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Influence of Inhibitors of Cytoskeletal Function on Triacylglycerol Accretion in Cultured Adipocyte Precursors

by

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ABSTRACT

Investigations in human subjects and animals aimed at relating energy intake, utilization and output, have repeatedly suggested that balance cannot be obtained by accounting only for the energy value of nutrients. While it has been proposed that diet-induced thermogenesis may be the responsible mechanism, a number of studies have questioned its significance in human subjects. A recently proposed novel hypothesis would explain both the "unaccounted for" energy and the known inter-individual variability, including differing susceptibility to obesity. According to the hypothesis, subjects vary in the degree of cellular bio-mechanical activity, including motility, on a genetic basis. A significant portion of the remaining energy would be channeled to chemical storage in the form of adipocyte triacylglycerol. To test this hypothesis at the isolated cell level, well characterized cell culture systems of human and rat adipocyte precursors (preadipocytes) were used. Omental cells for lean and massively obese persons, and rat perirenal preadipocytes, were exposed to agents which directly or indirectly suppress cytoskeletal functions, namely, cytochalasin D, demethylcolchicine, and trifluoperazine. At defined culture conditions and concentrations of each agent, accretion of triacylglycerol was observed in

all cell strains. Morphologic evidence, notably staining for neutral lipids with Oil-Red-O, was corroborated by estimation of glyceride-glycerol content using a fluorometric approach. Thus, despite the fact that adipose cells generally respond to injurious stimuli by mobilizing storage lipid, suppression of cytoskeletal functions leads to triacylglycerol accumulation in cultured preadipocytes.

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I. INTRODUCTION

In 1973, four clinical nutritionists, who had spent many years studying energy expenditure, came to the extraordinary conclusion that the energy requirements of human subjects, namely, the balance of intake and expenditure, were not known (Durnin et al., 1973). The large variation in the efficiency with which different individuals use nutrient energy has remained unexplained. Research on obesity has emphasized the regulation of food intake and its modification, while paying relatively little attention to the regulation of energy expenditure (Himms-Hagen, 1983). It is now generally accepted that some individuals do utilize excess energy while others can do so only to a limited extent. This variability in energy expenditure cannot be simply ascribed to different degrees of physical activity (Himms-Hagen, 1983).

A novel approach has been developed by Roncari (Roncari & Healy, 1984) in an attempt to solve this problem. Accordingly, this research program was based on the hypothesis proposing an inter-individual variation in the utilization of energy for cellular biomechanical work. Thus, differing quantities of energy would remain available for eventual storage in adipocytes in the form of triacylglycerol. This hypothesis will be described in a later section.

II. LITERATURE SURVEY

Studies conducted as early as the 1930's on the individual food intake of men, women and children indicate the perplexity and magnitude of energy balance variability (Widdowson, 1936, 1947; Widdowson & McCance, 1936). Widdowson and McCance (1936) repeatedly found that in any group of twenty or more subjects ranging from ages one through fifty with similar attributes, including body weight, and activities, food intake could vary as much as two-fold. More recent studies confirmed this variability in infants from the time of birth (Morgan et al., 1976; Morgan & Mumford, 1977), and in men and women aged 75 and over (Duke et al., 1980).

Individual differences in energy intake have been found to be less among groups of people living together, eating together and following the same program of exercise (Widdowson, 1983), for example, army officer cadets in training (Edholm et al., 1955) and army recruits (Edholm et al., 1970). Considerable variability is, however, still evident not only in energy intake but also in energy expenditure at rest and with various activities, especially when integrated over the whole day. One person may expend as much as 50% more energy than another in performing the same strenuous or physically undemanding task (Durnin & Passmore, 1967).

In addition to these observations, several studies of prolonged overeating and undereating demonstrate the existence of "unmeasured energy" (Q_X). It can be defined as energy utilization not accounted for by standard measurement of resting or stimulated metabolic rate, basal and adaptive thermogenesis, or excretion of "energy-rich" molecules.

The principle of conservation of energy states that energy input must equal energy output. Webb (1980) has deduced a set of equations to measure energy balance in many of these human feeding experiments. The majority of these studies measure each term directly; however, some have been derived through application of the formula:

$$Q_{FD} + Q_{ST} = Q_{UR} + Q_{FE} + Q_{HL} + Q_{WK} + Q_{\underline{\Delta}H} \qquad (1)$$

where Q_{FD} is the quantity of nutrient energy

Q_{ST} is the quantity of nutrient energy stored

Q_{UR} is the quantity of energy in urine

Q_{FE} is the quantity of energy in feces

Q_{HL} is the quantity of heat lost

Q_{WK} is the quantity of external work accomplished

$Q_{\underline{\Delta}H}$ is the quantity of body heat stored

Q_M is the quantity of metabolic expenditure

Q_M can be substituted for $Q_{HL} + Q_{WK} + Q_{\underline{\Delta}H}$.

Equal intake and expenditure result in food balance with no change in body stores (Q_{ST}). During overeating, any excess energy is stored, or "lost", in effect, from the energy exchange; Q_{ST} then carries a negative sign.

Q_X, the quantity of unmeasured energy, is also added to the equation as it is argued here that during conditions of food excess and food deficit this quantity is required for equivalency (Webb, 1980).

$$Q_{FD} + Q_{ST} = Q_{UR} + Q_{FE} + Q_M - Q_X \quad (2)$$

In the dietary studies to follow, Webb (1980) has defined Q_X as:

$$Q_X = Q_M - Q_{FD} - Q_{ST} + Q_{UR} + Q_{FE} \quad (3)$$

In most situations of prolonged food surplus and food deficit, the Q_X values were found to be negative. In other words, these dietary studies show that metabolic expenditure seriously underestimates the net energy exchange.

In a series of experiments, Miller and Mumford (1967) fed sixteen college students for periods of 4 to 8 weeks diets containing either 2.8 or 15% of the total nutrient energy as protein, providing 62 or 333 kilojoules (261 and 1400 kilocalories respectively, because 1 joule is equivalent to 4.18 calories) per day above their normal intake. Measurements were made of body weight, activity, nitrogen balance, urinary creatinine, digestibility of the food, basal metabolic rate, total body potassium, total body water, and subcutaneous fat. The mean weight gain of the low protein group was 1.1 kilograms (kg) compared to 5.0 kg expected if the excess energy is calculated as being converted to adipose tissue containing 66% fat. A larger weight gain of 3.7 kg, compared with the theoretical figure of 5.4 kg, was observed in the group following the high

protein diet. The provided measurements could not account for the low gain in weight by either set of participants. They failed to reveal:

(1) Increased activity,

(2) Decreased digestibility of food, or

(3) A change in body composition, and

(4) There was no increase in basal metabolic rate.

Similarly, studies conducted by Doyle et al. (1965), Ashworth et al. (1962), Sims and coworkers (1968), and Bray et al. (1974) disclosed relatively large discrepancies between nutrient energy intake and output.

In accordance with results reported on studies involving dietary surplus, research on undereating over extended periods indicates large values for unmeasured energy, as determined by Webb's (1980) energy balance equation. Buskirk et al. (1963) studied obese patients during a program of dietary restriction and scheduled exercise. Metabolic expenditure was measured in a special chamber with a ventilated hood for 24-hour periods in which sleeping, eating and walking on a treadmill were programmed to duplicate the schedule followed regularly by these patients on the hospital ward. The energy of the liquid diet and that contained in urine and feces were determined by calorimetry. The composition of weight loss was analyzed by assessing change in body volume, total body water and nitrogen balances. In this and other studies of nutrient energy deficit (Brozek et al., 1957; Gilder et al., 1967a, 1967b; Yang et

al., 1976; Yang, 1977), if the daily value of Q_X of several hundred kilojoules were multiplied by the time period over which these subjects were investigated, the unmeasured energy would accumulate to several hundred kilojoules per person.

Webb (1980) has compiled 52 sets of data from overfeeding and underfeeding studies lasting 3 weeks or longer resulting in a mean metabolic expenditure (Q_M) of 615 ± 120 kJ/d, and unmeasured energy (Q_X) of 168 ± 92 kJ/d, or 27% of metabolic energy.

Upon reviewing the data based on an analysis of several calorimetric studies of weight gain and loss, along with inter-individual variability in energy intake and expenditure, one could conclude that conventional accounting of energy balance is incomplete. Some investigators have attempted to explain the significantly large unmeasured energy values of 33 to 228 kJ reported in several dietary studies (Webb et al., 1980) as resulting from:

(1) Error in metabolic expenditure (Q_M)

(2) Error in measurement of the quantity of nutrient intake stored (Q_{ST})

(3) Existence of diet-induced thermogenesis, or Luxuskonsumption

(4) Unmeasured energy (Q_X) as a real quantity that defies direct measurement.

An inconclusive summary of these explanations will be presented in the ensuing paragraphs.

In Webb's (1980) analysis of energy exchange in man he indicates that metabolic expenditure (Q_M) could be a possible source of error. Investigators may incorrectly assume that direct and indirect calorimetry are equivalent methods of measurement. Webb (1980) found excellent agreement between direct and indirect calorimetry when measurements were made for 24-hour periods during rest and during one day with 4 hours of work. This precision, however, was not found when measurements were made on individuals during long periods of work and also men at rest but not sleeping at night (Webb, 1980).

