ml phosphoric acid, 14.0 ml distilled H<sub>2</sub>O, and 12.0 gms of sucrose. The distilled water was added to a 50 ml graduated cylinder containing 12.0 gms of sucrose. The graduate cylinder was shaken manually

until the sucrose was completely into solution. 0.2 ml of phosphoric acid was then added. The final solution volume equalled 23 ml.

#### Sodium Hydroxide Solution: Cathode Electrode Solution

0.2 gm of NaOH pellets was dissolved into 20 ml of deionized H<sub>2</sub>O contained in a 30 ml Pyrex beaker. The entire volume of NaOH solution was pumped on to the isoelectric column.

#### Density Gradient

In order to prevent the remixing of separated zones due to thermal convection, and to stabilize the carrier ampholytes and protein sample zones, a density gradient of sucrose was a necessary prerequisite for the present technique. Any non-ionic substance harmless to the sample and pH gradient can be used for preparing a density gradient.

A linear density gradient was prepared by mixing a light solution containing distilled water, ampholytes, and sample (FMS), and a dense solution containing distilled water, ampholytes, and a "dense" solute (sucrose) with an LKB Gradient Mixer (LKB Instruments, Inc., Rockville, Maryland) designed specifically for filling the electrofocusing column. With the Tygon tube and outlet tube clamped with pinch clamps the dense solution was added to the vessel that holds the spiral stirrer, followed by the addition of the light solution to the vessel that holds the tapered cone. The stirrer and cone were then placed into the vessels and the stirrer motor started.

The pinch clamp between the light and dense solution vessels of the gradient mixer was loosened, quickly followed by the loosening of the clamp on the outlet tubing leading from the dense solution of the gradient mixer to the pump. A flow rate of 1 ml per minute was used.

It took approximately 2 hours to fill the column. When the outlet tubing is first opened only liquid from the stirrer vessel flows through the outlet tubing. As the column is filled there is a continuous decrease in the density of the liquid until only the light solution passes through the outlet tubing, resulting in a gradually decreasing sucrose concentration from the bottom to the top of the isoelectric focusing column.

Dense Solution: 2% Ampholine

Three fourths of the total amount of carrier ampholytes (3.75 ml of 40% (w/v) ampholine solution), pH 3 to pH 10 was placed into a 100 ml graduated cylinder and diluted up to 42 ml with distilled water. 28 gms of sucrose were dissolved in this solution by gentle warming and stirring, which gave a final total volume of 60 ml to this mixture. Of this mixture 5.0 ml was discarded, the remaining 55 ml was added to the gradient mixer, placing it into the vessel that holds the spiral stirrer.

Light Solution: 2% Ampholine

One fourth of the total amount of carrier ampholytes pH 3 to pH 10 (1.25 ml of 40% (w/v) ampholine solution) was

placed into a 100 ml graduated cylinder and diluted up to 60 ml with distilled water. After this solution was thoroughly mixed, 5.0 ml was discarded, leaving a total of 55 ml of light solution which was added to the gradient mixer in the vessel that holds the tapered cone.

### Sample

15 to 20 mgs of FMS were added to the light solution of the gradient mixer just prior to filling the column.

### Experimental Protocol

The entire electrofocusing column (LKB 8100 Ampholine Column, LKB Instruments, Inc., Rockville, Maryland) was thermostated at 4°C by utilizing a cryostat (Tamson Thermostatic Bath (TM-9) and circulator (PBC-4, Neslab Instruments, Inc., Portsmouth, New Hampshire), which circulated coolant around both the inner and outer electrofocusing columns. The coolant consisted of equal volumes of distilled water and ethylene glycol.

The central electrode valve was opened by depressing the spring located at the top of the glass column tube. This helical spring is in the setting device connected to the upper end of a Teflon rod that serves as a lever for the valve at its lower end. The central electrode is wound around this Teflon rod which lies within the central tube. The valve makes it possible to shut the central tube off from the separation compartment when emptying. The helical spring keeps the valve normally closed.

The anode electrode solution was pumped into the central electrode compartment, at top speed, via the upper nipple, with an LKB 12000 Vario-Perpex pump (LKB Instruments, Inc., Rockville, Maryland). The central electrode served as the anode during the isoelectric focusing of FMS. The pump tubing was inserted all the way down to the bottom of the central electrode compartment. While filling, a few drops were allowed to come out of the bottom of the column via the small piece of capillary tubing, which was then clamped shut again.

In preparation to fill the separation compartment, the dense solution was added to the stirrer vessel on the LKB Gradient Mixer, followed by the addition of the light solution to the cone vessel. After the stirring motor was turned on, the peristaltic pump was hooked up to the gradient mixer and set at  $4 \times 10$ , equalling a filling rate of 72 ml per hour. The polyethylene tubing was inserted about 2 inches into the lower nipple and against the side of the glass; thereby, preventing the drops from falling and making the gradient uneven.

FMS was added to the light solution and the clamps between the light and dense solution vessels loosened. Then, quickly, the clamp on the outlet tubing leading from the dense solution of the gradient mixer to the pump was loosened. After approximately 2 hours the solution mixed on the LKB Gradient Mixer filled the electrofocusing compartment giving a linear density gradient. The pump and the gradient mixer were shut off.

The remaining space at the top of the electrofocusing column was filled with the cathode electrode solution which was pumped on top of the gradient at a pump speed setting of  $2.5 \times 10$  (a filling rate of 45 ml per hr). The column was filled until the cathode solution level reached the upper black line about 1 inch above the upper platinum electrode.

The electrodes were then connected to an electrophoresis power supply (Gelman Instrument Co., Ann Arbor, Michigan), the anode electrode to plus via the plug at the central electrode terminal located on the very top of the column, and the cathode electrode to minus via the plug at the upper electrode terminal located by its loop located at the top of the column.

After the electrodes were in proper position they were tied to the supporting rack to make them immovable. The power supply was turned on and the voltage increased to a constant voltage of just under 500 volts. Upon completion of this last step, the electrofocusing of FMS has begun; a pH gradient is gradually being created and FMS is starting to migrate towards the point in the gradient corresponding to its isoelectric point.

At first, the current on the milliamperere meter read 10 to 12 ma. This is because the water and the carrier ampholytes inside of the column are all intermixed. When the current (at a constant voltage of 500 volts) had decreased to a constant value of 2 ma most of the carrier ampholytes in the system had migrated at or near their isoelectric points. The FMS sample had also been focused at its isoelectric point to

a great extent; however, it took 48 hours to reach a steady final focusing.

Final movement to the equilibrium state is slow, and the contribution of FMS to the conductance extremely low. As a result the change in conductivity time was so small after the current had decreased to the constant value of 2 ma, that no further current change registered in the time remaining for final completion of the electrofocusing run. In general, the focusing requires 10 hours or more extra time after the current has decreased to a constant value. An electrofocusing run normally takes from 24 to 72 hours at a voltage of 300 to 500 volts, depending on the ampholine pH range used. Longer electrofocusing times are required for resolution of proteins in narrower pH ranges of ampholines.

When the electrofocusing was completed, the central electrode valve was closed. Extreme care was taken to release the spring pressure very carefully, to prevent the gradient from being disturbed by bubbles which would result in incorrect pH values of the effluent fractions due to their contamination with the anode electrode solution. The voltage was turned off and the jacks disconnected from the column. Then, at the fastest rate, the central electrode solution was pumped out of the column to prevent any leakage of the solution out of the central tube.

After a thorough rinsing of the polyethylene tubing, the tubing was connected from the fraction collector to the electrofocusing column. The connection tubing was as short

as possible to prevent mixing. With 80 collection tubes in the fraction collector rack, the fraction collector was turned on. The counter on the fraction collector was set at 20 drops per fraction (1.8 ml per tube).

The outlet clamp for the electrofocusing column was then opened and the column was emptied by means of an LKB peristaltic pump set at  $1.4 \times 10$  (a speed of approximately 22 ml per hour). It took about 6 hours to empty the entire column.

The effluent was monitored with an LKB Absorptiometer, set for monitoring the effluent at 280 nm.

The "Ambient Temperature" knob on the LKB Uvicord II Photometric Flow Analyzer was set at 1, for use at a coolant temperature 4 degrees centigrade. While the first fraction was being collected, the Uvicord was set at 2X the sensitivity scale. Once this was accomplished the "T%" was dropped back from 100% T to about 40-45% T so the entire column could be monitored. If this is not done the collection won't be able to be monitored. A low reading of 40 to 45% T will be recorded for the first couple of fractions due to significantly high values of absorption in that part of the gradient. However, after these first couple of fractions the absorption gets much lower, and T% much higher. If the recorder bar had been originally set at 100% T the low absorption fractions would cause the recorder bar to go "Off" scale, resulting in an unmonitored collection.

Measurement of the pH of the collected fractions provides a picture of the pH gradient, making it possible to determine the isoelectric point of each component of the sample with the accuracy obtainable with the pH meter being used.

The pI is dependent on the temperature, but there is no reliable factor which allows conversion of a pI measured at one temperature to the pI corresponding to another temperature. As a result the pH of the fractions from electrofocusing were measured on a Beckman Phasar-1 pH Meter at the same temperature as used during electrofocusing.

#### Data Analysis: Measurement of the Result

The LKB Uvicord II Photometric Flow Analyzer records the UV absorption curve on paper. The volume of the eluted liquid, or the corresponding length of the column in cm's, is plotted along the x-axis. The pH gradient is also plotted on the same diagram. The y-axis is divided into pH units, starting with the lowest and ending with the highest values of the pH gradient used.

#### Removal of Carrier Ampholytes from FMS Fractions

The FMS was separated from the sucrose and carrier ampholytes by means of dialysis using #3 Spectrapor Membranes (Spectrum Medical Industries Inc., Los Angeles, California). Since some of the Ampholine carrier ampholyte molecules have a molecular weight of more than 1000, they pass through the dialysis membrane rather slowly. Therefore, dialysis required

48 hours before all the carrier ampholytes had been removed.

The dialyzing sacs were closed by using Spectrapor Closures. Spectrapor closures were used instead of tying a knot in the wet tubing since knot tying can distort pores in wet tubing. String tying was not used because the string can cut tubing.

### Protein Determinations

The protein concentrations of the fractions of FMS obtained from electrofocusing were measured using the method of Lowry et al. (1951). This very sensitive method is based on the fact that a deep blue color is formed when protein in alkaline copper solution is treated with the phenol reagent of Folin and Ciocalteu (1927).

Two distinct steps are involved in the reaction. First, the protein reacts with copper in alkaline solution, and second, a reduction of phosphomolybdic and phosphotungstic acid by the copper-treated protein occurs.

### Preparation of Standard Curve

#### Reagents

1% Sodium Dodecyl Sulfate (SDS): 1 gram of  $\text{CH}_3(\text{CH}_2)_{11}\text{OSO}_3\text{Na}$  was dissolved in distilled water to a volume of 100 ml.

2% Sodium Carbonate in 0.1 N Sodium Hydroxide: 0.1 N NaOH was prepared by adding 0.4 grams of NaOH to enough distilled water to make 100 ml of solution. 2 grams of  $\text{Na}_2\text{CO}_3$  were mixed with a quantity of the 0.1 N NaOH solution sufficient to give 100

ml of solution. This reagent was prepared fresh just before use.

2% Sodium Tartrate: 2 grams of  $\text{Na}_2\text{C}_4\text{H}_4\text{O}_6 \cdot 2 \text{H}_2\text{O}$  were dissolved in distilled water and the solution diluted to 100 mls with distilled water.

1% Copper Sulfate: 1 gram of  $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$  was dissolved in distilled water and the solution was diluted to 100 mls with distilled water.

Phenol Reagent of Folin and Ciocalteu, 1N: A commercially prepared solution of the phenol reagent, 2 N, was purchased from Harleco, Philadelphia, Penn. A working solution was prepared from this, each day of use, by diluting a small volume of the stock solution with an equal volume of water to give a 1 N phenol reagent.

Standard Protein Solution: Crystallized human albumin, purchased from Dade Division of American Hospital Supply Corporation, Miami, Fla., was used for the preparation of a standard protein solution. When reconstituted with 3.0 ml of distilled water, the standard contains 80 mg of protein per ml.

Working Standard Protein Solution: A working standard protein solution of 80 ug/ml was prepared just before use by diluting 0.1 ml of the protein standard solution to 100.0 ml with distilled water.

### Procedure

Standards for the preparation of the standard curve were prepared by diluting various amounts of the working

standard solution to 1.0 ml with distilled water. One ml of distilled water was used as blank.

To 100 ml of the 2% sodium carbonate in 0.1 N sodium hydroxide, 1.0 ml of 2% sodium tartrate and 1.0 ml of 1% copper sulfate were added, and thoroughly mixed. Five ml of this mixed reagent was then added to each blank and standard. After mixing, they were left to stand at room temperature for 10 minutes.

One-half ml of the 1 N phenol reagent was added to each tube and mixed immediately. After 30 minutes or longer the absorbance was measured at 700 nm in a Beckman DB Spectrophotometer. The data for this standard curve appear in Table 5. A linear relationship between the absorbance at 700 nm and the concentration of protein within the range of 16 ug to 80 ug is shown in Figure 4.

#### Determination of FMS Protein Concentration

1 mg from each of the fractions of FMS obtained from electrofocusing was added to distilled water, and the volume then adjusted to 10.0 ml.

Various aliquots of the same FMS test solutions were diluted to a total volume of 1.0 ml with distilled water and analyzed for protein content. The amount of protein present was calculated by reference to the standard curve.

TABLE 5  
Standard Curve Data for  
Determination of Protein Concentration

Micrograms of Protein	No. of Determinations	Absorbance* 700 nm
16	5	0.014 $\pm$ 0.001
32	5	0.061 $\pm$ 0.002
48	5	0.114 $\pm$ 0.008
64	5	0.160 $\pm$ 0.005
80	5	0.216 $\pm$ 0.002

\* Mean  $\pm$  Standard Deviation

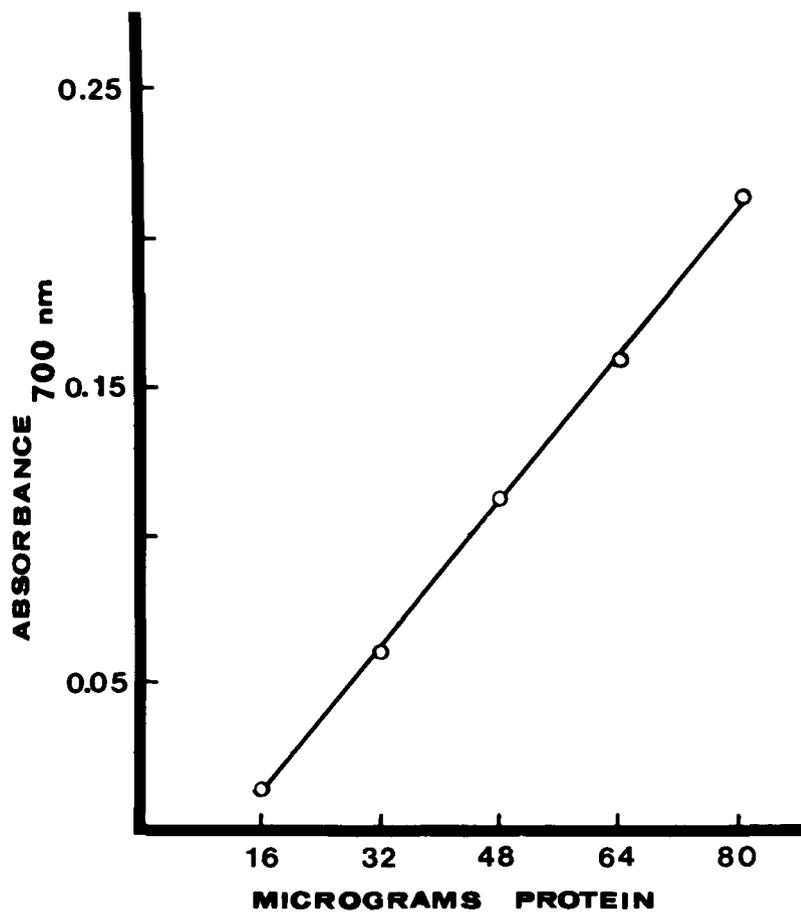


Figure 4. Standard Curve for Determination of Protein Concentration.