In all the preceding studies of overeating and undereating, Q_M was calculated by the most widely used and least cumbersome technique of indirect calorimetry. This method involves the measurement of respiratory gas exchange, namely oxygen consumption and carbon dioxide production, as well as urinary nitrogen as an index of protein metabolism. Standard values for oxygen, carbon dioxide and urinary nitrogen are relied upon to convert the energy derived by complete oxidation of these molecules into metabolic heat, using an equation developed by Weir (1949). In contrast to the continuous measurement of energy exchange by direct calorimetry developed by Atwater and Benedict (1905), indirect calorimetry is not a continuous method of measurement. Some investigators did, however, include 24-hour data on Q_M, and several employed frequent and thorough sampling of their subjects during restricted activities. In any case, the

question remains about the adequacy of sampling and about the reliability of calculating Q_M from indirect versus continuous measurement by direct calorimetry (Webb, 1980).

A second possible source of error is in the estimation of the quantity of nutrient intake stored (Q_{ST}), as a result of different methods of measuring body composition (Webb, 1980). The magnitude of the problem is reflected by the variation in published values for the caloric equivalents of weight loss reported in energy balance studies. The loss of a kilogram of fat appears to have resulted from deficits of 714 to 1905 kilojoules.

Webb (1980) states that body fat determined from body density is accurate only to ± 2% of body weight, for example, ± 1.4 kg for a 70 kg man or woman. The measurement of total body water to account for weight change resulting from alteration in levels of hydration, is expected to be accurate only to ± 2% to 3%, thus ± 1.0 to 1.5 kg for a person with 50 litres of body water (Webb, 1980). The limits of accuracy for determination of both body fat and total body water could account for variation in the quantity Q_{ST}, possibly contributing to a large unmeasured energy value.

Other small quantities of energy that may have been overlooked in balance calculations include loss of protein in hair, nails and dead skin, loss of ketone bodies during severe dietary restriction, loss of urea from skin and breath, loss of gaseous products of incomplete oxidation,

and the energy of solutes in sweat. However, realistically speaking, these losses are insufficient to explain the large values of Q_X found in those studies (Webb, 1980).

The literature on overeating describes an energy dissipating mechanism that was termed "Luxuskonsumption" by a German investigator, R.O. Neuman (1902). The concept of Lukusconsumption invokes that nutrient intake can be increased considerably without any corresponding weight gain, or that if weight gain ensues, it will be less than the value calculated from the excess consumed.

Several overfeeding experiments involving human subjects have appeared to support the existence of Luxuskonsumption. These include the work of Neuman (1902), Gulick (1922), Mann et al. (1955), Ashworth et al. (1962), Miller and Mumford (1967), Sims et al. (1968), Apfelbaum et al. (1977), Whipp et al. (1973), Garrow and Stalley (1975). In contrast, the findings of Passmore and coworkers (1955a, 1955b, 1963, 1967), Glick et al. (1977) and the 6-week-long carefully conducted experiments of Morgan and Durnin (1980) appeared to negate the concept of Luxuskonsumption. Garrow (1978) suggests that these conflicting reports might be reconciled if a threshold of overfeeding existed at which a Luxuskonsumption effect were activated. In the studies supportive of this phenomenon, more than 97 megajoules of excess energy was fed to subjects, while lesser amounts were consumed in the later investigations.

Hervey and Tobin (1983) believe that Luxuskonsumption would not have been found, had a serious attempt been made to directly measure energy expenditure over the intake period. These measurements were likely least reliable when the highest levels and longest durations of overfeeding were reported.

Energy expenditure in experiments by Rothwell and Stock (1979, 1980a, 1982) was derived from the difference between estimates of energy intake and change in body energy content, rather than from direct measurement. The impressive results of a study involving rats overfed on cafeteria diets, which are diets supplemented with a large choice of attractive foods high in energy and fat content, have been widely referenced as evidence for non-shivering thermogenesis in brown adipose tissue. The existence of this regulatory mechanism was based on a discrepancy between the intake and storage of energy. The energy intake of rats receiving the cafeteria diet was increased by 80% over a 3-week feeding period and thermogenesis reportedly dissipated 90% of the extra intake (Rothwell & Stock, 1979)!

In similar energy balance studies, Hervey and Tobin (1982) directly measured nutrient intake by bomb calorimetry of the food supplied, food scattered, and of excreta. Their experience and that of other investigators (Payne & Southgate, 1978) indicate that the energy obtained from food cannot be predicted accurately from manufacturers' data and food tables. The latter was the method employed in studies

conducted by Rothwell and Stock (1979, 1980a). These calculations often result in an underestimation of energy intake. A discrepancy in this value, in addition to incomplete recovery of sticky, greasy, energy-rich cafeteria foods from cages, along with a loss of moisture in studies conducted by Rothwell and Stock (1979, 1980a) would account for greater than 25% of the missing energy assumed to have been dissipated by thermogenesis (Hervey & Tobin, 1983).

Important inconsistencies in the relative amounts of fat and energy gains of rats, as reported by Rothwell and Stock (1979), may further augment missing energy. Compared to their own findings, Hervey and Tobin (1983) found the weight gained by Rothwell and Stock's control group (40%) was higher than would be expected for actively growing rats. This value was, however, less than the reported 100% extra gain of fat on cafeteria feeding (Hervey & Tobin, 1983). The ratio of the reported body energy gain to body weight gain, virtually all attributable to fat, for both control and cafeteria-fed groups were surprisingly similar at 17.4 and 17.0 kJ/g, respectively (Rothwell & Stock, 1979). In investigations conducted by Hervey and Tobin (1982) in similarly treated rats, values of 11.5 for controls and 19.8 kJ/g for cafeteria-fed animals were obtained. Assuming the latter set of numbers to be correct, they would reduce the missing energy reportedly ascribed to thermogenesis by Rothwell and Stock (1979) by about 30% (Hervey & Tobin, 1982, 1983).

Discrepancies were also found in determinations of energy expenditure in studies conducted by Rothwell and Stock (1979, 1980a, 1980b, 1982; Brooks et al., 1980; Rothwell et al., 1981). In more recent investigations (Rothwell & Stock, 1982b), measurement of energy expenditure by the energy balance method was compared with measurements of samples by oxygen consumption (indirect calorimetry). Rothwell and Stock used the mean of energy expenditure values obtained for 2-4 hour periods on days 9 and 13 to represent the average 24-hour energy expenditure for the 15-day experimental period. It is unlikely that this could have been a valid predictor of total expenditure, especially considering that a significant weight gain of 105-125 g occurred over the experimental period (Hervey and Tobin, 1983). This interpretation seems even less plausible in view of the authors' contention that the difference between the values for days 9 and 13 could be accounted for by a relationship of oxygen consumption to body weight ($kg^{0.75}$) (Hervey & Tobin, 1983). These findings also contradict earlier studies conducted by Rothwell and Stock (1979, 1980a, 1980b; Brooks et al., 1980; Rothwell et al., 1981) in which large increases in energy expenditure were postulated to exist over the entire cafeteria feeding period based on the basis of only moderate increments in oxygen consumption obtained from sample measurements, and large increases in energy intake accompanied by little or no additional weight gain (Hervey & Tobin, 1983).

Hervey and Tobin (1982) studied the energy balance problem in a systematic fashion by continuous measurement of energy expenditure. The investigations were carried out by indirect calorimetry and the measurement of energy intake and storage by bomb calorimetry of food, residues, excreta and carcass. They found that the sum of intake and storage exceeded turnover by 5-10 kJ/d, or about 5% of resting energy turnover (Hervey & Tobin, 1983). The discrepancy increased during cafeteria feeding (Armitage et al., 1981). Further studies in which rats were overfed by tube feeding also suggested lack of regulation of energy balance, body fat, or body weight (Hervey & Tobin, 1983). The increase in expenditure and the rate of weight gain were proportional to excess energy intake.

The work of Sims and associates (1968, 1973) represents a second series of widely quoted human experiments supporting the existence of Luxuskonsumption. Hervey and Tobin (1983) criticize the large discrepancies between energy intake and weight gain. They attribute the discrepancies to incompletion of the overfeeding schedule by participants because of anorexia and nausea, and they question the recorded intakes.

Some researchers (James & Trayhurn, 1981; Himms-Hagen, 1979) propose that brown adipose tissue is the effector of diet-induced thermogenesis in human obesity. Hervey and Tobin (1983) doubt this effect contending that a nonexistent mechanism requires no effector. In addition, most compre-

hensive studies of brown fat in human subjects indicate its virtual disappearance after one year of age (Hassi, 1977; Heaton, 1972).