The fractions of FMS obtained from electrofocusing were subjected to polyacrylamide gel electrophoresis utilizing a Hoefer Disc Electrophoresis Unit (Hoefer Scientific Instruments Co., San Francisco, California). Anionic polyacrylamide gels were used for the electrophoresis.

Reagents

Stock Solutions

Stock solutions were prepared as outlined in Table 6 using deionized water and were filtered and stored in brown glass bottles or bottles wrapped in foil in the refrigerator. Most of these stock solutions had a shelf life of up to three months.

Working Solutions

Working solutions were prepared as outlined in Table 7 fresh from the stock solutions on the day they were to be used.

Fixative, Fixative-Stain, and Destaining Solution

The fixative solution and the solution for washing and destaining the polyacrylamide gels were prepared in advance and stored at room temperature, Table 8. The Coomassie Brilliant Blue R-250 was prepared as an aqueous stock solution which was stirred and filtered prior to use. This stock solution was diluted 1:20 with 10% trichloroacetic acid (TCA) just prior to staining the gels. The fixative

TABLE 6  
Anionic Gel Stock Solutions

Separation Gel (Small-pore gel)		Sample and Stacking Gels (Large-pore gels)	
(A)*		(B)	
1 <u>N</u> HCl	approx. 48 ml	1 N HCl	approx. 48 ml
Trizma-Base	36.3 gm	Trizma-Base	5.98 gm
TEMED	0.46 ml	TEMED	0.46 ml
water to	100.0 ml	water to	100.0 ml
(pH 8.9)*		(pH 6.7)*	
(C)		(D)	
Acrylamide	30.0 gm	Acrylamide	10.0 gm
BIS	0.8 gm	BIS	2.5 gm
Potassium Ferri- cyanide	0.015 gm		
water to	100.0 ml	water to	100.0 ml
(E)		(F)	
Ammonium Persulfate	0.14 gm	Riboflavin	0.004 gm
water to	100.0 ml	water to	100.0 ml

\*pH adjusted by titrating with 1 N HCl before dilution to 100 mls.

TEMED - N,N,N',N'-Tetramethylethylenediamine

BIS - N,N'-Methylene-bisacrylamide

TABLE 7  
Anionic Gel Working Solutions

Separating Gel (Small pore gel)	Sample and Stacking Gel (Large-pore gel)	Buffer Solution
1 Part A	1 Part B	TRIZMA-BASE 6.0 gm
2 Parts C	2 Parts C	Glycine 28.8 gm
4 Parts E	1 Part F	Water to 1 liter
1 Part Water	4 Parts Water	
pH 8.9 (8.8-9.0)	pH 6.7 (6.6-6.8)	pH 8.3

TABLE 8

## Fixative, Staining and Destaining Solutions

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		Fixative Solution
TCA	10%	
		Fixative-Stain Solutions
		Coomassie Brilliant Blue
		Stock Solution:
		Coomassie Brilliant Blue 1 gm
		Water to 100 ml
		Working Solution:
		12.5-20.0% TCA* 20 ml
		Stock Solution 1 ml
		Destaining Solution
TCA	10%	

---

\* A further increase in sensitivity can be achieved by the use of a 20% TCA dye solvent.

stain solution was made up fresh from the stock solution before use.

### Procedure

Twelve Pyrex glass columns (10 cm long and having an inner diameter of 0.5 cm and an outer diameter of 0.7 cm) were acid cleaned and coated with a wetting agent (1 part Kodak Photo-Flo to 200 parts water). Base caps (vacutainer tops) were placed at one end of the gel columns which were then placed into a loading rack in which front-to-back vertical alignment of the gels could be assured.

Stock solutions A, C, and E were removed from the refrigerator and 4 ml of solution A, 16 ml of solution E, 8 ml of solution C, and 4 ml of deionized water were placed in separate flasks and allowed to warm up to room temperature. Approximately 20 mls of separation gel solution was the volume required to run twelve 10 cm long gels. The four components were then added to a single flask and swirled gently to prevent air bubble formation and to provide thorough mixing. This was the separation gel solution. Using a 1 ml tuberculin syringe, to which 5 cm of Tygon tubing was attached, exactly 1.6 ml of the separating gel solution was dispensed (without bubble formation) into each of the 12 vertical gel columns.

An Oxford pipette was then used to carefully layer 100  $\mu$ l (2-3 mm) of water on top of the gel solution. If the gel columns are not coated with a wetting agent prior to their

use, a bolus of water will form which will drop into the gel solution and dilute it. However, if the gel columns are coated, water will layer evenly on top of the unpolymerized gel solutions. The purpose of the water layering of gel solutions is that it prevents a gel-air meniscus and produces a flat gel surface which is essential for obtaining good protein band formation. It also prevents oxygen, which retards gel formation, from diffusing into the gels.

The separation gels were left undisturbed for one half hour during which time complete chemical polymerization occurred as was evident by the visibility of a distinct refractile line between the gel and water interface.

The gel columns were then removed from the loading rack and the water layer was removed by giving the column a quick flick of the wrist. The lip of the gel column was then touched to a piece of absorbent paper to remove the last traces of water.

Two milliliters of solution B, 4 ml of solution D, 2 ml of solution F, and 8 ml of water were pipetted into another set of small flasks. These solutions were warmed to room temperature and gently mixed in a flask that was completely wrapped in foil to protect this gel solution from premature photopolymerization. This solution was to serve as the photopolymerizing stacking and sample gel. A 1 ml syringe with Tygon tubing adapter was then used to add 0.3 ml of this solution to each gel column. This served as the

stacking gel. Once again, 100 ul of water was layered over this gel solution as previously described. The entire loading rack was positioned approximately 30 cm from a fluorescent light source. Fifteen minutes were allowed for photopolymerization to begin as evidenced by the appearance of opalescence in the stacking gel. When photopolymerization had begun, the light source was moved to within 5 cm of the columns and another 15 minutes were allotted to insure total photopolymerization. The water layer was then removed as previously described.

Exactly 1.0 mg of the FMS preparation to be tested was dissolved in 1.0 ml of the stacking and sample gel solution and 0.250 ml of the solution was added on top of the stacking gel and layered with 100 ul of water. The sample gel was allowed to photopolymerize for 30 minutes while positioned 5 cm from the fluorescent light.

Bromphenol blue (0.1 to 0.2 ml of a 0.001% solution), serving as a tracking dye, was incorporated into the sample gel solution prior to its addition to the gel column. This dye migrates faster than the fastest sample ion species.

The gel columns were then removed from the loading rack and inserted into the holes of the upper bath stoppers (sample gel uppermost). After all the gel columns were inserted in this manner, buffer was added on top of the sample gels, making certain that no air bubbles were entrapped in the remaining space of the upper gel column. The entire upper bath assembly containing the gel columns was then

lifted out and sufficient buffer solution was added to the separation gels such that a hanging drop of buffer solution remained at the bottom of all the gel columns. It is essential that no air bubbles are entrapped in the gel columns.

The lower bath (containing the anode) of the Hoefer Electrophoresis Apparatus was then filled with 1000 ml of buffer solution. The upper bath assembly was then carefully placed into position making certain that no air bubbles had formed at either end of the gel columns. Air bubbles result in uneven band formation because they prevent a uniform current flow through the gels. The upper buffer reservoir was then filled with 500 ml of buffer solution.

The lid was then placed on the upper bath. The power supply was connected. A current of 2.5 ma per gel column provided for good resolution of protein bands. Electrophoresis was carried out until the now discrete front of tracking dye band had migrated to within  $\frac{1}{4}$  inch of the separation gel end. This required approximately 1 hour and 30 minutes at 2.5 ma per gel column.

At the completion of electrophoresis, the power supply was turned off and the electrode jacks were disconnected. The gel columns were removed from the upper reservoir and were placed in a wash tray containing ice-cold water. The gels remained immersed in the ice-cold water for 5 minutes which was sufficient time for maximal gel contraction. A ten milliliter syringe fitted with an 18 gauge needle was

filled with ice-cold water. The gel columns were kept submerged while the tip of this needle (beveled edge towards the gel to avoid scratching the gel) was inserted between the glass column and the gel. The needle was inserted slightly beyond the sample gel of the gel column. Keeping the gel columns submerged and the needle flat against the glass surface, the entire gel column was rotated with one hand while the position of the needle was maintained. This freed the entire circumference of the sample gel from the glass column. Ice-cold water was continuously injected around the gel surface as the gel column was rotated. The water pressure freed the stacking and separating gels and in a few moments the intact gel was expelled from the separating gel end of the glass column.

The gels were quickly placed in a 10% solution of TCA for a minimum of one hour to insure that the protein fractions were fixed uniformly. The gels were then stained by immersion into 20 ml of the Coomassie Brilliant Blue working solution for another hour. Minute protein bands were well detailed in 20% TCA-Coomassie Brilliant Blue stain solutions. Destaining was done by three consecutive immersions of the gels into 10% TCA solution, each immersion lasting about an hour. The gels were then immersed into 10% TCA solution and the TCA solution was changed every day for approximately 3 days. When the section of a gel, containing no separated fraction was completely clear of stain, the gel was transferred to a solution comprised of equal parts of glycerol

and deionized water. This allows for the storing of gels without shrinkage.

#### Densitometry of Polyacrylamide Gels

Densitometry of the gels was performed with a Densicord Model 542 Densitometer (Photovolt Corporation, New York, N.Y.). The proper response setting was chosen and the stained separation gels were scanned over their entire length by the Densicord. The percentages of the various fractions from the densitometer trace were determined by establishing the area under the curve at all points.

#### Photography of Polyacrylamide Gels

A Polaroid camera with a close-up lens was a convenient method of photographing stained polyacrylamide gels.

## CHAPTER III

### Results

#### Effect of Body Weight and Age of the Rat on the Bulk Urinary Excretion, Total and Specific Activity of FMS

Eleven groups of 30 male albino rats varying in mean group weight from 152-416 grams were placed on a high fat diet with water ad libitum for a 7 day period. They were then transferred from their individual screen bottomed cages to individual metabolism cages, and their urine collected during a 24 hour fast under cold stress. The urine from the rats within a group was pooled and the crude FMS extracted, weighed, and bioassayed in vitro for lipolytic activity. Several approaches were employed for testing the lipolytic activity of the FMS preparations.

Depending upon group mean weight just prior to the 24 hour fasting period under cold stress, the assay data collected on each individual rat group was placed in one of three weight, hence age, categories: (Category 1) body weight range, "150-250" grams; Mean age, 7 weeks: (Category 2) Body weight range, "250-350" grams; Mean age, 14 weeks: (Category 3) Body weight range, "350-450" grams; Mean age, 18 weeks or greater.

Performance of in vitro bioassay, initially by the method Duncombe, afforded a convenient and rapid measurement of the quantitative release of FFA as index of the

lipolytic activity of the FMS preparation obtained from each rat group. Table 9 shows the data obtained for each of the three defined categories in terms of free fatty acids released from epididymal fat pads of the rats 130-150 grams in weight in a medium of Krebs-Ringer phosphate buffer (pH 7.4) containing 4% bovine serum albumin. Analysis of variance showed the observed variation between activity values of each of the categories to be significant ( $P < 0.05$ ). Student t-tests determined which specific categories show significance and at which level (Category I vs. II,  $P < 0.01$ ; Category II vs. III,  $P = 0.05$ ). Decreasing levels of significance in lipolytic activity are noted in association with increasing weight and age of rats from which the FMS is isolated.

After initial bioassay in vitro, the following in vitro bioassays utilized epididymal fat cell suspensions. Considerably increased lipolysis by FMS target cells was observed as reflected by sizably increased amount of FFA released in response to the same preparations of FMS. These results are attributable to an increased sensitivity of isolated fat cells as opposed to intact fat pads to lipolytic agents. Data on these bioassays depict the effect of weight and age on total urinary excretion of FMS under three classifications, the effect of weight and age on FMS bulk excretion (Table 10), FMS specific lipolytic activity (Table 11) and FMS total lipolytic activity (Table 12). Data in each of the three tables are

TABLE 9

Response of Adipose Tissue to FMS Isolated from Rats of Different Weight and Age

Category	Weight Range (gm)	Mean Age (wks)	FFA* (umoles)	P vs Difference Between Category Means	P vs Control
Control	-	-	0.361 $\pm$ 0.03**		
I	150-250	7	0.519 $\pm$ 0.05	P < 0.01	P < 0.001
II	250-350	14	0.435 $\pm$ 0.02	P = 0.05	P < 0.01
III	350-450	18	0.390 $\pm$ 0.005		P < 0.3

\* Expressed as umoles free fatty acids (FFA) released by 100 micrograms of test material acting on 250 milligrams of rat epididymal adipose tissue for 3 hours at 37°C.

\*\* Mean  $\pm$  standard deviation of the combined group means.

TABLE 10

Effect of Weight and Age on the Bulk Excretion of FMS

	Body Weight Category (gm)	Approx. Age (wks)	Mean Bulk Excretion (mg/animal) Category
	I. 150-250	7	1.9
	$\bar{M}$ 188		
G R O U P S	1) 152		0.6
	2) 193		0.2
	3) 233		2.5
	4) 174		4.1
	II. 250-350	14	2.0
	$\bar{M}$ 301		
G R O U P S	1) 260		0.2
	2) 329		2.8
	3) 329		1.4
	4) 253		4.1
	5) 336		1.7
	III. 350-450	18	4.2
	$\bar{M}$ 398		
G R O U P S	1) 379		5.0
	2) 416		3.3

TABLE 11

Effect of Weight and Age on the Specific Activity of FMS

Body Weight Category (gm)	Approx. Age (wks)	Specific Activity (umoles)		P vs Difference Between Category Means		P vs Control	
		Group	Category	FFA	Glycerol	FFA	Glycerol
Control				10.02	1.61		
I. 150-250	7			11.68	2.32		
M							
188							
1) 152		11.69	2.42				
2) 193		*11.59	2.29				
		11.56					
3) 233		11.38	2.15				
4) 174		12.20	2.42				
II. 250-350	14			10.91	2.15		
M							
301							
1) 260		*10.67	2.15				
		10.52					
2) 329		10.98	2.08				
3) 329		10.57	2.15				
4) 253		11.68	2.22				
5) 336		11.04	-				
III. 350-450	18			10.35	1.95		
M							
398							
1) 379		*10.39	2.02				
		10.48					
2) 416		*10.26	1.88				
		10.26					

Specific Activity is expressed as umoles FFA and glycerol released by 100 ug of FMS acting on rat epididymal fat cell suspension equivalent to 1 gram of lipid.

\* Two sets of determinations were run on these rat groups.

TABLE 12

Effect of Weight and Age on the Total Activity of FMS

Body Weight Category (gm)	Approx. Age (wks)	Total Activity umoles/animal	
		Group FFA	Group Glycerol
I. 150-250	7	18	4
$\bar{M}$ 188			
1) 152	7.0	1.5	
2) 193	*2.3	0.5	
	2.3	-	
3) 233	28.5	5.4	
4) 174	50.0	9.9	
II. 250-350	14	19	5
$\bar{M}$ 301			
1) 260	*2.1	.43	
	2.1	-	
2) 329	30.7	5.8	
3) 329	14.8	3.0	
4) 253	47.8	9.1	
5) 336	18.8	-	
III. 350-450	18	43	8
$\bar{M}$ 398			
1) 379	*52.0	10.1	
	52.4	-	
2) 416	*33.9	6.2	
	33.9	-	

Total Activity, calculated as weight of extract (mg) times specific activity.

\* Two sets of determinations were run on these rat groups.

delineated in terms of categorical means representative of the average of the sum of individual group means comprising each of the three categories, and mean bulk excretion level (Table 10), mean specific (Table 11) and total (Table 12) lipolytic activities for every animal group contained within each of the defined weight-age categories.

Analysis of variance revealed a significant variation in the degree of specific lipolytic activity amongst the three lots of FMS from the three defined weight-age categories, whether FFA ( $P < 0.001$ ) or glycerol ( $P < 0.01$ ) release was employed as index of FMS lipolysis. Thereupon, t-tests were conducted to ascertain at what significance level which specific categories are significantly different in lipolytic activity. Weight categories I and II were significantly different in specific lipolytic activity for both FFA and glycerol released by the isolated epididymal fat cells (FFA,  $P < 0.01$ ; glycerol,  $P < 0.05$ ). Between categories II and III the difference in FMS lipolysis showed significance for both indexes of lipolysis (FFA,  $P < 0.05$ ; glycerol,  $P < 0.01$ ).

Regarding the specific activity data, Students t-test performed on the mean specific lipolytic activity of means (the mean of the sum of the 11 group means) and the Controls showed a significant difference in lipolytic activity for both indexes of lipolysis (FFA,  $P < 0.01$ ; glycerol,  $P < 0.001$ ).

Figure 5 illustrates the relationship between 'Specific and Total Activity of FMS', dependent upon weight and age of the rat from which FMS is isolated. FFA and glycerol release are expressed in terms of umoles released per 100 micrograms of FMS acting upon epididymal fat cell suspension equivalent to 1 gram of cell lipid for 3 hours at 37°C.

Substantiated via the Analysis of Variance and Students t-test performed on the specific activity data obtained for each of the 11 rat groups, the histogram depicts a significant difference in Specific Lipolytic Activity amongst the three defined weight-age range categories: (1) Weight range, 150-250 grams; Mean age, 7 weeks; (2) Weight range, 250-350 grams; Mean age, 14 weeks; (3) Weight range, 350-450 grams; Mean age 18 weeks or greater. In addition, the histogram illustrates a highly negative correlation, to the same degree of significance as established by the already performed statistical analyses, existing between FMS Specific Activity and increasing weight-age of the rat.

A more pronounced effect exists between FMS Total Activity (Total Activity, calculated as weight of FMS extracted from the rat urine times specific activity) and increasing weight-age of the rat. However, for both indexes of lipolytic activity, instead of a highly negative correlation between increasing weight and age of the rat, the correlation is highly positive.

Decreasing 'Specific Activity' with increasing 'Total

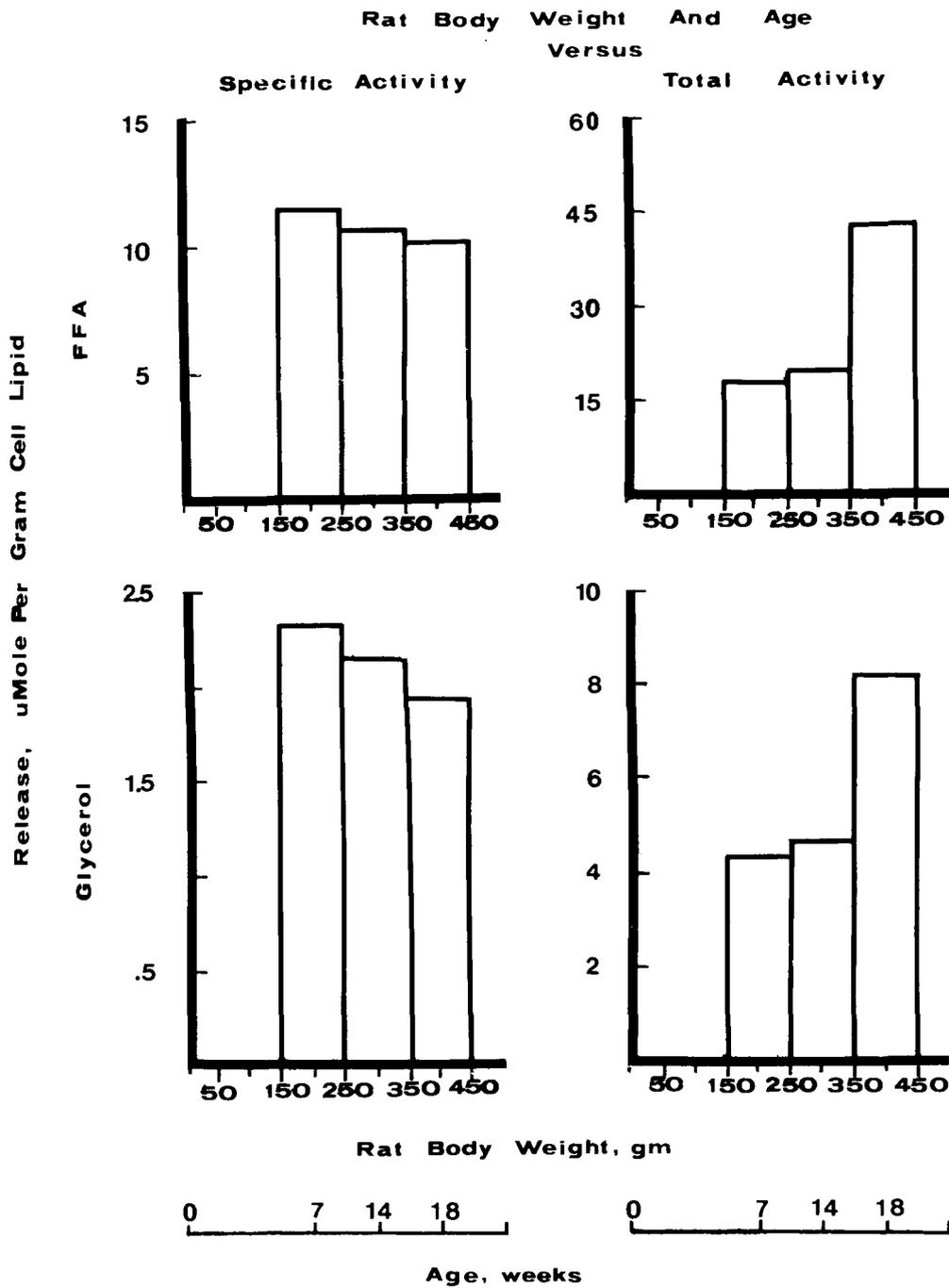


Figure 5. Variation of Specific and Total Lipolytic Activities with Rat Body Weight and Age.

Activity' was found in the 11 rat groups with increasing weight and age. The greatest specific activity was found in the FMS isolated from rats weighing 150-250 grams with a mean age of 7 weeks (Category 1). In contrast, total activity was found to increase with increasing weight and age. In comparison with rats weighing 150-250 grams and 7 weeks in age (Category 1), for rats weighing 350-450 grams with a mean age of 18 weeks or greater (Category 3), the total activity doubled, both for FFA and glycerol indexes of lipolysis.

In conjunction with the in vitro bioassays utilizing the increased sensitivity of isolated adipocytes, examination of the lipolytic response of adipocytes to FMS in the terms of their cell number and size was studied. Table 13 expresses in terms of FFA and glycerol release per  $10^6$  cells, specific lipolytic activity for each of the defined weight-age categories. It will be observed that fat cells have the greatest apparent sensitivity to FMS that is isolated from rats ranging 150-250 grams in weight. As the rats increased in weight and age the lipolytic response of the adipocytes to their FMS extracts apparently decreased.

Cellularity was determined on every cell suspension prepared for in vitro bioassay. Figure 6 shows the combined cell diameter frequency distributions of the individually prepared fat cell suspensions used in the various bioassays.

TABLE 13

Response of Adipocytes to FMS Isolated from Rats of Different Weight and Age

Category	Weight Range (gms)	Age (wks)	FFA		Glycerol	
			(umoles/10 <sup>6</sup> cells/hr)*	(umoles/10 <sup>6</sup> cells/hr)*	(umoles/10 <sup>6</sup> cells/hr)*	(umoles/10 <sup>6</sup> cells/hr)*
Control	-	-	195.61 x 10 <sup>-3</sup>	+ .009**	31.26 x 10 <sup>-3</sup>	+ .008**
1	150-250	7	227.30 x 10 <sup>-3</sup>	+ .005	45.04 x 10 <sup>-3</sup>	+ .002
2	250-350	14	212.79 x 10 <sup>-3</sup>	+ .008	41.74 x 10 <sup>-3</sup>	+ .001
3	350-450	18	202.89 x 10 <sup>-3</sup>	+ .002	37.86 x 10 <sup>-3</sup>	+ .001

A Coulter electronic counter was used to count and size the suspended epididymal fat cells. Cell preparations were made only from rats weighing between 130-150 gms. The mean diameter of the fat cells in this weight range, obtained by averaging the mean diameters from the individual cell suspensions used in the various assays, came to approximately  $44 \pm 0.4$  u.

\* Micromoles free fatty acids/glycerol released per 10<sup>6</sup> target cells by 100 ug of test material acting on rat epididymal adipose tissue fat cells equivalent to 1 gram of lipid in a medium of Krebs-Ringer phosphate buffer at 37°C for 1 hour.

\*\* Mean  $\pm$  standard deviation of the combined group means.

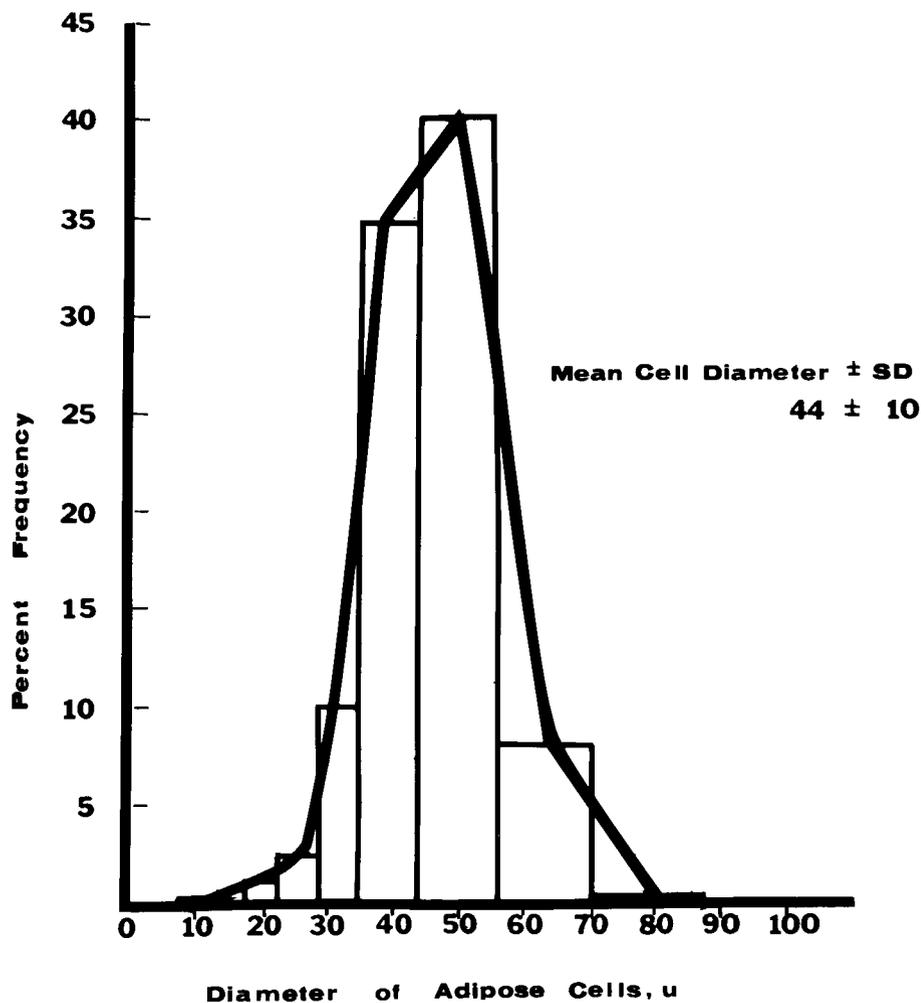


Figure 6. Frequency Distribution of Adipose Cell Diameters. Mean diameter is representative of 985728 free fat cells liberated by collagenase incubation from epididymal fat pads of 130-150 gm rats. Midpoint of classes and class limits and mean cell diameter  $\pm$  standard deviation are shown.

The mean cell diameter  $\pm$  standard deviation of the combined cell diameter frequency distributions came to  $44 \pm 10 \mu$ .

The mean diameter  $\pm$  standard error of the mean of the combined frequency distributions is  $44 \pm 0.17 \mu$ .

#### Purification of FMS by Ultrafiltration

Thirty male albino rats of the Sprague-Dawley strain were placed for 7 days on the high fat diet described previously. Their urine was then collected under a 24 hour fast at the controlled temperature of  $15^{\circ}\text{C}$ . The collected urine was pooled, and crude FMS was extracted by the four stage extraction process already described. Preparations of crude FMS were obtained from numerous urine collections from a number of groups of animals.

After assaying, on an individual basis, each lot of crude FMS obtained per 30 animals, all of the individual preparations of crude FMS were pooled by dissolving them in deionized water, and the solution was lyophilized.

The pooled crude FMS preparation was then divided into multiple units, each unit being carried through the ultrafiltration procedure, but separately. The results given in Table 14 present the mean lipolytic activities for the various fractions obtained from a number of these units that were carried through each stage of ultrafiltration. The aqueous solution of crude FMS of each unit was first purified by means of ultrafiltration using a PM-10 Diaflo membrane. The filtrate and the residue were each freeze-

Table 14

## Purification of FMS by Ultrafiltration

Source	Amount (mg)	Material	Amount (mg)	Lipolytic Activity* FFA (umoles)***	P vs Source	Total Activity** FFA (umoles)
		Control		.361 ± 0.03		
Urine		Crude FMS		.454 ± 0.01		
Crude FMS	439	PM-10 Filtrate	271	.496 ± 0.01	p < 0.001	134
		PM-10 Residue	156	.353 ± 0.14	p < 0.0025	55
PM-10 Filtrate	256	UM-2 Filtrate	201	.326 ± 0.02	p < 0.001	66
		UM-2 Residue	54	.578 ± 0.07	p < 0.001	31

\* Expressed as umoles free fatty acids (FFA) released by 100 ug of test material acting on 250 mg rat epididymal adipose tissue for 3 hours at 37°C.

\*\* 'Total Activity', calculated as weight of extract (mg) times 'Lipolytic Activity'.

\*\*\* Mean ± standard deviation, five determinations, with the exception of the 'Control' which is based on ten sets of five determinations.

dried and bioassayed in vitro for lipolytic activity by use of the assay utilizing the quantitative release of FFA from epididymal fat pads.

The mean results of these analyses, show that the active material was associated with the PM-10 filtrate. This filtrate in turn was ultrafiltered in the same manner through a UM-2 Diaflo membrane. Both the filtrate and the residue were again lyophilized and assayed in vitro for lipolytic activity. The activity was found to be associated with the UM-2 residue. These findings are in agreement with those of Kwok, 1974.

Further purification by ultrafiltration was accomplished by using a DM-5 Diaflo membrane which was substituted for the UM-2 membrane in one instance, and in another, incorporated into the purification protocol (Kwok, 1974) after ultrafiltration by the PM-10 and UM-2 membranes. From the results of these analyses shown in Table 15 it is apparent that the active material was associated with the DM-5 residue under both of these ultrafiltration routes.

Crude FMS carried through the UM-2 ultrafiltrative yielded a product (UM-2 residue) with an increased in vitro specific activity of about 27% (Table 15). When DM-5 ultrafiltration was applied to this residue, the specific activity of the product (DM-5 residue) was about 60% greater than that of crude FMS. When UM-2 ultrafiltration was omitted and the PM-10 filtrate was subjected directly to

TABLE 15

## Purification of FMS by DM-5 Ultrafiltration

Source	Amount (mg)	Material	Amount (mg)	Lipolytic Activity* FFA (umoles)***	P vs Source	Total Activity** FFA (umoles)
		Control		.361 ± 0.03		
		PM-10 Filtrate	15	.496 ± 0.01		7.4
PM-10 Filtrate	15	DM-5 Filtrate	11	.413 ± 0.004	p < 0.001	4.5
		DM-5 Residue	4	.941 ± 0.03	p < 0.001	3.8
		UM-2 Residue		.578 ± 0.07		31
UM-2 Residue	54	DM-5 Filtrate	10	.466 ± 0.08	p < 0.0125	4.7
		DM-5 Residue	44	.728 ± 0.01	p < 0.001	32

\* Expressed as umoles free fatty acids (FFA) released by 100 ug of test material acting on 250 mg rat epididymal adipose tissue for 3 hours at 37°C.

\*\* 'Total Activity', calculated as weight of extract (mg) times 'Lipolytic Activity'.