There is little doubt that studies carried out to date have been unsuccessful in accounting for differences in energy utilization between lean and obese individuals. Controversy exists as to the nature of the quantity of unmeasured energy reported by several investigators (Webb, 1980; Webb et al., 1980; Neuman, 1902; Gulick, 1922; Mann et al., 1955; Ashworth et al., 1962; Miller et al., 1967; Sims et al., 1968; Apfelbaum et al., 1977; Whipp et al., 1973; Garrow & Stalley, 1975; Rothwell & Stock, 1979, 1980a, 1980b, 1982a, 1982b). The question remains regarding this unknown quantity: does it represent error or the existence of an energy dissipating mechanism? It is possible that this quantity does not relate to external energy expenditure as we know it and has, thereby, evaded direct measurement. Hence, a hypothesis based on internal or cellular energy interconversion as a mechanism to explain inter-individual variability in terms of body fat content, could be attractive.

Since the unrecognized mode of energy expenditure may involve utilization for the biomechanical processes of cells and subcellular structures, these will now be described briefly. Biomechanical functions are mediated by microfilaments, microtubules and related structures. Microtubules consist of α and β tubulins and their associated proteins, while microfilaments are composed of actins and

their associated proteins. In turn, microtubules, microfilaments and intermediate filaments constitute an interconnecting matrix, collectively termed "cytoskeleton". The numerous energy-expending cellular processes requiring these structures include beating of cilia and flagella, organelle transport, contraction of muscle, movement of chromosomes during cell division, migration of cells along the substratum, endocytosis including phagocytosis and exocytosis. Adenosine triphosphate (ATP), the energy "currency" of cells, fuels a number of cellular motility processes including cytokinesis, the work performed during muscle contraction, cellular locomotion and internalization in receptor-mediated endocytosis. Intracellular systems generating mechanochemical force include the actin-myosin ATPase, dynein-ATPase and the recently described kinesin (Vale et al., 1986).

Some of the biomechanical processes, and the mediating mechanisms will now be described. In cytokinesis, the final stage of mitosis, actin-myosin interactions have been implicated in the separation of daughter cells (Zimmerman et al., 1981). The hydrolysis of ATP to ADP and P_i produces the energy required for constriction of the cleavage furrow.

Muscle contraction involves a series of steps initiated by a myosin molecule binding ATP, causing weakening of the actin-myosin bonds and possible release of the myosin head from actin (Eisenberg & Hill, 1985). ATP is then hydrolyzed to ADP plus P_i, the cleavage products remaining bound to the

myosin. This process results in an energized myosin headpiece which has rotated to a position perpendicular to the actin filament. The movement is facilitated by flexible regions or hinges on the myosin molecule (Eisenberg & Hill, 1985). Stimulation of the muscle by a rise in internal calcium concentration then triggers the binding of each myosin headpiece to an adjacent actin filament. Subsequently, both ADP and P_i are released and the myosin conformation changes, pulling the actin filament 45° past the thick filament (Goody & Holmes, 1983). In the absence of additional ATP, the muscle returns to the rigor complex, in which the myosin head is inflexibly locked to the thin filament (Goody & Holmes, 1983).

The release of the bound ADP and P_i is a strongly exergonic step and the free energy released is used to power the conformational change in the myosin. With each cycle, which requires the hydrolysis of one ATP molecule per myosin head, the actin filament is pulled a distance of about 7 nm (Eisenberg & Hill, 1985). Each myosin molecule hydrolyzes approximately 5-10 ATP molecules per second during muscle contraction, with -30,660 kJ released per mole of ATP hydrolyzed (Eisenberg & Hill, 1985).

Cellular movement along a substratum is facilitated by the extension of actin rich lamellipodia from the leading edge of the cell (Chan, 1981). Adhesion plaques containing actin-binding proteins such as vinculin and α-actinin connect the ventral surface of the lamellipodia to the

substratum, while the body of the cell advances. At the same time ruffled extensions on the dorsal surface of the cell move backwards and collapse. The tail end of the cell remains attached to the substratum by a retraction fiber. As the cell moves forward this thin strand elongates and finally severs, with the major portion retracting into the cell body (Chan, 1981). This contractile process is dependent on a supply of ATP and is associated with the loss of actin microfilament bundles and their final replacement by a meshwork of microfilaments (Chan, 1981).

Receptor-mediated endocytosis involves the selective uptake of extracellular proteins and small particles following high specificity binding to receptor proteins on the cell surface. Internalization of the ligand requires energy.

Dynein-ATPase is utilized for ciliary and flagellar movement and may be one of the force-generating mechanisms required during cell division.

Ciliary and flagellar movement are dependent on the two dynein-ATPase arms attached to the outer doublet microtubules (a subfiber) in the axoneme. Dyneins make then break bonds with adjacent microtubules, causing each doublet to slide relative to its neighbour. An interconnection of radial spokes to central microtubules generates the beating motion of flagella (Gibbons, 1981). In the movement of a cilium, the power stroke is fueled by the hydrolysis of ATP.

A wave of bending moves along the cilium from its base, pushing it forward (Gibbons, 1981).

The amount of force necessary to move one chromosome to the spindle pole during anaphase A, at typical speeds of 1 µm/min is relatively small and requires the free energy of hydrolysis of 20 ATP molecules to ADP (Mitchison et al., 1985). In the sliding microtubular model of chromosome movement, adjacent polar microtubules slide past each other to effect spindle elongation. Kinetochore microtubules slide along polar microtubules to move chromosomes towards the poles. The generator of force for sliding is presumed to be dynein arms that bridge the two sets of microtubules with the hydrolysis of ATP powering chromosome transport (Hirokawa et al., 1985; Mitchison et al., 1985). A dynein ATPase effected polar microtubule sliding model has also been suggested to explain anaphase B (Mitchison et al., 1985).

New circumstantial evidence suggests that kinesin, a cytosolic protein, is a motor for fast axonal anterograde (towards the nerve terminal) transport of organelles along microtubules in the presence of ATP (Vale et al., 1986). A speculative role for kinesin has also been suggested in mitosis. In axonal transport the motor is believed to be located on the organelle bound by a putative membrane receptor protein rather than on the microtubule (Vale et al., 1985; Gilbert et al., 1985).

In anaphase B, kinesin is believed by some investigators to cross-link microtubules of opposite polarity in the spindle interzone (Vale et al., 1986). This protein, by generating a shear-force between these microtubules, may serve as a motor that pushes the spindle poles apart (Vale et al., 1986). Kinesin attached to vesicles or kinetochores might also induce translocation of vesicles or chromosomes away from the poles towards the positive ends of the microtubule (Vale et al., 1986). Such movements might be important for the congression of chromosomes to the equator during prometaphase (Pickett-Heaps et al., 1982) and for the ATP-dependent translocation of chromosomes towards positive ends of centrosomal microtubules in vitro (Mitchison & Kirschner, 1985).

The hypothesis interrelating these biomechanical processes with storage of chemical energy will now be presented.

III. HYPOTHESIS

The hypothesis proposes that subjects vary in the degree to which their cells utilize energy for biochemical work (i.e. cell movement, muscular contraction) (Roncari & Healy, 1984). After energy utilization for fixed metabolic needs (i.e. maintenance of electrochemical gradients, solute transport, "work against gravity"), for biomechanical work as well as release in the form of heat, surfeit energy would be used for synthesis and storage of energy-rich molecules. Adipose tissue triacylglycerols are quantitatively the most important of these energy-rich molecules.

According to the hypothesis, moreover, the inter-individual variability in energy deposition would reside in the degree of energy entrapment for biomechanical work. Despite similar nutrient energy intake and metabolic needs, individuals whose cells have the lowest inherent degree of biomechanical work would have the largest quantity of energy available for storage as fat tissue triacylglycerol, and would be most likely to develop obesity. This mechanism may also mediate progression to massive corpulence. Conversely, subjects with the highest inherent degrees of biomechanical work would have the least quantity of energy available for chemical storage. Thus, these individuals would have the greatest tendency to be thin, and would display the greatest

resistance to obesity when exposed to nutritional excess and a sedentary lifestyle. Since the expression of these processes would have a complex genetic basis, all subjects (with the possible exception of monozygotic twins) would vary in this regard.

IV. OBJECTIVE

To test the new hypothesis related to energy disposition in cultured preadipocytes. The specific aim was to determine whether agents that inhibit the function of the cytoskeletal elements, microtubules and microfilaments, lead to increased triacylglycerol accretion.

V. RATIONALE

The fundamental new hypothesis clearly requires testing by a number of complementary methods. At the isolated cell level, one prediction of the hypothesis would be that impairment of cytoskeletal function would leave more energy available for storage in the form of chemical energy. I have thus explored this possibility using a characterized system of cultured rat and human preadipocytes.

VI. MATERIALS AND METHODS

A. Materials

Male Sprague-Dawley Rats: Biosciences, University of Calgary, Alberta

Growth Media (for Human and Rat Adipocyte Precursors) contains: MEM Alpha Medium, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid), Fetal Bovine Serum: Gibco Laboratories, Life Technologies Inc., Grand Island, New York

Hanks' Balanced Salt Solution and Demecolcine (N-methyl-N-desacetylcolchicine: Gibco Laboratories.