\*\*\*Mean ± standard deviation, five determinations, with the exception of the 'Control' which is based on ten sets of five determinations.

DM-5 ultrafiltration, the specific activity of the product (DM-5 residue) was about 107% greater than that of crude FMS.

This difference in percentage increase in specific lipolytic activity per unit weight of crude FMS between the two DM-5 residues prepared via alternate routes of ultrafiltration may be due to the increased time spent by crude FMS in aqueous solution in the process of ultrafiltration, viz. a three step ultrafiltration procedure as opposed to a two step ultrafiltration. Beaton (1964) found a significant loss of activity with FMS in aqueous solution (pH 3.1) in a 24 hour period with no marked further loss between 24 and 48 hours.

Another possible explanation is that an artifact in the type of membrane used during ultrafiltration may cause a structural change in the FMS fraction being ultrafiltered. For example, DM-5 Series membranes are less hydrophilic than UM membranes and have exceptional resistance to detergents, salts, and phosphates, which affect the UM structure. Since the increase in time spent by FMS in aqueous solution is relatively small when the ultrafiltration sequence includes the UM-2 ultrafiltration step, the latter possibility appears to be a more plausible explanation.

In making a comparison of the total activity data, shown in Table 15, between the UM-2 residue and its products of DM-5 ultrafiltration, it is noted that the total activity of the UM-2 residue was 31 and of the products was 4.7

(ultrafiltrate) and 32 (residue). The sum of the total activities of products (36.7) exceeds that of the original UM-2 residue. The same type of observation is noted in Table 15 when comparing total activity data between the PM-10 filtrate and its products of DM-5 ultrafiltration. These discrepancies are not accounted for. They may be due to the removal of inhibitory substances or to the freeing of active material from its bound form during the DM-5 ultrafiltration process.

Ultrafiltration via the DM-5 membrane added information as to the approximate molecular weight of FMS. Although ultrafiltration membranes do not yield quantitative molecular weight fractionation, their rejection characteristics are suitable for separation of proteins into reasonably accurate molecular weight ranges.

In agreement with the work of Kwok (1974), fractionation of crude FMS into substances greater than and less than 10,000 in molecular weight by PM-10 ultrafiltration and subsequent bioassays indicated that the fraction with molecular weight greater than 10,000 did not possess fat-mobilizing activity, (Table 14). When the active fraction, the PM-10 filtrate, was further fractionated in the same manner through a UM-2 Diaflo membrane, with a cut-off at 1,000 in molecular weight, the active material was associated with the UM-2 residue. Thus, active material isolated from rat urine has a molecular weight between 1,000 to 10,000 daltons.

The newly patented DM-5 Diaflo membrane has a cut-off at 5,000 in molecular weight. Fractionation of the PM-10 filtrate by DM-5 ultrafiltration, followed by subsequent in vitro bioassay indicated that the fraction with molecular weight greater than 5,000 (DM-5 residue) possessed the fat-mobilizing activity (Table 15).

Fractionation of crude FMS first by PM-10 ultrafiltration followed by UM-2 ultrafiltration produced a substance containing fat mobilizing activity with a molecular weight between 1,000 to 10,000. When this material with fat mobilizing activity was then fractionated into substances greater than and less than 5,000 in molecular weight by use of the DM-5 Diaflo membrane, subsequent in vitro bioassay indicated that the fraction with molecular weight greater than 5,000 (DM-5 residue) possessed the greatest fat mobilizing activity per unit weight. These data approximate FMS as isolated from rat urine to have a molecular weight between 5,000 to 10,000 daltons.

Consideration of the results in Table 15 indicate that even though both routes of ultrafiltration via the DM-5 Diaflor membrane have indicated through subsequent in vitro bioassay that the DM-5 residues possessed the fat mobilizing activity, the DM-5 filtrate obtained from ultrafiltration of the UM-2 residue, contained lipolytic activity similar in degree to that of crude FMS. In contrast, the inactive fractions, namely, the PM-10 residue and the UM-2 filtrate obtained from PM-10 and UM-2 ultrafiltration, possessed

lipolytic activity of the same magnitude as that of the control sample. This suggests the possibility that the FMS isolated from rat urine not only has a molecular weight between 5,000 to 10,000, but that the molecular weight of the substance containing fat mobilizing activity may be close to 5,000. Some of the fat mobilizing substance may be leaking through the other side of the DM-5 Diaflo-membrane, that has a cut-off of 5,000 in molecular weight, during the ultrafiltration process.

#### Purification of FMS by Gel Filtration

Kekwick and Pawan (1967) reported the achievement of some purification of FMS by passage of an ultrafiltrate, derived from ultrafiltration with a Visking Dialysis membrane, through a column of Sephadex G-50 directly followed by passage through a column of BioGel P-10, and pooling the fractions which exhibited maximal U-V absorption. When in our laboratory, a 0.01% aqueous solution of UM-2 residue was subjected to gel filtration employing these same two gel types, in the same sequential arrangement, the elution peak and the activity peak representing the transmittance of the effluent at 280 nm were found to coincide with each other (Kwok, 1974).

To date, there is no report of an elution profile, data, or any descriptive information, on the purification of FMS by gel filtration employing the exclusive use of BioGel P-10 or Sephadex G-50. Nor has an explanation

or reason been given as to why the purification of FMS by gel filtration necessitates passage of the source material through these two functionally similar gel types. Based on the estimated molecular weight of FMS, both BioGel P-10 and Sephadex G-50 cover the same recommended fractionation range desirable for optimum separation and/or purification of FMS.

Consequently, consideration was given to the possibility of whether or not efficient gel chromatographic purification of FMS could be achieved with the elimination of one of these chromatographic columns.

BioGel P-10 has an exclusion limit 10,000 less in molecular weight than Sephadex G-50 and covers the approximate molecular weight range of 1,500 to 20,000 over which separation of molecular substances, contained within the UM-2 residue (1,000 to 10,000 in molecular weight), could be expected to occur. Therefore, initial column chromatography was performed by passage of the UM-2 residue through a prepared column of BioGel P-10 with elution by deionized water.

The elution profile of the UM-2 residue on BioGel P-10 appears in Figure 7. Fractions 6 to 9 and 20 to 23, corresponding to elution Peak I and Peak II respectively, were each pooled, lyophilized and assayed in vitro for lipolytic activity by measuring the quantitative release of FFA from rat epididymal fat pads. As can be seen in Table 16 Peak I

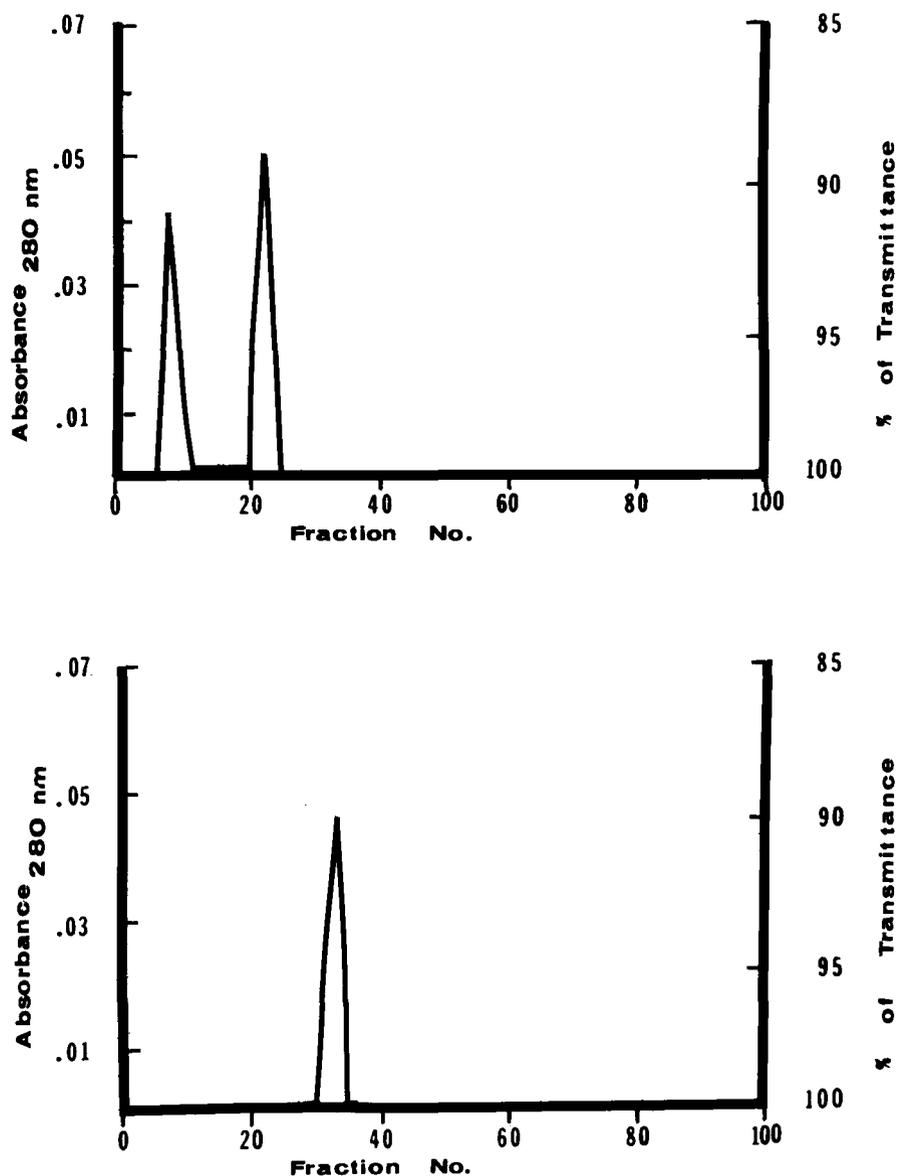


Figure 7. Top: Gel Filtration of UM-2 residue on Bio-Gel P-10. Bottom: Gel Filtration on Sephadex G-50 followed by Bio-Gel P-10 of a combined preparation of Peak I and Peak II previously fractionated by passage of UM-2 residue through the column of Bio-Gel P-10 (top). In both procedures, top and bottom: column size 2.5 x 45 cm; eluant deionized water; temperature 5°C; fraction size 5 ml. Percent transmittance monitored at 280 nm.

TABLE 16

## Purification of FMS by Gel Filtration

Material	BioGel P-10 Effluent	FFA, umoles*	P vs. Source
Control		0.361 $\pm$ 0.03	
UM-2 Residue		0.578 $\pm$ 0.07	
UM-2 Residue	Peak I	1.120 $\pm$ 0.05**	p < 0.001
	Peak II	.355 $\pm$ 0.03	p < 0.001
DM-5 Residue***		0.835 $\pm$ 0.02	
DM-5 Residue	Peak I	1.922 $\pm$ 0.03	p < 0.001

\* Expressed as umoles free fatty acids (FFA) released by 100 ug of test material acting on 250 mg rat epididymal adipose tissue for 3 hours at 37 degrees centigrade.

\*\* Mean  $\pm$  standard deviation, five determinations.

\*\*\*Composite mixture of the two DM-5 residues cited in Table 15, 50% (w/w).

contained lipolytic activity. The second peak possessed no lipolytic activity.

The in vitro specific lipolytic activity revealed approximately two fold purification of the fat mobilizing substance (FMS) contained within the UM-2 residue. Purification of crude FMS through gel filtration of UM-2 residue on the BioGel P-10 column revealed a product whose specific lipolytic activity had increased by 150%.

Gel filtration of the UM-2 residue through the prepared column of BioGel P-10 resulted in two major peaks, only one of which, upon in vitro bioassay, contained lipid mobilizing activity. However, passage in our laboratory (Kwok, 1974) of the same material (UM-2 residue) or passage by Kekwick and Pawan (1967) of their material, of similar fat mobilizing content (Visking Dialysis membrane, ultrafiltrate), through Sephadex G-50 and BioGel P-10 in sequential arrangement, demonstrated the elution of a single major peak coinciding with the activity peak. In an attempt to provide more information concerning this difference between elution patterns, the following comparative gel chromatographic procedure was performed.

The remainder of the lyophilized material from Peak I and the remainder of the lyophilized material from Peak II, were recombined in equal proportions (w/w), and dissolved in deionized water. With deionized water as eluant, this

aqueous mixture was subjected to gel filtration by passage through a prepared column of Sephadex G-50, directly followed by passage through the same BioGel P-10 column employed in the previous UM-2 chromatographic procedure. As can be seen in Figure 7, the elution profile contained only one major peak.

This resultant difference between elution profiles on UM-2 residue filtration via exclusive use of the BioGel P-10 column and, upon recombination of the two UM-2 residue eluant peak fractions from this BioGel column, the elution profile on their refiltration via the sequential arrangement on Sephadex G-50 and BioGel P-10, does provide supportive evidence for better resolution of the component substances contained within the UM-2 residue. This is demonstrated by the two separate peaks on the UM-2 residue BioGel P-10 elution profile, in contrast, to their resultant elution profile after their recombination and sequential refiltration through Sephadex G-50 directly followed by BioGel P-10 which contains only one major peak.

The use of BioGel P-10 may provide better purification of the fat mobilizing substance contained within the UM-2 residue than filtration through Sephadex G-50 directly followed by BioGel P-10. This purification improvement is suggested by the appearance of two separate peaks on the BioGel P-10 elution profile, of which only one contained lipolytic activity.

However, the disappearance of the second major peak from the elution profile resulting from the sequentially arranged columns may be due to the adsorption of this inactive substance, represented as the second peak (Fig. 7), onto the Sephadex G-50 column before entrance of the effluent onto the BioGel P-10 column. As a result, an insufficient quantity of this inactive component, if any, would be available to produce a major peak upon its final elution off of the sequentially following BioGel P-10 column. The same end would be accomplished; this being the removal of this inactive substance from the UM-2 residue.

The possibility also exists, that this inactive substance cannot be dissociated from active FMS by gel filtration under the experimental conditions imposed by the sequential arrangement of the two gel types. Interpretation has been made subject to inherent limitation because, upon completion of the sequential gel filtration procedure, an insufficient quantity of material was available to provide a quantitative bioassay. However, upon attainment of the experimental results on the DM-5 residue gel filtration procedure next to be described, further pursuit of the sequential filtration procedure was not an utmost necessity.

Beaton et al. (1966b) upon fractionation of their ultrafiltrate derived from ultrafiltration under a Visking cellulose tubing (pore size 24 Å) on Sephadex G-25 (fractionation range 1,000 to 5,000 in molecular weight), demon-

strated the presence of three active fractions associated with the ultrafiltrate. In our laboratory Kwok (1974) passed an aqueous solution of UM-2 residue through a Sephadex G-25 column with elution by distilled water and found the activity to be associated only with the first ten 5 ml fractions.

The results obtained by our laboratory (Kwok, 1974) suggested that the lipolytically active material may be approximately equal to, or greater than 5,000 in molecular weight. Further evidence was provided in support of this hypothesis, when after DM-5 ultrafiltration (cut-off 5,000 in molecular weight) of the PM-10 filtrate or the UM-2 residue, the lipid mobilizing substance was found to be primarily contained within the DM-5 residue.

As a consequence of these experimental findings, this DM-5 residue was subjected to further purification by gel filtration, with elution by deionized water, using the Bio-Gel P-10 column. The elution pattern of the DM-5 residue on BioGel P-10 appears in Fig. 8.