Oil-Red-O, Cytochalasin D, Trifluoperazine, Triolein Triglyceride Standard, Lipid Control E-Mixed Triglyceride Standard (from serum), Imidazole-Hydrochloride, Adenosine Triphosphate, Phosphoenolpyruvate, β-Nicotinamide Adenine Dinucleotide (Reduced), β-Nicotinamide Adenine Dinucleotide (Oxidized): Sigma Chemical Company, St. Louis, Missouri

Lactate Dehydrogenase, Pyruvate Kinase, Esterase (Hog Liver), Glycerol Kinase (Candida mycoderma), Lipase (Rhizopus arrhizus): Boehringer Mannheim, West Germany.

Sodium Dodecyl Sulfate: Fisher Scientific Company, Fair Lawn, New Jersey.

Nutralipid 10% Fat Emulsion: Kabi Vitrum AB (Sweden) for Pharmacia Canada Inc., Dorval, Quebec.

Bio-Rad Protein Assay Kit: Bio-Rad Laboratories Ltd., Mississauga, Ontario.

All other chemical reagents were of highest analytical grade.

B. Source and Isolation of Human Omental Adipocyte Precursors

After informed consent, omental adipose tissue samples were obtained from lean and obese subjects at the time of elective surgery. All subjects were females. Lean patients weighed up to 110% of reference values based on the 1983 Metropolitan Life Height and Weight tables. The massively obese patients were defined arbitrarily by a body weight higher than 170% of reference. All lean and obese subjects were over 20 years of age.

Adipocyte precursors were isolated and cultured by established methods, reported previously in detail (Van et al., 1976; Roncari & Van, 1978; Van & Roncari, 1978). Omental adipose tissue was resected at the beginning of each operation and immersed in Hanks' balanced salt solution warmed to room temperature, pH 7.4.

Adipose samples were rinsed with Hanks' balanced salt solution and all visible blood vessels and fibrous tissue was dissected and discarded. The samples were then cut into small pieces, followed by mincing and digestion with 1 mg/ml of collagenase in Hanks' balanced salt solution, pH 7.4, at 37°C for 30 minutes (or until complete disaggregation of the

tissue occurred). A ratio of 1:3 (volume/volume) adipose tissue sample and incubation medium were found to be optimal in yielding a uniformly digested preparation of cells.

Following digestion, tissue remnants (collagen matrix and blood vessels) were discarded and the liberated adipocytes were allowed to separate by flotation for five to ten minutes. The adipocyte precursor suspensions (source of stromal-vascular fraction) were transferred to 15 ml centrifuge tubes (Corning, Corning, New York) and centrifuged at 700 x g for ten minutes. After centrifugation, the stromal-vascular fraction pellet was suspended in 10 ml of growth media consisting of complete MEM alpha medium buffered to pH 7.4. with 15 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) supplemented with 15% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin and transferred to two 25-cm^2 Falcon tissue culture flasks (Falcon Plastics, Fisher Scientific, Fair Lawn, New Jersey) for propagation. Cells were cultured to monolayer confluence in a humidified incubator (LunAire CO_2 incubator, Ingram & Bell) at 37°C, pH 7.4 in the presence of 5% CO_2. Within hours the cultures were washed with Hanks' balanced salt solution, pH 7.4 warmed to 37°C, followed by the addition of fresh growth medium. After 24 hours this washing procedure was repeated. The growth medium was then changed every second day. The cell culture reached confluence, and was thereby ready for subculture, within six to eight days.

C. Source and Isolation of Rat Perirenal Adipocyte Precursors

Male Sprague-Dawley rats weighing 100-175 g fed Purina Rat Chow were sacrificed for experimental protocol by decapitation. Adipocyte precursors (also referred to as preadipocytes) were isolated from perirenal fat pads and immersed in Hanks' balanced salt solution warmed to room temperature at pH 7.4 (Van & Roncari, 1978).

Immediately following collection, the adipose tissue was minced and digested with collagenase (1 mg/ml) in Hanks' balanced salt solution, pH 7.4 at 37°C for approximately 15 minutes (or until complete disaggregation of tissue occurred). Incubation involved the use of a magnetic stirring plate placed inside an oven with a constant temperature of 37°C. A ratio of 1:3 (volume/volume) adipose tissue sample to incubation medium was also found to be optimal in yielding a uniformly digested preparation of rat cells. Usually three rats were sacrificed to yield a suitable quantity of adipose tissue for primary culture.

Following digestion, the liberated adipocytes were allowed to separate by flotation for five to ten minutes. The adipocyte precursor suspension (source of stromal-vascular fractions) was transferred to 15 ml centrifuge tubes and centrifuged at 700 x g for ten minutes. After centrifugation, the stromal-vascular fraction pellet was suspended in growth medium consisting of complete Alpha Medium buffered to pH 7.4 with 15 mM HEPES supplemented with

15% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin and transferred to two 25-cm^2 Falcon tissue culture flasks for propagation.

Cells were cultured to monolayer confluence in a humidified incubator at 37°C, pH 7.4 in the presence of 5% CO_2. Within 24 hours, the rat adipocyte precursors were washed three times with Hanks' balanced salt solution, pH 7.4, warmed to 37°C followed by the addition of fresh growth media. After 24 hours, this washing procedure was repeated. The growth medium was then changed every second day. The cell culture is ready for subculture when monolayer confluence is reached in approximately four to five days.

D. Human and Rat Adipocyte Precursor Subculture

Growth medium is removed by aspiration from the pre-adipocyte culture. The cells are then washed once with Hanks' balanced salt solution without calcium. Two millilitres of a solution of Hanks' balanced salt solution without calcium were mixed with equal parts warm (37°C) trypsin-Na_2EDTA and added to a 25-cm^2 flask, or 4 ml to a 75-cm^2 flask (Falcon Plastics, Fisher Scientific, Fair Lawn, New Jersey). The flasks were placed in the 5% CO_2 incubator for a few minutes or until the cells were all completely detached from the substratum. After adding 1 ml of growth medium, the cell suspension was then transferred to 15 ml centrifuge tubes and centrifuged at 700 x g for ten minutes. After centrifugation the trypsin-Na_2EDTA and growth medium

overlying the pellet were aspirated off. The remaining pellet is resuspended in approximately five millilitres of fresh growth medium and is pipetted to mix. Counts are made using a hemocytometer (Bright Line Hemocytometer, A.O. Scientific Instruments, Buffalo, NY) and Coulter Counter, Model ZM (Coulter Electronics Ltd, England) as described. Flasks and/or welled plates are then seeded to the desired density, and are cultured in a humidified incubator at 37°C, pH 7.4 in the presence of 5% CO_2. Fresh growth medium is added every one to two days (Van et al., 1976; Roncari & Van, 1978; Van & Roncari, 1978).

Rather than resuspension, pellets of approximately 1 x 10^6 cells were also frozen to be thawed for use in experiments conducted at a later date. The pellets were pipette mixed with 1 ml of freezing solution containing 90% fetal bovine serum and 10% dimethyl sulphoxide. The mixture was then transferred to a freezing vial (Fisher Scientific, Fair Lawn, New Jersey), labelled and kept at room temperature for 30 minutes prior to freezing at -2°C. After 24 to 48 hours the frozen vials were transferred to a -70°C freezer for up to one week, followed by immersion in liquid nitrogen for storage up to one year.

To thaw, a frozen vial of preadipocytes was first removed from the liquid nitrogen storage tank and swirled by hand in a 55°C water bath for a few minutes. The contents of the vial were then transferred to a 15 ml centrifuge tube and 5 ml of growth medium was added and pipetted to mix.

The mixture was centrifuged at 700 x g for five minutes, followed by removal of the supernatant by aspiration. Five millilitres of growth medium was added to the pellet and pipette mixed. The cell suspension was transferred to a 25-cm^2 flask and grown to the desired density level.

E. Cell Counting Procedure

Cell counting is required in order to seed flasks and wells with a consistent number of cells for experimental reproducibility. Counts are obtained after preadipocytes have detached from the substratum, are centrifuged and suspended in growth medium, as described in the subculture procedure. Three counts were obtained using a Coulter Counter, Model ZM following dilution of 100 µl aliquots of cell suspensions in 20 ml of Hematall, isotonic diluent.

The mean of these automatic cell enumeration determinations was compared with the mean of three counts using a hemocytometer (Bright Line Hemocytometer, A.O. Scientific Instruments, Buffalo, New York). Further dilutions with growth medium were frequently required to achieve the desired seeding densities for control and experimental conditions.

F. Cell Seeding

The inoculum size resulting in appropriate cell growth, but without reaching confluence, was established for all the experimental conditions, which will be described. Seeding

at fairly low cell density (nonconfluence) prevented spontaneous lipid accretion found occasionally to occur in the case of obese human omental adipocyte precursors grown to confluence. Human omental preadipocytes were grown in six well plates (Falcon Plastics, Fisher Scientific, Fair Lawn, New Jersey) and 75-cm^2 flasks seeded at 10,000 cells/well for experimental plates, 500, 700, 1500 and 2500 cells/well for control plates, 250,000 cells/flask for experimental flasks, and 25,000, 50,000 and 75,000 for control flasks. This study was to determine the size of flask or welled plate most conducive to a consistent growth pattern over the five day experimental period for morphological studies involving suppression of replication by cytoskeletal inhibiting agents. In addition, an optimal seeding density for treated and control flasks/plates was to be determined. Controls were established by matching all density in flasks/plates with treated flasks/plates at the end of the five day experimental period.