Fractions 19 through 22, corresponding to the elution peak were pooled, lyophilized, and the residue assayed in vitro for lipolytic activity by measurement of the quantitative release of FFA from rat epididymal fat pads. As can be seen in Table 16, there is a significant difference in lipolytic activity between the DM-5 residue and the substance of the elution peak ( $p < 0.001$ ). In vitro specific

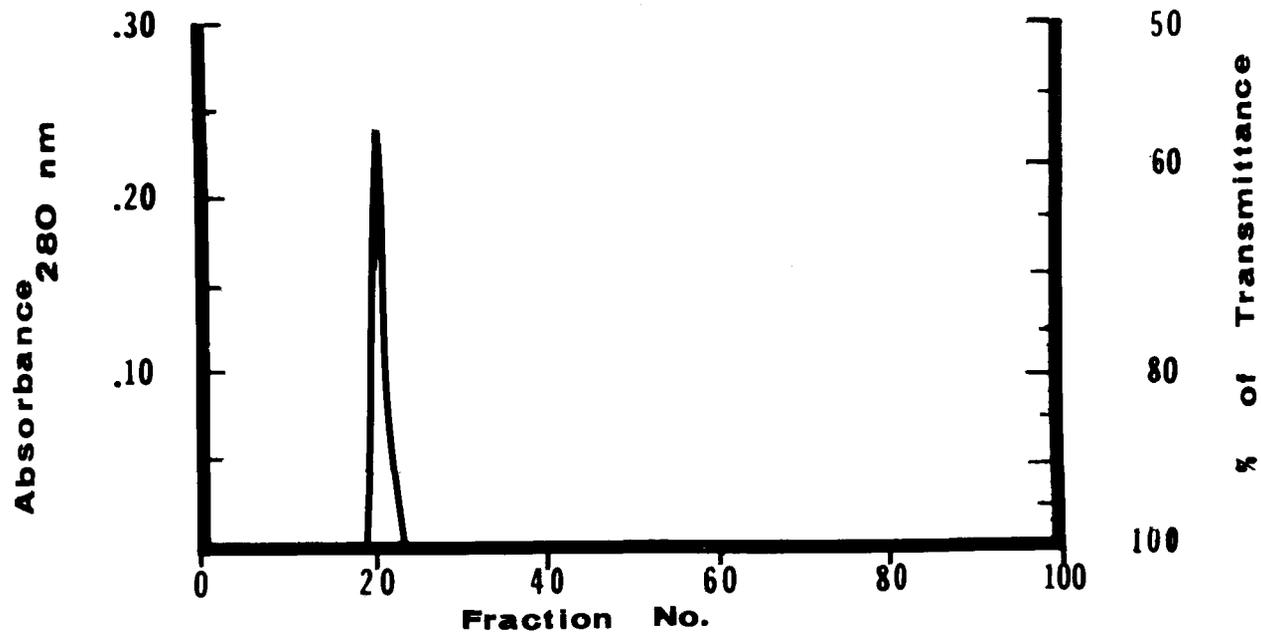


Figure 8. Gel Filtration of DM-5 Residue on Bio-Gel P-10. Column size 2.5 x 45 cm; eluant deionized water; temperature 5°C; flow rate 1.8 ml/minute; fraction size, 5 ml. Percent transmittance monitored at 280 nm.

lipolytic activity increased 130% per unit weight of the composite DM-5 residues obtained via DM-5 ultrafiltration. The extension of the purification process to include molecular sieve chromatography exclusively via BioGel P-10 with the elimination of Sephadex G-50, enhanced the specific lipolytic activity of crude FMS on a weight-for-weight basis by 147% for the UM-2 residue and 323% for the DM-5 residue.

#### Isoelectric Focusing of FMS

FMS at various stages of its purification was subjected to isoelectric focusing. Fig. 9 depicts the UV absorption curve and superimposed pH curve obtained by eluting and subsequent monitoring of the electrofocused FMS Fraction I, FMS Fraction II, and FMS Fraction III bands and an unnamed band associated with FMS Fraction III from the electrofocusing apparatus in which the FMS UM-2 residue preparation has been resolved. The electrofocusing run required 48 hours. The pH of the collected fraction at the elution peaks corresponds to the pI of the various resolved FMS Fractions. The electrofocusing was run at 4°C. The pH readings were determined at 4°C.

Fig. 10 depicts the electrofocused FMS Fraction I and FMS Fraction II bands from the apparatus in which the FMS DM-5 residue preparation has been resolved.

Fig. 11 shows resolution of the FMS Fraction I and FMS Fraction III bands from the active eluant peak fractions

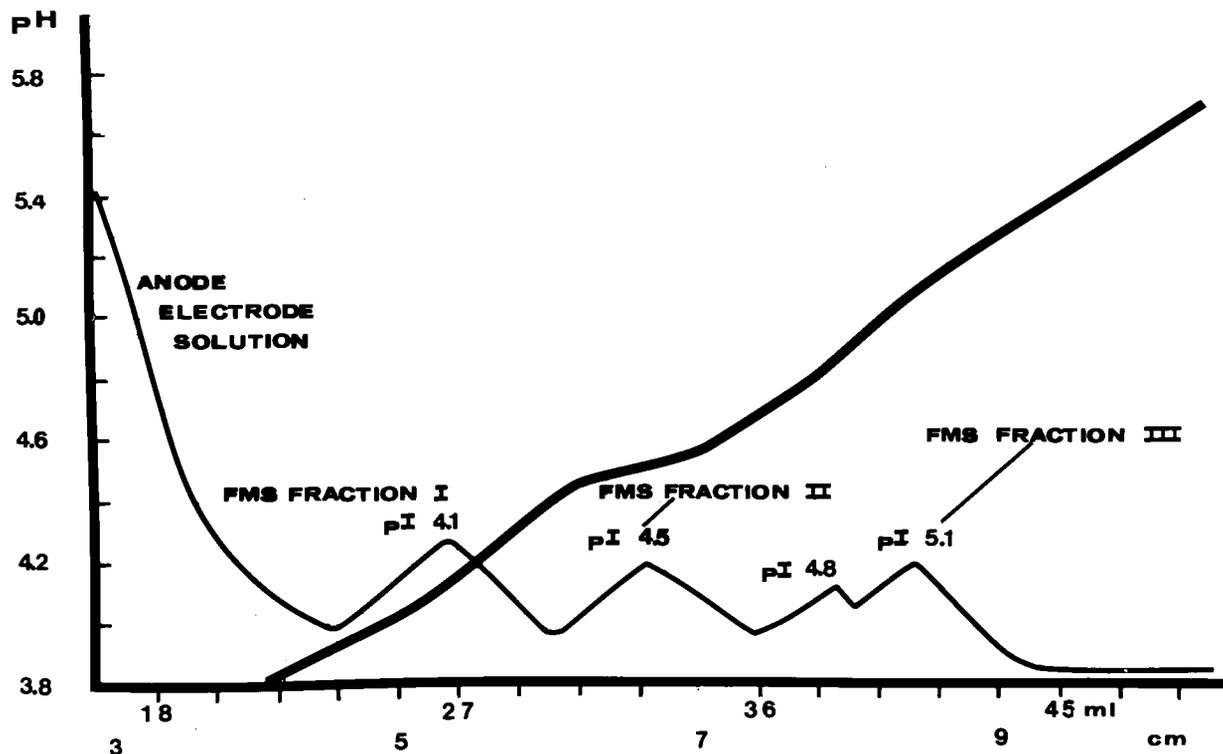


Figure 9. Separation of FMS UM-2 Residue by LKB 8100 Ampholine Electrofocusing Column in Which FMS Fraction I, FMS Fraction II, and FMS Fraction III Have Been Resolved. The curve with peaks shows the absorption of the eluate at 280 nm. The steadily increasing curve is a plot of the pH gradient superimposed. Isoelectric point values shown at the various peaks were read from the pH gradient curve.

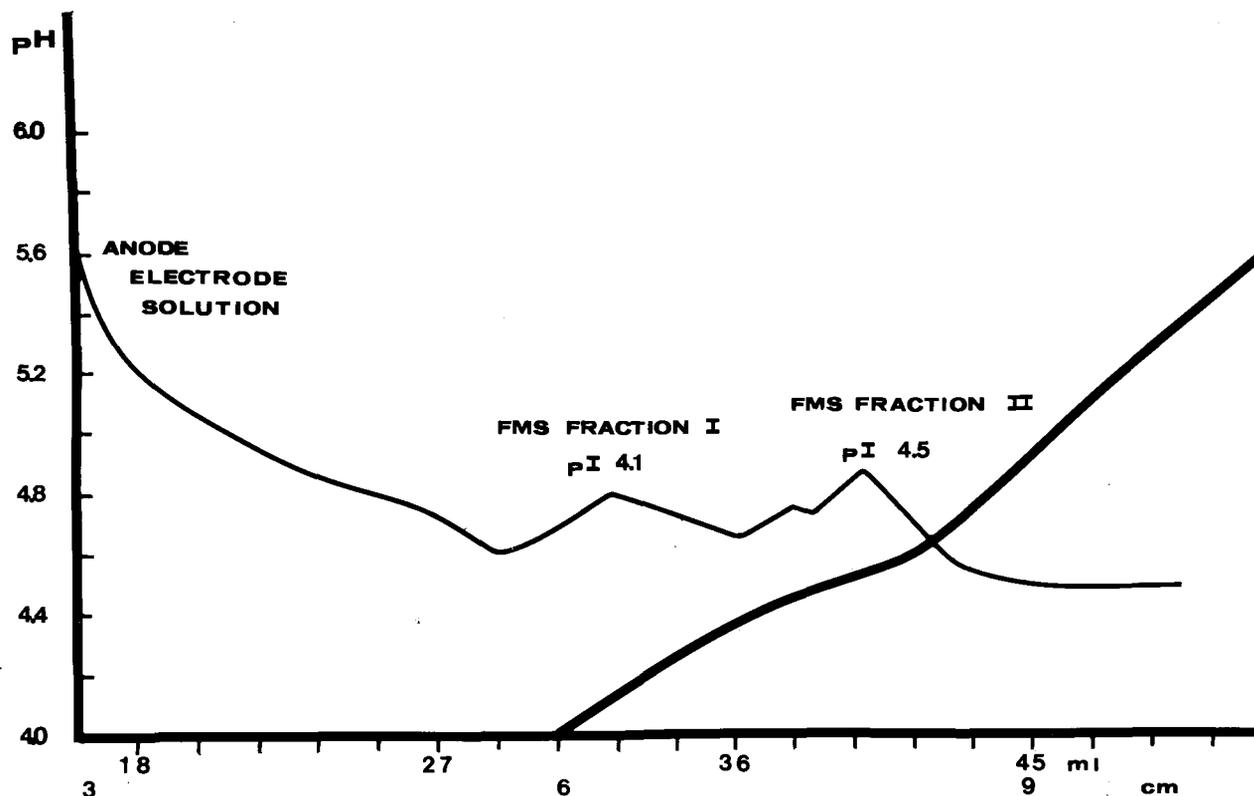


Figure 10. Separation of FMS DM-5 Residue by LKB 8100 Ampholine Electrofocusing Column in Which FMS Fraction I and FMS Fraction II Have Been Resolved. The curve peaks shows the absorption of the eluate at 280 nm. The steadily increasing curve is a plot of the pH gradient superimposed. Isoelectric point values shown at the various peaks were read from the pH gradient curve.

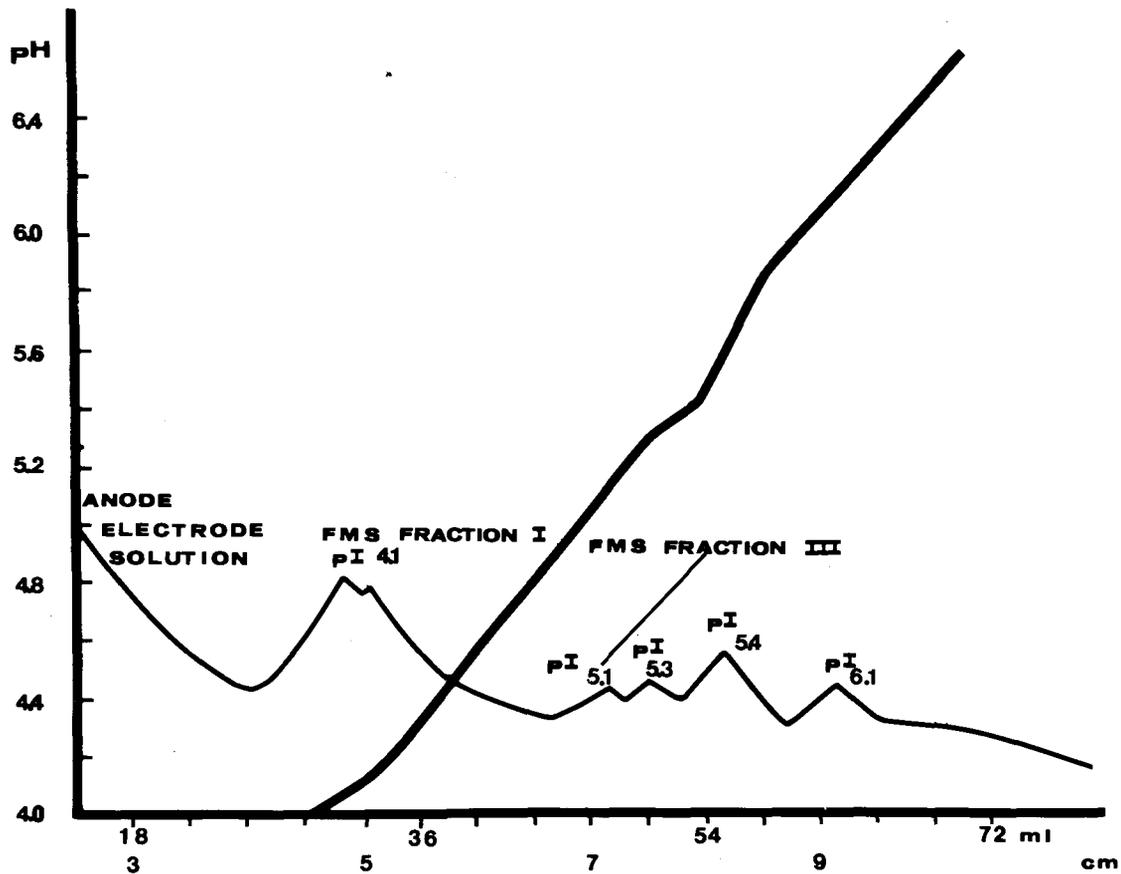


Figure 11. Separation of the Active Eluant Peak Fraction Obtained from Passage of FMS UM-2 Residue Through a Sephadex G-50 Bio-Gel P-10 Column by LKB 8100 Ampholine Electrofocusing Column in Which FMS Fraction I and FMS Fraction III Have Been Resolved. The curve with peaks shows the absorption of the eluate at 280 nm. The steadily increasing curve is a plot of the pH gradient superimposed. Isoelectric point values shown at the various peaks were read from the pH gradient curve.

obtained from passage of FMS UM-2 residue through a Sephadex G-50 BioGel P-10 column. FMS Fraction III is directly followed by three unidentified bands corresponding to the pI of 5.3, 5.4, and to the pI of 6.1. These bands present though not shown on the UM-2 residue electrofocusing map (Fig. 9) proved to be non-active lipolytically.

No matter which FMS preparation is resolved, the highly sensitive resolving power of the isoelectric focusing technique clearly demonstrates that the FMS Fractions (lipolytically active bands) will always end up at the point in the gradient where the pH is equal to the isoelectric point of the protein component.

#### In Vitro Bioassay of FMS Fraction I, FMS Fraction II and FMS Fraction III

All electrofocused bands eluted from the electrofocusing column were initially assayed in vitro for lipolytic activity by the very rapid and simple modified method of Duncombe (1964). One hundred micrograms of test material was incubated with 250 milligrams of rat epididymal fat pad in a medium of Krebs-Ringer phosphate buffer containing 4 per cent bovine serum albumin.

Table 17 shows the results for the initial bioassay of FMS Fractions I, II, III, and unidentified bands. For comparative purposes, the results are expressed in terms of umoles FFA released per gram of epididymal fat pad upon incu-

TABLE 17

Lipolytic Activity of Electrofocused Bands from the  
Electrofocusing Column in which FMS Preparations  
Have Been Resolved

Material	No. of Determinations	<u>In Vitro</u> * Lipolytic Activity
Control	15	1.44 ± .12**
FMS Fraction I	15	7.24 ± .38
FMS Fraction II	10	7.29 ± .26
FMS Fraction III	5	7.14 ± .54
Unidentified Bands (Include those of: pI 5.3, 5.4 and 6.1)	5	1.43 ± .37

\* Expressed as umoles free fatty acids released by 100 ug of test material acting on 1 gram of rat epididymal adipose tissue from 3 hours at 37°C.

\*\* Mean ± standard deviation, five determinations, composite of data obtained from lipolytic assays of the fractions from the three electrofocusing profiles.

bation for 3 hours at 37°C being acted upon by 100 micrograms of test material. It is observed that there is no difference in lipolytic activity between FMS Fraction I, II and III.