G. Bio-Rad Assay for Protein Determination

Protein concentrations were determined by the Bio-Rad Protein Assay, a dye-binding assay, which is based on the absorbtion of proteins bound to the dye, Coomassie Brilliant Blue (Bradford, 1976; Sedmak et al., 1977; Spector, 1978). Bovine serum albumin was used as standards, and absorption was quantified with a Gilford Response TM spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio) under linear conditions.

H. Treatment of Human Omental and Rat Perirenal Adipocyte Precursors with Agents that Suppress Cytoskeletal Functions

1. Preparation of Agents

(a) Cytochalasin D

A stock solution of cytochalasin D was prepared by dissolving 1 mg of the agent in 1 ml of 95% ethanol. This 1 mg/ml stock solution was diluted with growth medium (MEM alpha medium buffered to pH 7.4 with 15 mM HEPES supplemented with 15% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin) over the concentration 0.01 µM to 1 µM (0.005 µg/ml to 0.5 µg/ml). The agent was added to adipocyte precursor cells in the following concentrations: 0.01 µM (0.005 µg/ml), 0.02 µM, 0.05 µM, 0.1 M, 0.2 µM, 0.4 µM, 0.5 µM, 0.6 µM, 0.8 µM and 1 µM.

(b) Demecolcine (N-methyl-N-desacetylcolchicine)

A 10 µg/ml stock solution of demecolcine was diluted with growth medium over the concentration range 0.01 µM to 1.3 µM (0.005 µg/ml to 0.5 µg/ml). The drug was added to adipocyte precursor cells in the following concentrations: 0.01 µM (0.005 µg/ml), 0.03 µM, 0.06 µM, 0.13 µM, 0.21 µM, 0.26 µM, 0.52 µM and 1.3 µM.

(c) Trifluoperazine

A stock solution of 10 mM trifluoperazine was prepared by dissolving the drug in a minimum volume of 95% ethanol. Prior to addition to cells the stock solution was diluted further with weakly acidified (pH 6.0), sterile growth

medium. The drug was prepared in the concentration range 0.1-1000 μM (0.048-480 μg/ml). Trifluoperazine was added to adipocyte precursors in the following concentrations: 0.1 μM (0.048 μg/ml), 1 μM, 10 μM, 18 μM, 25 μM, 35 μM, 50 μM and 100 μM.

2. Preparation of Nutralipid

Nutralipid 10% is a sterile fat emulsion prepared for intravenous administration. It is composed of 10 g/dl soybean oil, whose triacylglycerols contain predominantly unsaturated fatty acids, including linoleic (50%), oleic (26.5%), palmitic (10.5%), linolenic (8.5%) and stearic (3.5%) in addition to 12 mg/ml egg phospholipids and 244 mM glycerol in water. The pH of the emulsion is adjusted with sodium hydroxide to approximately 8. Particle size and biological properties are similar to those of natural chylomicrons. Nutralipid 10% was diluted 5-fold with growth medium to a 2% fat emulsion. It was then added with or without drugs/agents as an additional source of fatty acyl chains.

3. Experimental Protocol Including Agents and Nutralipid Addition

Frozen preadipocytes were thawed, transferred to 25-cm^2 flasks as previously described, and allowed to grow to confluence. The cells were then taken to the next passage as described. The adipocyte precursors were again grown to confluence, detached with trypsin-Na_2EDTA, counted, and used to seed plates and flasks to the desired density, as

previously specified. The designated concentration of cytochalasin D, demecolcine, and trifluoperazine, in the absence or presence of 2% Nutralipid, were added every two days over a five day period to nonconfluent human omental and rat perirenal adipocyte precursors. Most experiments used cells in second subculture with the addition of agent and/or Nutralipid within one to two days after seeding. Fresh cells (not previously frozen) were used in the first subculture for a limited number of experiments.

At the end of the five day experimental period, the cells were prepared for morphological studies, as well as determination of glyceride-glycerol by a fluorometric procedure. Preparation included cell centrifugation, counting, and freezing in 2 ml of Hanks' Balanced Salt Solution at -20°C.

Details of the experimental protocols are outlined in Tables 1, 2 and 3. Every observation or determination, i.e., morphological or biochemical, was conducted in duplicate or triplicate. For each agent used, moreover, a control devoid of it was prepared by harvesting at similar cell density. In addition, the experiments in which Nutralipid was added included in each case control flasks with Nutralipid but devoid of agent.

TABLE 1

Studies With Cytochalasin D

Cell Type (Second Subculture)	Number of Subjects	Concentrations Used (μM)
Human Omental Adipocyte Precursors from Lean Subjects	2	0.4
	2	0.2
	1	0.02, 0.1, 0.2, 1.0
	+ Nutralipid	
	1	0.02, 0.1, 0.2, 1.0
	4	0.01, 0.05, 0.2, 0.5, 1.0
Human Omental Adipocyte Precursors from Obese Subjects	2	0.4
	2	0.2
	1	0.2, 0.4, 0.6, 0.8, 1.0
Rat Perirenal Adipocyte Precursors	8	0.01, 0.05, 0.2, 0.5, 1.0
	+ Nutralipid	
	8	0.01, 0.05, 0.2, 0.5, 1.0

TABLE 2

Studies With Demecolcine

Cell Type (Second Subculture)	Number of Subjects	Concentrations Used (μM)
Human Omental Adipocyte Precursors from Lean Subjects	2	0.13
	1	0.03, 0.13
	+ Nutralipid	
	1	0.03, 0.13
	4	0.01, 0.06, 0.13 0.21, 0.26
Human Omental Adipocyte Precursors from Obese Subjects	2	0.13
	1	0.06, 0.13, 0.26, 0.52, 1.3
Rat Perirenal Adipocyte Precursors	8	0.01, 0.06, 0.13, 0.21, 0.26
	+ Nutralipid	
	8	0.01, 0.06, 0.13, 0.21, 0.26

TABLE 3

Studies With Trifluoperazine

Cell Type (Second Subculture)	Number of Subjects	Concentrations Used (μM)
Human Omental Adipocyte Precursors from Lean Subjects	2	0.1, 10.0, 100.0
	1	10.0, 50.0, 100.0
	4	1.0, 10.0, 25.0, 50.0, 100.0
	+ Nutralipid	
	1	10.0, 50.0, 100.0
	4	1.0, 10.0, 25.0, 50.0, 100.0
Rat Perirenal Adipocyte Precursors	8	18.0, 25.0, 35.0 25.0, 35.0

I. Morphological Studies

1. Phase-Contrast Microscopy

Live cells were observed directly by phase-contrast microscopy using a Nikon inverted Diaphot-TMD microscope (Nikon Canada Inc., Mississauga, Ontario).

2. Staining for Neutral Lipid with Oil-Red-O

Preadipocytes are washed one to three times with Hanks' Balanced Salt Solution without calcium, after removal of growth medium by aspiration. The cells were fixed overnight in 4% formaldehyde solution (5 ml for 25-cm^2 flask, 10 ml for 75-cm^2 flask, 2 ml for 6 well plate, and 1 ml for 12 well plate). A solution of Oil-Red-O stain was prepared according to the procedure of Preece (1972). The fixative was removed by aspiration, and the cells were rinsed with 60% isopropanol for thirty seconds. Plates or flasks of adipocyte precursors were stained for two to three hours, using volumes of Oil-Red-O that were the same as those specified for the fixative. They were then rinsed with 60% isopropanol for a few seconds, and washed in running water for two to three minutes.

While a firm correlation has been proven between staining for neutral lipids with Oil-Red-O and triacylglycerol accumulation in preadipocytes (Wier & Scott, 1986; Green & Meuth, 1974), verification was also sought in these investigations.

J. Fluorometric Assay for Glyceride-Glycerol

1. Principle

The fluorometric procedures described by Nemeth and coworkers (1986) measures the disappearance of nicotinamide adenine dinucleotide, reduced (NADH) or the appearance of nicotinamide adenine dinucleotide, oxidized (NAD^+) with a sensitivity 10- to 100-fold greater sensitivity than that of the UV-spectrophotometric assay (Eggstein & Kuhlmann, 1974).

This method incorporates a lipid extraction procedure, the spectrophotometric assay described by Wahlefeld (1974), the fluorometric assays of Burch et al. (1970), and Lowry and Passonneau (1972). In a single reaction step, the extracted triacylglycerols are hydrolyzed with a lipase and an esterase. The glycerol produced is directed to an NADH-NAD^+-mediated reaction, as outlined in Figure 1.

Triacylglycerol concentration can be determined fluorometrically by measuring either the disappearance of NADH or the appearance of NAD^+ after its conversion to a fluorescent product (Lowry & Passonneau, 1972), as shown in Figure 1.