FMS Fractions I, II, and III were further subjected to in vitro bioassay utilizing the more specific assay system which employs isolated fat cells for direct analysis on target cell-FMS interaction. Table 18 expresses in terms of FFA release the lipolytic response of FMS target cells to 100 micrograms of FMS Fraction I, FMS Fraction II, and FMS Fraction III on a per unit cell lipid basis.

The lipolytic activity of each of the three FMS fractions was significantly different from that of crude FMS ( $p < 0.001$ ). The lipolytic activity of the crude FMS was significantly different from that of the Controls ( $p < 0.05$ ). Analysis of variance (F-test) on FMS Fraction I, II, and III shows that on a per unit cell-lipid basis the lipolytic activity does not differ significantly amongst the three fractions of FMS ( $p > 0.2$ ).

Table 19 expresses on a per unit cell-lipid basis the lipolytic response of FMS target cells to 100 micrograms of FMS Fraction I, FMS Fraction II, and FMS Fraction III in terms of glycerol release. Whereas net changes in the amount of free fatty acids released into the incubation medium represent the difference between the amount formed by lipolysis and the amount reesterified, glycerol is a more probable reflection of the rate of lipolysis. The correla-

TABLE 18

FFA Release as Index of Lipolytic Activity of FMS  
at the Initial and Final Stages of Purification

FMS	No. of Determinations	In Vitro* Lipolytic Activity
Control	6	10.3 ± 0.55**
Crude FMS***	15	11.0 ± 0.60
Fraction I <sup>†</sup>	5	31.2 ± 0.71
Fraction II	5	31.8 ± 0.56
Fraction III	5	31.3 ± 0.74

\* Expressed as umoles free fatty acids released by 100 ug of test material acting on rat epididymal adipose tissue fat cell suspension equivalent to 1 gram of lipid for 3 hours at 37°C.

\*\* Mean ± standard deviation, for the given number of determinations.

\*\*\* Composite of FMS extracted from each of the three weight-age categories.

† Composite fractions from three isoelectric focusing profiles.

TABLE 19

Glycerol Release as Index of Lipolytic Activity of FMS at the Initial and Final Stages of Purification

FMS***	No. of Determinations	In Vitro* Lipolytic Activity
Control	6	1.16 $\pm$ 0.00**
Crude FMS	10	2.18 $\pm$ 0.16
Fraction I	5	9.41 $\pm$ 0.03
Fraction II	5	9.35 $\pm$ 0.07
Fraction III	5	9.36 $\pm$ 0.01

\* Expressed as umoles of glycerol released by 100 ug of test material acting on rat epididymal adipose tissue fat cell suspension equivalent to 1 gram of lipid for 3 hours at 37°C.

\*\* Mean  $\pm$  standard deviation, for the given number of determinations.

\*\*\*FMS material as described in Table 18.

tion between the rate of lipolysis on each of the three FMS Fractions for these two criteria of lipolytic activity is significantly positive, showing no reesterification is taking place.

Adipose tissue cellularity was determined on every fat cell suspension prepared for bioassay by the use of the Coulter electronic counter. Table 20 expresses in terms of glycerol and FFA release per  $10^6$  target cells, the lipolytic activity of FMS Fraction I, II and III.

#### Determination of Total Protein Content of FMS Fraction I, FMS Fraction II, and FMS Fraction III

The protein content of the three FMS Fractions were measured using the method of Lowry et al. (1951). Table 21 displays the data obtained for these analyses.

Specific lipolytic activities for FMS Fraction I, FMS Fraction II, and FMS Fraction III, in terms of umoles of FFA or glycerol released per 100 ug of FMS protein acting upon rat epididymal adipose tissue cell suspension for 3 hours at  $37^{\circ}\text{C}$ , are given in Table 22. For both indexes of lipolytic activity, FMS Fraction I and FMS Fraction II contain the greatest degree in vitro specific lipolytic activity.

#### Polyacrylamide Gel Electrophoresis of FMS

FMS Fraction I, FMS Fraction II, and FMS Fraction "III" (Fraction III plus pI 4.8 band) were examined for homogene-

TABLE 20

Response of Adipocytes to FMS at the Initial  
and Final Stages of Purification

FMS**	No. of Determinations	FFA (umoles/10 <sup>6</sup> cells/hr) *	Glycerol (umoles/10 <sup>6</sup> cells/hr) *
Control	6	200.76 x 10 <sup>-3</sup> ± 0.01***	31.26 x 10 <sup>-3</sup> ± 0.000***
Crude	15	215.08 x 10 <sup>-3</sup> ± 0.01	42.33 x 10 <sup>-3</sup> ± 0.003
Fraction I	5	609.77 x 10 <sup>-3</sup> ± 0.01	183.91 x 10 <sup>-3</sup> ± 0.001
Fraction II	5	621.89 x 10 <sup>-3</sup> ± 0.01	182.74 x 10 <sup>-3</sup> ± 0.001
Fraction III	5	611.73 x 10 <sup>-3</sup> ± 0.01	182.93 x 10 <sup>-3</sup> ± 0.000

A Coulter electronic counter was used to count and size the suspended epididymal fat cells. Cell preparations were made only from rats weighing between 130-150 gms. The mean diameter of the fat cells in this weight range, obtained by averaging the mean diameter from the separate cell suspensions used in the various assays, came to approximately 44u.

\* Micromoles of free fatty acids or glycerol released per 10<sup>6</sup> target cells by 100 ug of test material acting on rat epididymal adipose tissue fat cells equivalent to 1 gram of lipid in a medium of Krebs-Ringer phosphate buffer at 37°C for 1 hr.

\*\* FMS material as described in Table 18.

\*\*\* Mean ± standard deviation.

TABLE 21  
Total Protein Content of  
FMS Fractions I, II and III

FMS	Number of Determinations	Percent Protein*
Fraction I	5	59 $\pm$ 5
Fraction II	5	60 $\pm$ 6
Fraction III	5	100 $\pm$ 2

\* Mean  $\pm$  standard deviation.

TABLE 22  
 Specific Lipolytic Activity of FMS Fraction I,  
 FMS Fraction II, and FMS Fraction III Protein

FMS	Determinations	FFA* (umoles)	Glycerol* (umoles)
Fraction I	5	52.88 ± 0.71**	15.95 ± 0.03**
Fraction II	5	53.03 ± 0.56	15.58 ± 0.07
Fraction II	5	31.30 ± 0.74	9.36 ± 0.01

\* Expressed as umoles FFA or glycerol released by 100 ug of FMS protein acting on rat epididymal adipose tissue cell suspension equivalent to 1 gram of lipid for 3 hours at 37°C.

\*\* Mean ± standard deviation, five determinations.

ity by polyacrylamide gel electrophoresis. The results are photographically shown in Figure 12 and 13. Figure 12 represents analysis with 250 micrograms of sample per gel. Figure 13 displays the 150 microgram sample gels of FMS Fraction I, II, and "III" whose staining intensities were subjected to quantitative densitometry with the Densicord Model 542 densitometer in conjunction with a Keuffel and Esser Model 620005 compensating polar planimeter.

FMS Fraction I and FMS Fraction II separated into one major and one minor band. FMS Fraction "III" separated into two major and five minor bands.

Figure 14, 15, and 16, show respectively, densitometer scans of FMS Fraction I, FMS Fraction II, and FMS Fraction "III" 150 microgram sample gels. The 250 microgram sample gels were not subjected to quantitative densitometry due to broadened bands (overloaded bands) that distorted the pattern on the densitometer tracing.

The FMS Fraction I electrophoresed gel's slowest moving component, Component I, contained 97% of the total staining intensity, while Component II contained the remaining 3% (Figure 14). Identical quantitative staining intensity values were computed for the major and slowest-moving band, Component I, and the minor band, Component II, present in the FMS Fraction II electrophoresed gel (Figure 15).

The staining profile on the FMS Fraction "III" electrophoresed gel revealed that two bands, Component II (40%)

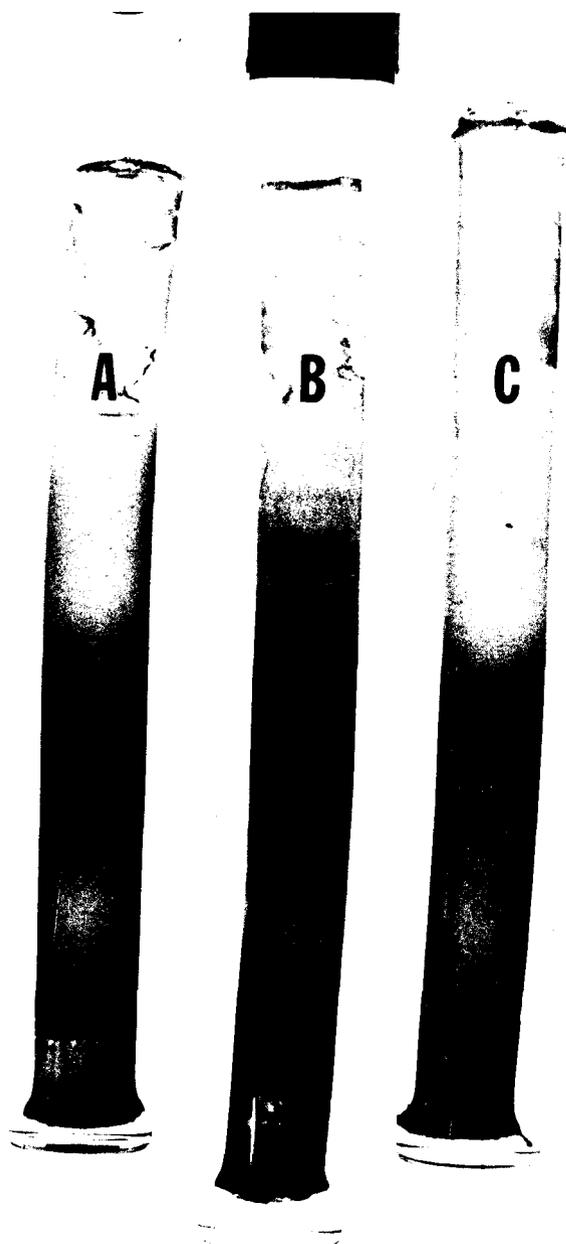


Figure 12: Polyacrylamide Gel Electrophoretic Patterns of (A) FMS Fraction II, (B) FMS Fraction III, and (C) FMS Fraction I, with 250 ug FMS Sample Application. Detection with fixative-dye 20% TCA-Coomassie Brilliant Blue stain solution.

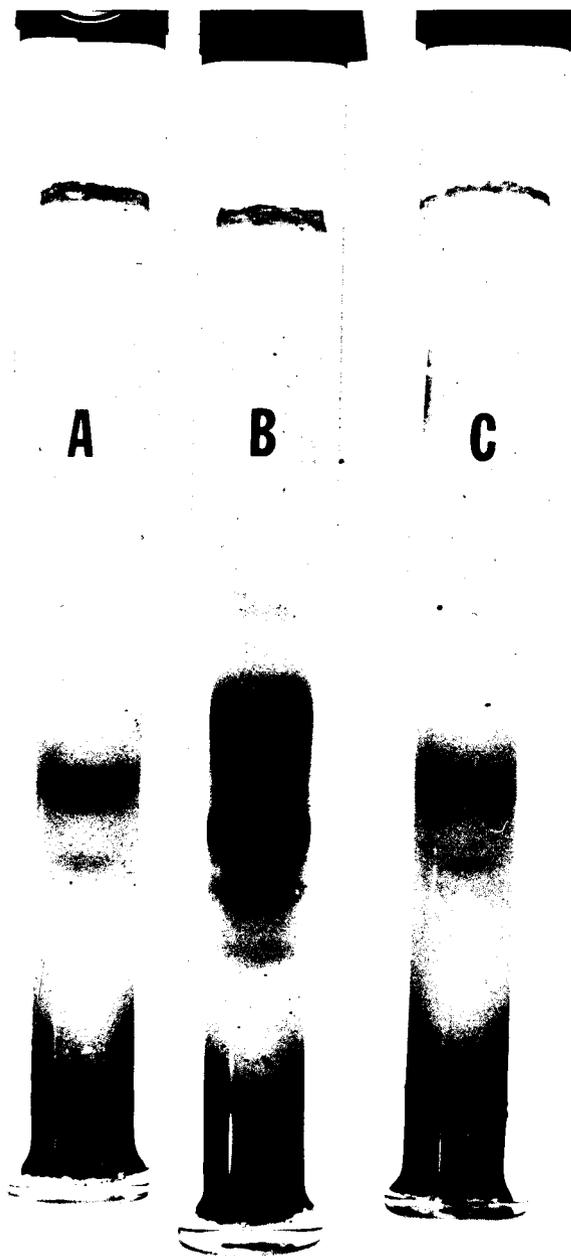


Figure 13. Polyacrylamide Gel Electrophoretic Patterns of (A) FMS Fraction II, (B) FMS Fraction III, and (C) FMS Fraction I, with 150 ug FMS Sample Application. Detection with fixative-dye 20% TCA-Coomassie Brilliant Blue stain solution.

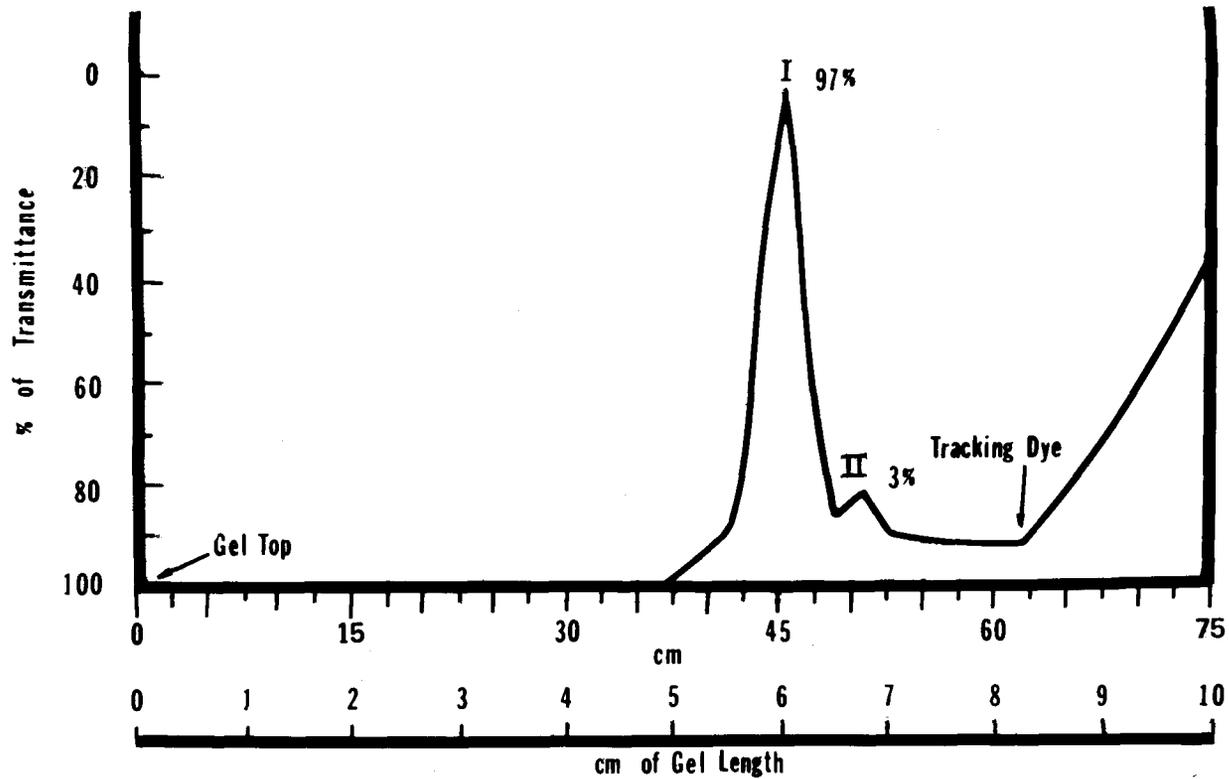


Figure 14. Chart Record of Percent Transmittance from Densicord Model 542 Densitometer Scan of FMS Fraction I Electrophoresed Gel after Staining with Coomassie Blue. Quantitation revealed that component I accounted for 97% of the staining profile and component II 3%.