2. Dole-Meinertz Method for Extraction of Neutral Lipid (Dole & Meinertz, 1960)

This procedure extracts quantitatively hydrophobic lipids including triacylglycerol, cholesterol esters and 90% of long-chain fatty acids. Treated and untreated control adipocyte precursors were pelleted as in the subculture procedure, suspended in 2 ml of water and vortexed to mix. Ten millilitres of extraction mixture (.1 volume 1 N sul-

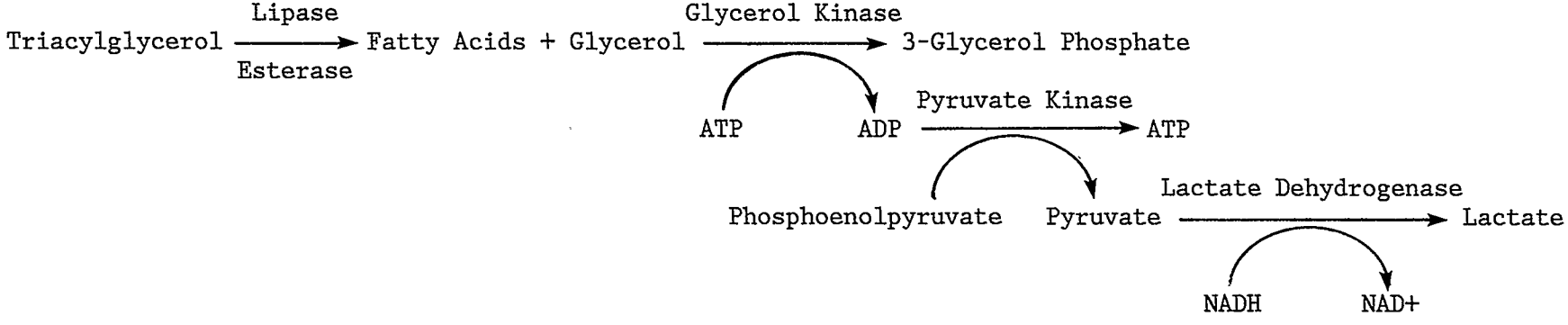


Figure 1. Enzymatic Pathways Used in the Primary Reaction of the Spectrofluorometric Assay to Measure Triacylglycerol.

phuric acid, 1 volume heptane, 4 volumes isopropyl alcohol) were added to the cell suspension in a 50 ml centrifuge tube, shook hard and allowed to stand at room temperature for approximately five minutes. Eight millilitres of nanopure water and 12 ml of heptane were added, followed by vigorous shaking. The system separates into two phases within five minutes. Aliquots of the upper heptane phase, which contains the lipids, were transferred and prepared for the fluorometric assay, as will be described.

3. Preparation of Reagents

The following concentrations were used in the assay reagent for the combined enzymatic hydrolysis of triacylglycerol and the determination of glycerol (Figure 1): 100 mM potassium phosphate buffer, pH 7.4; 4 mM magnesium chloride; 0.1 mg/ml sodium dodecyl sulfate; 0.02% (weight/volume) bovine serum albumin; 0.2 mM adenosine triphosphate; 0.35 mM phosphoenolpyruvate; 6 U/ml lactate dehydrogenase; 1 U/ml pyruvate kinase; 0.6 U/ml esterase (hog liver); 0.6 U/ml glycerol kinase; 200 U/ml lipase (Rhizopus arrhizus); NADH in appropriate concentrations for each procedure. The assay reagent was also prepared without glycerol kinase as a reagent blank.

Special consideration was given to the preparation of phosphoenolpyruvate, adenosine triphosphate, and NADH. For maximal stability, the phosphoenolpyruvate solution, 500 mM, was prepared as a trisodium salt to maintain neutrality (or slight alkalinity) and stored at -70°C. Adenosine tri-

phosphate, 500 mM, was neutralized with two equivalents of sodium hydroxide (2.76 g of ATP in 10 ml of 1 M NaOH) and stored at -20°C. A 20 mM stock solution of NADH was made up in 100 mM sodium carbonate buffer, pH 10.3, and was stored at -70°C in small aliquots (50 to 100 µl) that were heated to 100°C for 5 minutes just prior to use to destroy NAD^+.

4. Standard Assay Procedure

The choice of a fluorometric procedure was dictated by the level of sensitivity desired. The optimal ranges of triacylglycerol measured by the two procedures are approximately 5-50 µg of triacylglycerol (about 5-50 nanomoles of triacylglycerol or glycerol) for the disappearance of NADH, and 0.5-5 µg (about 0.5-5 nmol triacylglycerol or glycerol) for the appearance of NAD^+ (converted to a fluorescent product). In view of the amounts of triacylglycerol present in the adipocyte precursors indicated by preliminary experiments, the procedure yielding greater sensitivity was chosen (appearance of NAD^+).

Triacylglycerol extracts and standards, as well as glycerol standards, were pipetted into 12.5 x 45 mm fluorometric tubes (Fisher Scientific, Fair Lawn, NJ) and, where applicable, the heptane was evaporated with a stream of nitrogen at room temperature. Standard curves were developed for both glycerol and triacylglycerol (lipid control E), the latter extracted and processed by procedures identical to those used for the test samples.

5. Appearance of NAD^+

Two hundred microlitres of the assay reagents containing 200 µM NADH were mixed with each sample and standard, and incubated for 30 minutes at 25°C. The unreacted NADH was subsequently destroyed by adding 20 µl of 1 M HCl and incubating the mixture for 10 minutes at 25°C. The NAD^+ was converted to a fluorescent product by the addition of 2 ml of 6 M sodium hydroxide containing 10 mM imidazole base (mixed just prior to use) and incubation for 20 minutes at 60°C. The tubes were cooled to room temperature before the fluorescence was measured.

6. Calculations

The fluorescence attributed to triacylglycerol was calculated as the difference between the fluorescence of the samples containing the complete assay reagent and that for the blank. Triacylglycerol concentrations were then determined from the fluorescence values of the standard curve. Concentration was expressed in nanomoles of triacylglycerol, using 885 g as the average molecular mass.

VII. RESULTS

All the agents that were selected, namely, cytochalasin D, demecolcine, and trifluoperazine, resulted reproducibly in neutral lipid accumulation in both human omental and rat perirenal precursors under the specified experimental conditions. Details of the various experimental conditions are outlined in Tables 4-6. The morphological changes are shown in Figures 2-11.

As shown in Figure 2, some accumulation of lipid occurred after treatment with cytochalasin D, but, of the concentrations tested, neutral lipid was deposited only at 0.2 μM in the case of adipocyte precursors from lean subjects. By comparison, Figure 3 illustrates that, for cells from massively obese persons, accumulation occurred at all concentrations of cytochalasin D that were tested. In addition, precursors from the obese revealed more lipid accretion and cell rounding. Figure 3B shows an arborized pattern of cell processes, suggesting decreased adhesion and other perturbation of membrane structure and function.

A. Cytochalasin D

As shown by the representative pictures in Figure 4, when the culture medium was supplemented with Nutralipid, which contains triacylglycerols as specified under Materials

TABLE 4

Influence of Cytochalasin D, Demecolcine and Trifluoperazine on Neutral Lipid Accretion in Omental Adipocyte Precursors from Lean Subjects

Agent and Number of Experiments (N)	Concentrations at which Staining for Neutral Lipid with Oil-Red-O Found to be Present (μM)	Most Effective Concentration (μM)
Cytochalasin D (N = 9)	0.2	0.2
Demecolcine (N = 8)	0.01 - 1.3	0.13
Trifluoperazine (N = 7)	1 - 100	25
Cytochalasin D + 2% Nutralipid (N = 5)	0.01 - 1	0.2 - 1
Demecolcine + 2% Nutralipid (N = 5)	0.03 - 0.26	0.26
Trifluoperazine + 2% Nutralipid (N = 5)	1 - 100	25

TABLE 5

Influence of Cytochalasin D, Demecolcine and Trifluoperazine on Neutral Lipid Accretion in Omental Adipocyte Precursors from Massively Obese Subjects

Agent and Number of Experiments (N)	Concentrations at which Staining for Neutral Lipid with Oil-Red-O Found to be Present (μM)	Most Effective Concentration (μM)
Cytochalasin D (N = 5)	0.2 - 1	0.2
Demecolcine (N = 3)	0.06 - 1.3	0.13

TABLE 6

Influence of Cytochalasin D, Demecolcine and Trifluoperazine on Neutral Lipid Accretion in Rat Perirenal Adipocyte Precursors

Agent and Number of Experiments (N)	Concentrations at which Staining for Neutral Lipid with Oil-Red-O Found to be Present (μM)	Most Effective Concentration (μM)
Cytochalasin D (N = 8)	Ø	Ø
Demecolcine (N = 8)	Ø	Ø
Trifluoperazine (N = 8)	1 - 35	25
Cytochalasin D + 2% Nutralipid (N = 8)	0.01 - 0.5	0.2
Demecolcine + 2% Nutralipid (N = 8)	0.01 - 0.13	0.13
Trifluoperazine + 2% Nutralipid (N = 8)	1 - 35	25

Figure 2. Treatment of Human Lean Omental Adipocyte Precursors with Cytochalasin D. The cells were treated for five days, fixed and stained with Oil-Red-O. A. Human Lean Omental Adipocyte Precursors in second subculture (2°) - Control. B. Human Lean Omental Adipocyte Precursors 2° 0.05 µM. C. Human Lean Omental Adipocyte Precursors 2° 0.2 µM. The black bar represents 25 µM (original magnification x 200).