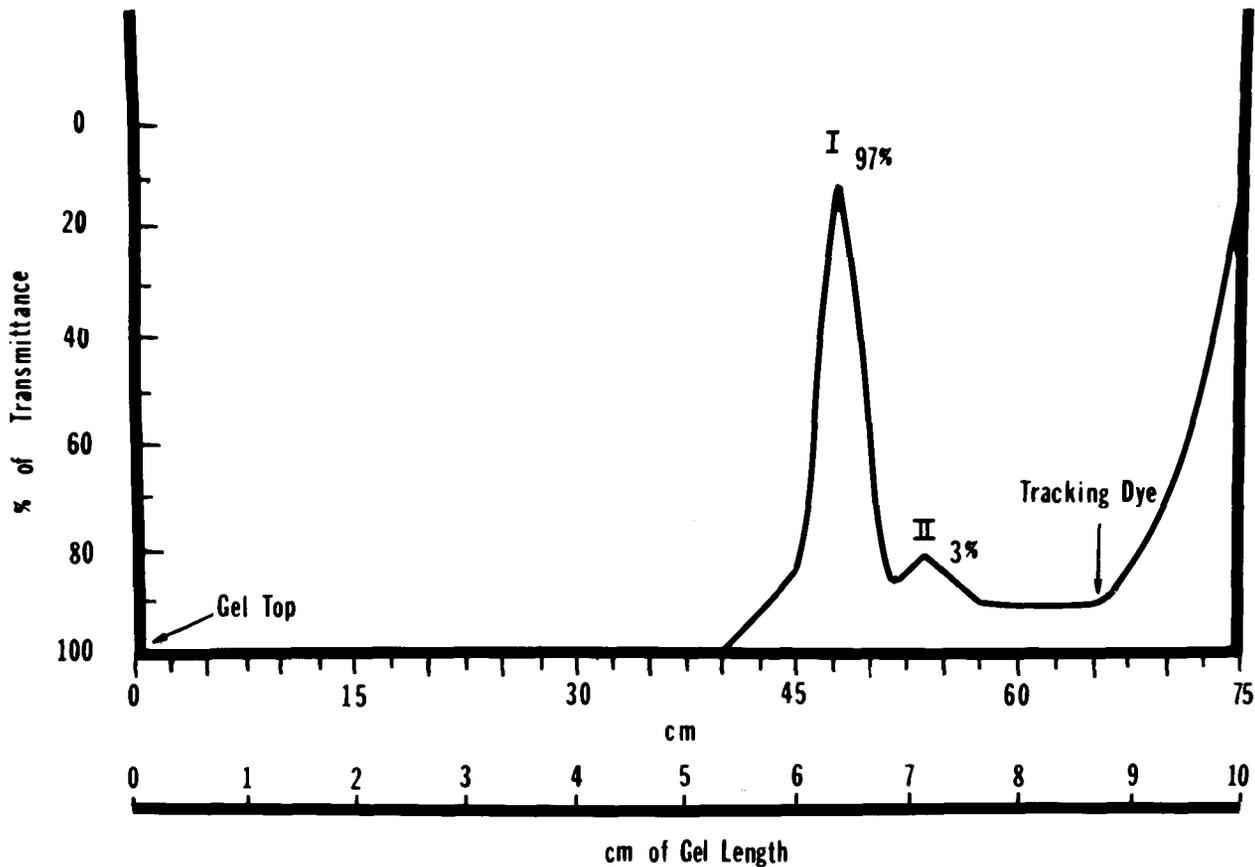


Figure 15. Chart Record of Percent Transmittance from Densicord Model 542 Densitometer Scan of FMS Fraction II Electrophoresed Gel after Staining with Coomassie Blue. Quantitation revealed that component I accounted for 97% of the staining profile and component II 3%.

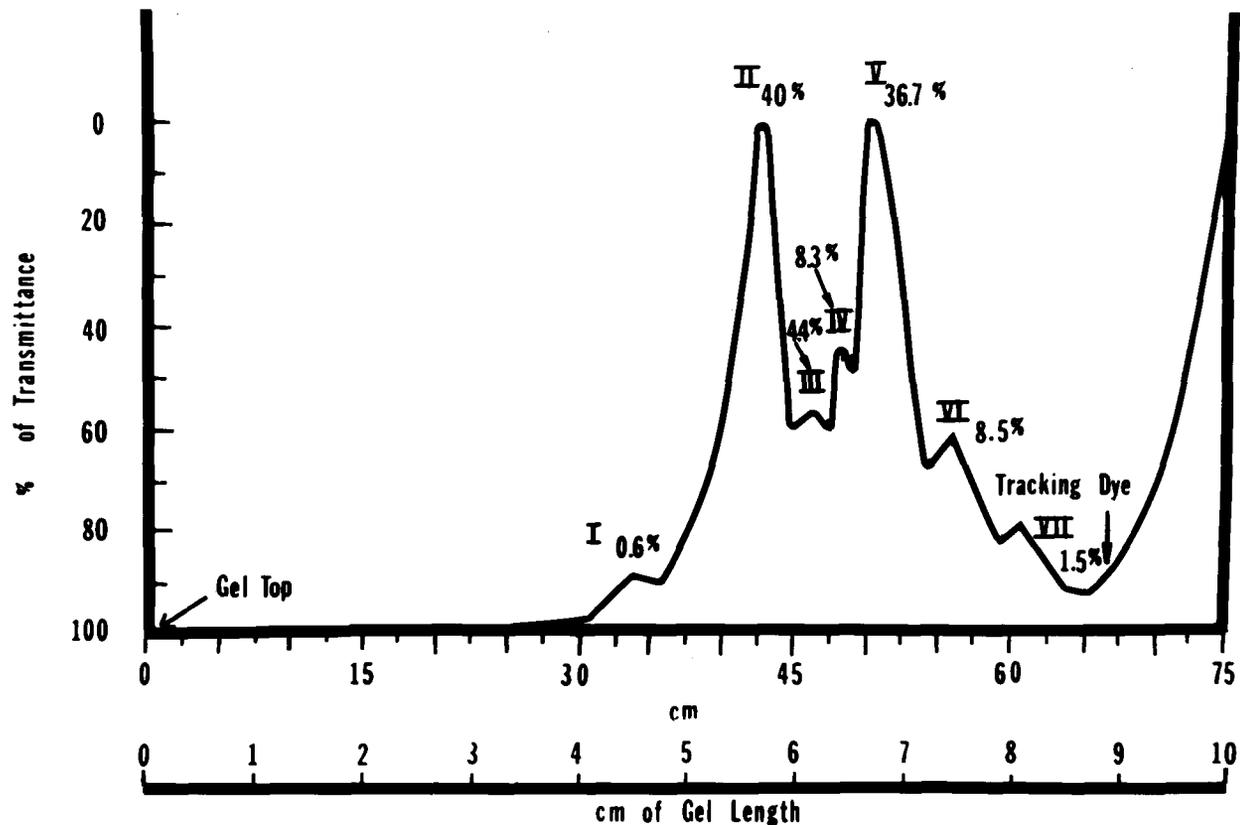


Figure 16. Chart Record of Percent Transmittance from Densicord Model 542 Densitometer Scan of FMS Fraction "III" Electrophoresed Gel After Staining with Coomassie Blue. Quantitation revealed that component II (40%) and component V (36.7%) account for over three-fourths (76.7%) of the total staining profile. Components I, III, IV, VI, and VII comprise the remaining 23.3%.

and V (36.7%), accounted for over three-fourths of the total staining intensity. The various minor bands accounted for the remaining 23.3%: Component I, 0.6%; Component III, 4.4%; Component IV, 8.3%; Component VI, 8.5%; and Component VII, 1.5%.

The tracking dye band of Coomassie blue was allowed to migrate to a predetermined mark shown 75 cm from the origin respective to the chart recording, to allow mobility measurement comparison, upon alignment of the series of gels. Approximately the same distance of migration is observed for the one major and one minor band (FMS doublet) of FMS Fraction I and II upon gel electrophoresis.

## CHAPTER IV

### DISCUSSION AND CONCLUSIONS

A Fat Mobilizing Substance (FMS) shown to accelerate lipolysis of triglyceride in adipose tissue has been isolated from the urine of various mammalian species, including man, during fasting and in other conditions associated with increased catabolism of fats (Chalmers et al., 1958, 1960; Stevenson et al., 1964; Beaton et al., 1966; Pawan, 1971).

FMS appears to be a polypeptide and is presumed to act by stimulating adenylate cyclase (Kwok, 1974). The precise chemical nature of this substance is not known, and to date, none of its physical characteristics have been reported. Progress in this area has been hampered by the fact that only milligram quantities have been obtained by current purification procedures, resulting in a lack of ready availability of purified material suitable for basic work.

Numerous studies have shown that a loss of body fat results from a decrease in the size of adipose tissue cells. Injected FMS causes a mobilization and reduction of the body fat content (Chalmers et al., 1960; Kekwick and Pawan, 1968); hence, a reduction in the size of these fat cells.

Urinary FMS is a reflection of the production of this substance in the body and its excretion by the kidneys.

Observations of higher mean urinary FMS levels in a group of non-obese as compared with the mean values in obese subjects (Pawan, 1971) and that infant and young children excreted more fat mobilizing activity than did young male adults during an 18 or 24 hour fast (Stevenson et al., 1965), together with the knowledge that adipose tissue relative to body weight increases with age and is markedly increased in the obese condition, suggest that FMS may be part of a physiological homeostatic control of body fat with weight and age possibly bearing a relationship to the degree of FMS production and secretion.

Accordingly, this study was concerned with obtaining further information relevant to the characterization of this lipolytic factor. The focus was directed to (1) development of an assay system based on FMS-target cell interaction from fat cells of known size and lipid content, (2) introduction of a glycerol in vitro bioassay procedure as an index affording comparative criterion to the existing in vitro measurement of FFA release for the lipolytic response of FMS, (3) direction of attention towards the degree in which age and weight factors affect the quantity and quality of FMS urinary excretion, and (4) improvement of the efficiency of purification of this active principle.

In the first part of this study, crude FMS was isolated according to the time-honored method involving benzoic acid precipitation of FMS from the urine of fasting rats, water

extraction, alcoholic precipitation, and lyophilization of the precipitate after redissolving in distilled water.

Initially the colorimetric method developed by Duncombe (1964), as modified by Kwok (1974), was applied to determine the quantitative release of FFA from epididymal fat pads of the rat into the medium. This proved to be the most rapid and convenient method.

Accurate comparative interpretation to previous works which express FMS lipolytic activity data in terms of release of FFA per 250 milligram rat epididymal fat pad being acted upon by 100 micrograms of FMS, rests upon the conditional state of the epididymal fat pads utilized within the assay system. Previous studies in our laboratory (Kwok, 1974) utilized fasted rats which predisposes the tissue to a higher initial concentration of FFA. In this study, however tissue fragments were excised from epididymal fat pads of normal fed rats; hence a reasonable explanation, for the relatively higher control values in former studies of Kwok (1974) in comparison to those obtained in this study. The amount of FFA released into the medium is greater from adipose tissue fragments excised from epididymal fat pads of fasted rats than from adipose tissue of normal fed rats. Although control lipolytic activity values were different in degree for each adipose tissue conditional state, the activity ratio of control/crude FMS was the same. Moreover, Beaton et al., (1966a) applying the same experimental design

and mode of data expression and utilizing epididymal fat pads from normal fed rats, obtained similar values on control and crude FMS data to those obtained in this study.

The increased sensitivity of isolated fat cells to lipolytic agents was demonstrated in this study by applying an assay system utilizing isolated fat cells to measure the lipolytic response of FMS. In addition, this method of assay provided an opportunity to study the lipolytic activity of FMS as a function of cell size, number and known lipid content. The Coulter electronic counter reliably measured the number of cells and provided the means to obtain the volume and diameter of the target cells.

Thought was given to the cellular nature of the adipose tissue to be employed within the assay systems, since it has become increasingly evident that differences in adipose tissue mass due to the number of the adipose cells in the tissue and the size of these adipocytes can have an effect on the metabolic activity of this tissue.

Fat cell size in the rat increases throughout growth. Fat cells may lose their sensitivity to the lipolytic effects of FMS as they enlarge. In applying this concept to the in vitro systems used in the various approaches to assay FMS, older rats of increased weight would have larger fat cells, which could be less sensitive to the lipolytic effects of FMS than the smaller fat cells from younger rats lesser in weight. Consequently, cell preparations were

made only from rats weighing between 130-150 grams.

This study showed that the individually prepared cell suspensions obtained from each rat in this particular weight range display a marked degree of intra-suspension heterogeneity, in adipocyte diameter and volume. Also displayed, was their marked degree in diameter and volume inter-suspension homogeneity. The mean cell diameter for any one cell suspension never deviated an entire micron from the experimentally determined mean of means cell diameter of 44  $\mu$ , with predictable, similar cell frequency distributions procured on all the independently prepared cell suspensions derived from different rat stock within this 130-150 gram weight range. Values on mean cell diameter and volume determined within this study are in essential agreement with data published by other groups e.g., Livingston et al., 1974. Within this particular weight range the rat maintains a reliable and reproducible mean cell diameter and cell volume.

Normal fed rats of the same weight range (130-150 grams) were utilized in each and every approach employed in the testing of the various FMS preparations. Therefore, comparisons could be made between the cellular responsiveness of intact epididymal adipose tissue and that of isolated fat cell suspensions to the lipolytic action of FMS. A sizeable increase in lipolytic activity was observed in all assays utilizing isolated fat cells significantly above

the activities obtained with the bioassays employing intact epididymal fat pads.

In addition, to the quantitative release of FFA by FMS-target cells, the establishment in this study of glycerol release as a reliable index of FMS lipolysis was initiated not only to remove the inherent problem of relying on only one criterion for in vitro measurement of FMS lipolytic activity, but to make it possible to draw certain inferences concerning changes in the rate of lipolysis and/or reesterification. During lipolysis, glycerol is released and not metabolized to any significant extent. The free fatty acids can be activated and reesterified (Vaughan, 1962). The relationship existing between the data obtained on these two parameters of lipolytic activity, shows no reesterification of the free fatty acid had taken place during FMS induced lipolysis in any of the in vitro bioassays performed for the testing of FMS lipolytic activity. Also, it can be affirmed that the glycerol released into the medium was produced from triglycerides and not by hydrolysis of  $\alpha$ -glycerophosphate or from smaller glycerides. Glycerol produced by hydrolysis of  $\alpha$ -glycerophosphate or from lower glycerides would be associated with the formation of only 0 to 2 umoles of FFA per umole glycerol, instead of the 3 umoles per umole of glycerol produced from triglycerides observed in this study.

When the lipolytic activity elicited by FMS isolated from groups of male rats of variable weight and age was studied, it was found that an increase in weight and age was accompanied by a concomitant decrease in FMS specific activity and an increase in total activity. The lipolytic response of isolated adipocytes measured by their quantitative release of FFA and glycerol was found to display a highly positive correlation to the release of FFA by epididymal fat pads in response to the lipolytic action of FMS.

On the basis of FFA released per 250 milligram epididymal fat pad being acted upon for 3 hours at 37°C, a direct comparison to the results of Beaton et al. (1966) could be made. In essential agreement with Beaton, the greatest specific lipolytic activity was found in FMS isolated from male rats weighing between 150-250 grams with a mean age of 7 weeks. Beaton, in observing the specific activity excreted by 24 male rats at five different age intervals as they increased in age from 26 to 78 days, found that the greatest specific activity was excreted at 50 days of age, 147 grams.

In contrast to Beaton et al. (1966), total lipolytic activity was found to increase with increasing weight and age. For male rats weighing between 350-450 grams with a mean age of 18 weeks, the total activity nearly doubled

that excreted by male rats weighing between 150-250 grams at a mean age of 7 weeks.

Beaton (1966) reported the greatest total lipolytic activity to be at 37 days of age (102 grams) with a non-appreciable variance between 37, 50 and 62 days of age (102, 147, 197 grams). FMS excretion decreased markedly between 62 and 78 days of age (297 grams).

A plausible explanation does exist for this significant difference in total activity data between Beaton and the experimental findings of this thesis. This difference may be the consequence of a difference in experimental protocol applied by Beaton et al. (1966) and that used in our laboratory. Beaton fasted 24 male rats at 26 days of age and subjected this same group of animals to the fast at 37, 50, 62, and 78 days of age. He employed neither a high fat diet nor a fast under cold stress as was applied in this experiment. In this current study, 11 different rat groups of 30 each were used to investigate the effects of weight and age on FMS excretion.

More importantly, the current experimental protocol eliminated the recycling of the same test animals. In contrast to 11 different groups of rats being subjected to fast under cold stress on a one time basis, Beaton subjected one groups of rats repeatedly to a fast at different

age intervals; this may have led to an exhaustion of the FMS supply available, or to a defect in FMS production in these animals.

This possible explanation can be substantiated with use of other data published by Beaton et al. (1966) in the same report. When Beaton tested the effect of duration of fasting on excretion of FMS by the rat, he found a high total activity for rats weighing 270 grams during a 24 hour fast, when the rats were used on a one time basis; in contrast, he found a low total activity in rats 78 days in age (mean weight 297 grams) and fasted for 24 hours, rats that were repeatedly subjected to this experimental condition. Further, Beaton et al. (1966) in a study of the effect of the sex of the animal on FMS excretion showed a high total activity for male rats 90 days of age (mean weight 329 grams), higher than any total activity reported at any age for the repeatedly used rats in his study of age effects on FMS excretion.

Because of the small quantities of starting material available, each step in the attempted procedures for purification was assessed to determine the most suitable combination of extraction, ultrafiltration, and gel filtration for the isolation of purer material.

Some purification can be achieved by Diaflo ultrafiltration. The products of DM-5 ultrafiltration appear to produce retentates of higher specific activity and products

of higher yield in total activity than the preceding ultrafiltration products of either PM-10 or UM-2 ultrafiltration. Also there are decreased losses in total activity when the DM-5 ultrafiltration is directly preceded by PM-10 ultrafiltration as opposed to DM-5 ultrafiltration preceded by UM-2 ultrafiltration of the PM-10 filtrate. Consequently, it appears profitable to incorporate DM-5 ultrafiltration into existing FMS ultrafiltration purification protocol with the possible elimination of the UM-2 ultrafiltration step.

Although Kekwick and Pawan (1967) and in our laboratory, Kwok (1974), have achieved further purification of their respective active ultrafiltration products by gel filtration through a column Sephadex G-50 directly followed by BioGel P-10, passage through both of these two functionally similar gel types appears not to be of necessity when the DM-5 residue is being further purified. It seems that DM-5 ultrafiltration removes the same inactive material, contained in the UM-2 residue and in the PM-10 filtrate, as does the Sephadex G-50 chromatographic column. This, suggests elimination of Sephadex G-50 gel filtration from the existing FMS purification protocol in dealing with the further purification of the DM-5 residue.

It can be further deduced, that passage through both Sephadex G-50 and BioGel P-10 gel chromatography columns may not be essential for further UM-2 residue purification

by gel filtration. With the separation of the UM-2 residue into an active and inactive peak fraction upon gel filtration by exclusive use of BioGel P-10, preparation of a Sephadex G-50 column to remove the same inactive material should be unnecessary.

Relative to the DM-5 residue, a relatively small amount of activity was present in the DM-5 filtrate. It now appears that the source of this activity is FMS Fraction III isolated from the isoelectric analysis of the UM-2 residue. FMS Fraction III in combination with non-lipolytic material is contained within the PM-10 filtrate and UM-2 residue but is removed by DM-5 ultrafiltration; and, as a consequence, appears to be found in the DM-5 filtrate. As shown on the UM-2 residue electrofocusing map, FMS Fraction III as contained in the UM-2 residue is closely associated with non-lipolytic material which could not be removed from FMS Fraction III when the UM-2 residue was directly subjected to isoelectric focusing without being previously purified by gel filtration.

This inactive material along with FMS Fraction III is removed upon DM-5 ultrafiltration of either the PM-10 filtrate or UM-2 residue. Consequently, this inactive material, as well as FMS Fraction III, are still contained within the DM-5 filtrate. This seems in part to logically account for the relatively low specific lipolytic activity associated with the DM-5 filtrate.

The ease and convenience of DM-5 ultrafiltration in comparison to preparation of the Sephadex G-50 chromatographic column, coupled with DM-5 ultrafiltration higher specific and total activity yields than PM-10 or UM-2 ultrafiltration, suggest that DM-5 ultrafiltration of the PM-10 filtrate followed by gel filtration on BioGel P-10 to be the most suitable combination of ultrafiltration and gel filtration for future purification of FMS Fraction I and FMS Fraction II.

FMS Fraction III contained within the DM-5 filtrate, following DM-5 ultrafiltration of the PM-10 filtrate, must be further purified by either BioGel P-10 or Sephadex G-50 before being subjected to electrofocusing under the current experimental protocol employed for isoelectric analysis. To obtain purified FMS Fraction III BioGel P-10 filtration would be the method of choice if no electrofocusing were to be carried out. Consequently, first, the DM-5 residue would be applied to the BioGel P-10 column, then, the DM-5 filtrate would be applied following the elution of FMS Fraction I and FMS Fraction II. As experimentally shown, the DM-5 residue would produce one major elution peak containing both FMS Fraction I and FMS Fraction II. In agreement with the UM-2 residue BioGel P-10 filtration, the DM-5 filtrate would produce one activity peak (Fraction III) and an inactive peak fraction.

A limitation of ultrafiltration and gel filtration in the isolation and purification of FMS is the inability of gel filtration to separate FMS Fraction I from FMS Fraction II. Their isolation can be achieved by isoelectric focusing of the DM-5 residue without prior gel filtration. Gel filtration may become obsolete for isolation of FMS Fraction III if a narrower ampholine range is used during isoelectric analysis of the DM-5 filtrate.

Obviously, ultimate proof of the best applicable purification route, rests in determination of FMS amino acid sequence, synthesis of a peptide with the proposed structure, and demonstration that the synthetic material has the full biological potency ascribed to FMS.

In addition to serving as techniques for further purification of crude FMS, ultrafiltration and isoelectric focusing added to existing information on the approximate molecular weight of FMS. In agreement with Kwok (1974), UM-2 ultrafiltration of the PM-10 filtrate yielded a substance containing fat mobilizing activity with a molecular weight between 1,000 to 10,000 daltons. When this fat-mobilizing material was further fractionated into substances greater than and less than 5,000 in molecular weight by use of the Diaflo ultrafiltration apparatus fitted with the newly patented DM-5 Diaflo membrane, subsequent, in vitro bioassay, revealed that the fraction with molecular weight greater than 5,000 possessed the greater fat mobilizing

activity. In accordance with the subsequent isolation of FMS Fraction I and FMS Fraction II upon resolution of the DM-5 residue on the electrofocusing apparatus, FMS Fraction I and FMS Fraction II, have to be between 5,000 and 10,000 in molecular weight.

Although relative to the DM-5 residue, the DM-5 filtrate contained negligible lipolytic activity, it did contain lipolytic activity similar in degree to that of crude FMS, thus indicating the presence of a lipolytically active fraction with a molecular weight between 1,000 and 5,000.

In conjunction with the cumulative data obtained from the separate electrofocusing of DM-5 and the UM-2 residues, it appears that FMS Fraction III is contained within the DM-5 filtrate and has a molecular weight between 1,000 to 5,000. Furthermore, present data, along with previous work done by other researchers and in our laboratory (Kwok, 1974) seem to justify the conclusion that FMS Fraction III appears to be a distinctive polypeptide of molecular weight to the upper range of an estimate of 1,000 to 5,000 daltons. For example, in our laboratory Kwok, (1974) upon fractionation of UM-2 residue through Sephadex G-25, molecular weight range between 1,000 and 5,000, found the activity to be associated with and initially after the void volume of the column.

Isoelectric focusing, proved important as a separation technique in the isolation and further purification of FMS.

The lipolytically active principle as resolved on the electrofocusing apparatus showed three active variants, namely: FMS Fraction I, FMS Fraction II, and FMS Fraction III. The structural difference between these three FMS fractions that account for their resolution during electrofocusing were not established.

As in other polypeptide hormones, the biological and immunological activities may reside in structural sub-entities that may be similar but may also differ in important structural details not yet clarified. Many highly purified proteins produce multiple bands, because of the presence of conformational isomers, isozymes, or partially filled sites for the essentially irreversible binding of metals, coenzymes and other small molecules. Such mechanisms may account for the microheterogeneity of FMS shown in the isoelectric separation of FMS Fraction I and FMS Fraction II.

FMS Fraction I and FMS Fraction II are resolved on the electrofocusing apparatus as two distinct FMS variants, but are eluted as one major activity peak upon DM-5 or UM-2 residue gel filtration on BioGel P-10 and upon UM-2 residue gel filtration on Sephadex G-50 directly followed by BioGel P-10. In addition, FMS Fraction I, and FMS Fraction II, are in the same, comparatively constricted, molecular weight range set by their identical fractionation properties upon ultrafiltration, display a non-significant difference in specific lipolytic activity and showed the same protein

content upon analysis. Furthermore, FMS I and FMS II displayed identical staining properties upon examination by gel electrophoresis.

With respect to FMS Fraction III, the heterogeneity of FMS shown on the electrofocusing apparatus may reflect some change incurred prior to excretion or generated during the isolation and purification of FMS. It might be argued that FMS Fraction III is identical to the first and second FMS Fractions with the non-protein component of FMS Fraction I and FMS Fraction II being removed from FMS Fraction III. The hypothesis is supported by the results of protein analysis of the fractions.

Electrofocusing yielded some data towards the estimation of the isoelectric point (pI) of FMS Fraction I, II, and III.

The pI (isoelectric point) values corresponding to FMS Fraction I and FMS Fraction II were respectively, pI 4.1 and pI 4.5, in the preparative separation of DM-5 residue or UM-2 residue.

FMS Fraction III yielded a pI value of 5.1 in the resolution of UM-2 residue and of the active eluant peak fractions obtained from passage of FMS UM-2 residue through a Sephadex G-50-BioGel P-10 column.

Upon analysis of the three FMS fractions by polyacrylamide gel electrophoresis and densitometric quantitation, no difference was detected between FMS Fraction I and FMS

Fraction II electrophoresed gels. The combination of the electrophoretic and molecular filtration effects resolved both FMS Fraction I and II into two discrete bands. FMS Fraction I contained one major and one small fast moving subsidiary band. This same doublet was also contained in FMS Fraction II. The two discrete bands exhibited identical staining intensity values and identical migration distances.

A third active FMS fraction resolved in the isoelectric analysis of UM-2 residue was subjected to PAGE. As shown on the UM-2 residue electrofocusing map (Figure 9), FMS Fraction III, pI 5.1, and an inactive fraction, pI 4.8, comprise FMS Fraction "III". The degree of heterogeneity is unaccounted for.

In favorable agreement with the results in this experiment on PAGE, Kekwick and Pawan reported obtaining one major component peak, in the ultracentrifuge, with a small subsidiary peak in testing the homogeneity of their partially purified FMS extract. This same extract gave a single band when subjected to PAGE. With reason, this single band migration on the polyacrylamide gels can be made subject to debate since the experimental conditions associated with their PAGE were not defined. The relative position, sharpness, and intensity of minor bands can be easily affected by changes or differences in the conditions of PAGE: pH, buffer composition, ratio of acrylamide monomer to bis-acrylamide, etc.

In contrast to the results on PAGE analysis of FMS found in this study, Kwok (1974), in our laboratory, upon subjecting his purified FMS preparation (UM-2 residue Sephadex G-50 BioGel P-10 active eluant peak fraction) to PAGE, observed a single major protein band. However, the difference in results can be attributed to non-identical conditions under which these PAGE analyses were performed. The results in this study were dependent upon an electrophoresis run for a longer period of time and gels that measured 10 cm in length as opposed to 6.5 cm. Also, the applied purified preparation was different in its source. The purification scheme applied in this study, may have removed or modified factors which previously interfered or did not allow full separation of FMS.

It now appears that previous reports on isolation of FMS as a polypeptide molecule of assumingly single molecular form are somewhat premature. There is little doubt that this Fat-Mobilizing Substance, 'FMS', consists of three active variants representing three molecular forms which are presumably derivatives of the same protein molecule.

It is recognized that the experiments in this study give less definitive answers regarding the nature of these three active variants than curiosity desires; however, it is hoped that they will draw attention on this subject of study and cause further experimentation by others to realize the full potential of FMS as possibly part of a physiological homeostatic control of body fat.

## SUMMARY

A Fat Mobilizing Substance (FMS) has been prepared from the urine of fasting rats by a four stage extraction procedure involving benzoic acid precipitation, water extraction, alcoholic precipitation and lyophilization. New approaches were established for testing the lipolytic activity of FMS in addition to the quantitative release of free fatty acids (FFA) from epididymal fat pads of the rat into a medium containing bovine serum albumin. To avoid misleading information on the metabolic potential of FMS on its target tissue induced by differences in the cellularity of the tissue amongst animals of different body weight, the cellular nature of the tissue employed within the assay systems was delineated.

An assay system was instituted employing isolated fat cells and based upon the release of glycerol and FFA from fat cells of known size and lipid content. Fat cell preparations were derived solely from rats 130-150 grams in weight and the mean cell diameter was found to be  $44\mu \pm 10$ . The preparations demonstrated a marked degree of diameter and volume inter-suspension homogeneity. Therefore, data were expressed in terms FFA released per  $10^6$  target cells and per unit cell lipid. Considerably increased lipolysis by FMS target cells was observed in all assays utilizing

isolated fat cells; this was reflected by the sizeable increased amounts of FFA released in response to the same FMS preparations initially tested employing intact fat pads from animals within the same weight range.

For the purpose of removing the inherent problem of relying on only the one suspect parameter, FFA, as an index of FMS in vitro lipolysis glycerol release was introduced as a comparative criterion. The relationship existing between the two parameters showed no reesterification of the FFA had taken place during FMS induced lipolysis and affirmed that the glycerol released into the medium is produced from triglycerides and not primarily from glycerophosphates or lower glycerides. A highly positive correlation was demonstrated between the two methods of assay.

When the lipolytic activity elicited by FMS isolated from variable weight range and age male rat groups was studied, it was found that an increase in weight and age was accompanied by a concomitant decrease in FMS specific activity and an increase in total activity.

It now appears that previous reports on isolation of FMS as a polypeptide molecule of assumingly single molecular form are somewhat premature. Investigation of FMS with isoelectric focusing revealed FMS consists of three active variants representing three molecular forms which are presumably derivatives of the same protein molecule, identifiable: FMS Fraction I, pI 4.1; FMS Fraction II, pI 4.5; and

FMS Fraction III, pI 5.1.

FMS Fraction I and II demonstrated identical staining properties upon densitometric quantitation of their polyacrylamide gels. FMS Fraction I resolved and separated into one major (97%) and one small (3%) fast moving subsidiary band. This same FMS doublet having identical staining intensity values with identical migration distances was also contained in FMS Fraction II. FMS Fraction II gels displayed an unaccounted for heterogeneity.

FMS Fraction I and II appear to have molecular weights between 5,000 and 10,000 daltons, display no difference in specific lipolytic activity, and chemically show identical protein content (60%). FMS Fraction III yielded lower values of specific lipolytic activity per FMS protein and appears chemically to be a 100% polypeptide of a molecular weight to the upper range of an approximated molecular weight of 1,000 to 5,000 daltons.

FMS Fraction I, II, and III were purified by ultrafiltration and column chromatography and isoelectric focusing. From this study, it is suggested that future purifications use the following scheme: (1) initial PM-10 ultrafiltration of Crude FMS (2) DM-5 ultrafiltration of the PM-10 filtrate, followed by (3a) gel filtration on BioGel P-10 of the DM-5 residue to be the most suitable combination of ultrafiltration and gel filtration for future purification of FMS Fraction I and II, and (3b) gel filtration of the

DM-5 ultrafiltrate should be the most suitable for FMS Fraction III.

A limitation of ultrafiltration and gel filtration in the isolation and purification of FMS is the inability of gel filtration to separate FMS Fraction I from FMS Fraction II. Their isolation can be achieved by isoelectric focusing of the DM-5 residue without prior gel filtration.

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