Figure 3. Treatment of Human Obese Omental Adipocyte Precursors with Cytochalasin D. The cells were treated for five days, fixed and stained with Oil-Red-O. A. Human Obese Omental Adipocyte Precursors in second subculture (2°) - Control. B. Human Obese Omental Adipocyte Precursors 2° 0.4 µM. C. Human Obese Omental Adipocyte Precursors 2° 1.0 µM (original magnification x 200).

Figure 4. Treatment of Human Lean Omental Adipocyte Precursors with Cytochalasin D and 2% Nutralipid. The cells were treated for five days, fixed and stained with Oil-Red-O. A. Human Lean Omental Adipocyte Precursors in second subculture (2°) - Control. B. Human Lean Omental Adipocyte Precursors 2° 0.05 µM. C. Human Lean Omental Adipocyte Precursors 2° 0.2 µM (original magnification x 200).

and Methods, treatment with cytochalasin D resulted in appreciable accumulation of neutral lipid relative to cells treated with Nutralipid alone.

In addition, much more accretion occurred than in preadipocytes exposed to the cytoskeletal inhibitor in the absence of Nutralipid. As illustrated in Figure 4C, higher concentrations of cytochalasin D also resulted in cell rounding.

Figure 5 illustrates the marked promotion by cytochalasin D of neutral lipid accretion, in the presence of Nutralipid, in rat perirenal adipocyte precursors. Indeed, the perirenal precursors revealed relatively large, coalescing lipid globules.

As shown in Figures 4C and 5C, of the cytochalasin D concentrations tested in the presence of Nutralipid, the most effective was 0.2 μM, in terms of promotion of neutral lipid accretion, in both human and rat adipocyte precursors. Cells treated at this concentration retained an apparently normal morphology and remained attached to the substratum. Higher levels of cytochalasin D resulted in disruption of cellular integrity and in detachment.

B. Demecolcine

As shown in Figure 6, some accumulation of neutral lipid occurred after treatment with demecolcine at all concentrations tested in the case of adipocyte precursors from lean subjects. Figures 6B and 6C reveal the polygonal

Figure 5. Treatment of Rat Perirenal Adipocyte Precursors with Cytochalasin D and 2% Nutralipid. The cells were treated for five days, fixed and stained with Oil-Red-O. A. Rat Perirenal Adipocyte Precursors in second subculture (2°) - Control. B. Rat Perirenal Adipocyte Precursors 2° 0.1 µM. C. Rat Perirenal Adipocyte Precursors 2° 0.2 µM. The black bar represents 25 µM (original magnification x 200).

Figure 6. Treatment of Human Lean Omental Adipocyte Precursors with Demecolcine. The cells were treated for five days, fixed and stained with Oil-Red-O. A. Human Lean Omental Adipocyte Precursors in second subculture (2°) - Control. B. Human Lean Omental Adipocyte Precursors 2° 0.06 µM. C. Human Lean Omental Adipocyte Precursors 2° 0.26 µM (original magnification x 200).

configuration of treated cells. A similar degree of lipid accumulation occurred in preadipocytes from both lean and obese subjects (cells from latter not shown).

As indicated by the representative pictures in Figure 7, when the culture medium was supplemented with Nutralipid, treatment with demecolcine resulted in appreciable accumulation of neutral lipid in preadipocytes from lean subjects, relative to cells treated with Nutralipid alone. In addition, much more accretion occured than in precursors exposed to this inhibitor of microtubule function in the absence of Nutralipid.

Figure 8 illustrates the marked promotion of demecolcine on neutral lipid accretion, in the presence of Nutralipid, in rat perirenal adipocyte precursors. Indeed, the perirenal precursors revealed relatively large, coalescing lipid globules.

The largest accumulation of neutral lipid was effected by demecolcine under the following conditions: 0.13 μM of the microtubule inhibitor in human adipocyte precursors in the absence of Nutralipid (Figure 6C), 0.26 μM in the presence of 2% Nutralipid (Figure 7C), and 0.13 μM in rat preadipocytes in the presence of 2% Nutralipid (Figure 8C). Under these conditions the cells remained attached to the substratum and appeared intact.

Figure 7. Treatment of Human Lean Omental Adipocyte Precursors with Demecolcine and 2% Nutralipid. The cells were treated for five days, fixed and stained with Oil-Red-O. A. Human Lean Omental Adipocyte Precursors in second subculture (2°) - Control. B. Human Lean Omental Adipocyte Precursors 2° 0.13 µM. C. Human Lean Omental Adipocyte Precursors 2° 0.26 µM (original magnification x 200).

Figure 8. Treatment of Rat Perirenal Adipocyte Precursors with Demecolcine and 2% Nutralipid. The cells were treated for five days, fixed and stained with Oil-Red-O. A. Rat Perirenal Adipocyte Precursors in second subculture (2°) - Control. B. Rat Perirenal Adipocyte Precursors 2° 0.06 µM. C. Rat Perirenal Adipocyte Precursors 2° 0.13 µM (original magnification x 200).

C. Trifluoperazine

As shown in Figure 9, exposure of the human adipocyte precursors from lean persons with trifluoperazine resulted in slight accumulation of neutral lipid. Under these conditions, the cells remained fibroblast-like in appearance. By comparison, as shown by the representative pictures of Figure 10, exposure to both the phenothiazine and Nutralipid resulted in appreciable accretion of neutral lipid. In certain cells, the nucleus was displaced peripherally, and some rounding occurred (Figure 10C).

The representative pictures of Figure 11 illustrate accumulation of neutral lipid in rat adipocyte precursors exposed to both trifluoperazine and Nutralipid. In contrast to cells incubated with Nutralipid alone, those exposed to the phenothiazine revealed coalescing lipid droplets and rounding (Figures 11B, 11C). At a trifluoperazine concentration of 25 μM, formation of long, thin cytoplasmic processes was accentuated.

The largest accumulation of neutral lipid occurred at a trifluoperazine concentration of 25 μM in both human and rat adipocyte precursors in the absence or presence of 2% Nutralipid (Figures 9C, 10B, 11C). At this level of trifluoperazine, the cells remain attached to the substratum and appeared morphologically intact.

It was also observed that trifluoperazine resulted in the least morphological changes in either human or rat adipocyte precursors when compared to cytochalasin D,

Figure 9. Treatment of Human Lean Omental Adipocyte Precursors with Trifluoperazine. The cells were treated for five days, fixed and stained with Oil-Red-O. A. Human Lean Omental Adipocyte Precursors in second subculture (2°) - Control. B. Human Lean Omental Adipocyte Precursors 2° 10 µM. C. Human Lean Omental Adipocyte Precursors 2° 25 µM (original magnification x 200).

Figure 10. Treatment of Human Lean Omental Adipocyte Precursors with Trifluoperazine and 2% Nutralipid. The cells were treated for five days, fixed and stained with Oil-Red-O. A. Human Lean Omental Adipocyte Precursors in second subculture (2°) - Control. B. Human Lean Omental Adipocyte Precursors 2° 25 µM. C. Human Lean Omental Adipocyte Precursors 2° 50 µM (original magnification x 200).

Figure 11. Treatment of Rat Perirenal Adipocyte Precursors with Trifluoperazine and 2% Nutralipid. The cells were treated for five days, fixed and stained with Oil-Red-O. A. Rat Perirenal Adipocyte Precursors in second subculture (2°) - Control. B. Rat Perirenal Adipocyte Precursors 2° 10 µM. C. Rat Perirenal Adipocyte Precursors 2° 25 µM (original magnification x 200).

demecolcine and colchicine. Trifluoperazine, moreover, suppressed cell replication to an appreciably lesser extent than the other agents.

In summary, all agents chosen because of their known properties to inhibit cytoskeletal function (cytochalasin D and demecolcine), or because such an effect was presumed (trifluoperazine through its inhibition of the calmodulin-Ca^{2+} complex), resulted in accumulation of neutral lipid in both cultured human and rat adipocyte precursors at certain concentrations (Figures 2-11).

In the case of each agent, supplementation of the culture medium with Nutralipid resulted in appreciably greater neutral lipid accumulation (Figures 4B, 4C, 5B, 5C, 7B, 7C, 8B, 8C, 10B, 10C, 11B, 11C), compared to cells treated with agent alone (Figures 2A, 2B, 6A, 6B, 9A, 9B). While Nutralipid, in the absence of inhibitor, resulted in the presence of some stainable neutral lipid, both human and rat preadipocytes retained their spindle-like shape (Figures 4A, 5A, 7A, 8A, 10A, 11A). In contrast, the combination of Nutralipid and each agent brought about much greater accumulation, in some cases leading to cell rounding (Figures 4B, 4C, 5B, 5C, 7B, 7C, 8B, 8C, 10B, 10C, 11B, 11C).

While triacylglycerol is the predominant lipid that accumulates in preadipocytes, and an excellent correlation has been established between staining for Oil-Red-O in these cells and the presence of triacylglycerol (Wier & Scott, 1986; Green & Meuth, 1974), its nature was also

confirmed in these studies by direct chemical determination of glyceride-glycerol. In view of the above correlation, it was decided to emphasize the morphological studies, rather than the biochemical assays. Using the triacylglycerol standard, linearity was achieved between about 1 and 4 nanomoles of glyceride-glycerol. On the basis of the standard plots, the representative value for glyceride-glycerol content for control preadipocytes from lean subjects was 123 nmol/mg protein (mean of two determinations), while the glyceride-glycerol content for cells treated with cytochalasin D, in the absence of Nutralipid was 219 nmol/mg protein (mean of two determinations). It was similarly confirmed for demecolcine and trifluoperazine that the nature of the accumulating neutral lipid was indeed triacylglycerol.

VIII. DISCUSSION

Despite similar nutrient energy intake and levels of physical activity, there is a great degree of inter-individual variability in body fat content, with the possible exception of monozygotic twins (Roncari & Healy, 1984). Most health care professionals would agree with the observation that many obese patients eat no more and sometimes less than those who are not overweight and often experience great difficulty in reducing their body weight in spite of drastic reduction in food intake. These clinical findings are supported by many detailed energy balance studies of prolonged undereating and overeating, indicating large discrepancies in measures of individual intake and individual expenditure (Doyle et al., 1965; Ashworth et al., 1962; Sims et al., 1968; Bray et al., 1974; Miller & Mumford, 1967; Apfelbaum et al., 1977; Whipp et al., 1973; Garrow & Stalley, 1975).

In most energy balance studies investigators measured the energy intake from food directly by bomb calorimetry. This approach involves ignition of the food sample placed in a heavy steel container called a "bomb" and calculation of the energy value by noting the heat dissipated into a known volume of water surrounding the bomb. Body energy stores were determined by the estimation of body fat from body

density measurements and the estimation of lean body mass from measurements of total body water. Energy expenditure was determined by indirect or direct calorimetry and losses in the urine and feces were measured by bomb calorimetry. Webb (1980) compiled 52 sets of data from studies lasting three weeks or longer, where all the terms required to calculate energy balance were directly measured as described or could be readily estimated. He found that a certain quantity of unmeasured energy with a mean value of 168 ± 92 kJ/d (27% of metabolic energy) was required in order to balance the calculations.

A discussion of the nature of this unmeasured energy considers error and noninclusion of small quantities like loss of protein in hair, nails, and desquamated skin, which are thought to be insufficient to explain this unknown quantity.

Some investigators suggest that Luxuskonsumption, an energy dissipating mechanism purported to exist in conditions of chronic overeating, can fully account for the unmeasured energy term. However, Hervey and Tobin (1982, 1983) thoroughly and convincingly criticize the evidence presented by Rothwell and Stock (1979, 1980a, 1980b, 1982a, 1982b) and others (Sims et al., 1968, 1973; Rothwell et al., 1981; Brooks et al., 1980) supporting the existence of Luxuskonsumption from overfeeding experiments based on large increases in energy expenditure predicted from differences between estimates of energy intake and changes in stored

energy. Hervey and Tobin (1982) indicate a number of serious errors, cumulative with time, arising from this indirect method of measurement of energy expenditure. They suggest that studies that employ direct measurement and complete energy balance would provide more credible evidence for the existence of Luxuskonsumption.

Brown adipose tissue is believed to be the effector for Luxuskonsumption, an energy dissipating mechanism (Rothwell & Stock, 1979, 1980a, 1980b). Brown fat most probably plays a critical role in adaptive thermogenesis cold-adapted and hibernating animals (Seydoux, 1983). In nonshivering thermogenesis, sympathetic outflow from the central nervous system stimulates brown adipose tissue depots with a very high rate of lipid oxidation and heat production, resulting in an increased resting metabolic rate. The most comprehensive studies of brown adipose tissue in human subjects, however, show its virtual nonexistence after one year of age (Hassi, 1977; Heaton, 1972). It would therefore be difficult to conclude that this tissue has a significant role in humans, and that it is responsible for the purported Luxuskonsumption.

A form of energy adaptation is known to occur in response to changes in nutrient intake. During periods of prolonged restriction of food intake, such as occurs during starvation or in obese patients placed on hypocaloric weight reducing regimens, there is a progressive decrease in the resting metabolic rate (Keys et al., 1950; James et al.,

1978). However, the basal or resting metabolic rates (RMR) (uncorrected for surface area) of obese people tends to be greater than that of lean people when they are in energy balance (Hoffmans, 1979; James et al., 1978). It has been suggested that the increase in RMR in the obese may be due to increased lipolysis from enlarged adipocytes resulting in enhanced free fatty acid oxidation and esterification (Nestel & Whyte, 1968). If overeating was assumed to account for the degree of obesity (excessive body mass index), an elevated RMR could at least partially explain an increase in energy expenditure under conditions of dietary surplus.

It would thus appear that studies of energy balance to date have been unsuccessful in accounting for differences in energy utilization between lean and obese persons. Controversy exists as to the nature of a quantity of unmeasured energy reported by many investigators. This unknown amount may not represent error nor the existence of Luxuskonsumption and brown adipose tissue. Perhaps it is a quantity that does not relate to an external measure of energy expenditure and has thereby evaded direct measurement. Hence, a hypothesis based on internal energy interconversions as a mechanism to explain inter-individual variability in terms of body fat content, would be attractive.

The novel hypothesis proposed by Roncari (Roncari & Healy, 1984) invokes that, in addition to energy required for obligate functions constituting basal or resting metabo-

lism, mechanisms based on biomechanical processes utilize energy at varying levels between different persons. A substantial portion of the remaining energy would then be stored in chemical form, mainly as triacylglycerol in adipocytes. The variability in cytoskeletal activity mediating the biomechanical processes would have a genetic basis. According to the hypothesis, therefore, an interrelationship exists between energy utilization for biomechanical work and storage as chemical energy. The former would represent an unrecognized quantity or mechanism of energy expenditure with a variable level of activity between individuals. This mode of energy utilization would constitute the independent variable in each person. The dependent variable, utilizing the remaining or excess energy, would be the synthesis and storage of energy rich molecules.

Individuals would thus respond to sustained "excessive" energy available by three general patterns:

(1) A small fraction of the population utilizes the least quantities of free energy for biochemical work leaving the largest amounts available for triacylglycerol synthesis and storage. This pattern could lead to the development of massive obesity (body weight at least 170-200% of reference) characterized by both enlargement and hyperplasia of adipocytes.

(2) In contrast, the uncommon second pattern of response would include individuals with the highest degree of biomechanical work, resulting in the least quantity of

energy available for chemical storage. These persons would have the highest propensity to be thin and would display the greatest resistance to obesity in the presence of nutritional excess and sedentary habits.

(3) The most common response to these "life-style" factors would be the utilization of relatively less free energy for biochemical cellular processes leaving a larger quantity available for triacylglycerol synthesis and deposition. The consequent moderate "hypertrophic" obesity is characterized by enlarged adipocytes, but a normal total complement of these cells.

According to the new hypothesis, susceptibility to the development of massive obesity could result from a dampening of certain biomechanical processes by a mutant protein of the plasma membrane, or the cytoskeleton, present in an abundant cell type or in several cell types. Consequently, energy expenditure by the cell would be reduced leading to the availability of excessive energy for storage as adipocyte triacylglycerol, and ultimately the development of massive corpulence. For example, a reduced quantity of or an altered kinesin membrane receptor on organelles, or a paucity of kinesin, could result in decreased utilization of energy to a degree leading to prodigious expansion of fat tissue.

Because of interconnections and amplifying functional interactions between the cytoskeleton and cellular membranes, a single mutant protein may result in a cascade-like

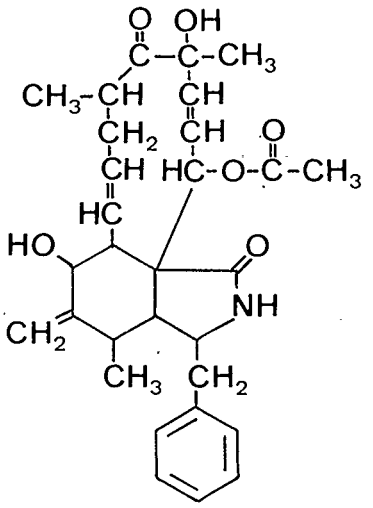
effect. This amplification could lead to a widespread dampening of biomechanical activities. A mutation leading to small but sustained impairment of biomechanical activities could have profound long-term influences in terms of bioenergetics. According to available knowledge regarding energy requirements for biomechanical functions, even a difference of only 1%, when sustained over a few years, could account for a large disparity in body fat content between subjects.

In contrast, mutations resulting in excessive mechanical activity would leave the least quantities of energy available for chemical storage and would thus predispose to the other extreme, unusual leanness. Between the two extremes of massive obesity and unusual leanness, the hypothesis proposes a continuous, "normal" distribution of biomechanical activity with a reciprocal relationship to chemical energy storage. Such heterogeneity might result from genetic polymorphism related to structural or regulatory genes for cytoskeletal elements. Novel endocrine and neural systems that regulate biomechanical functions might also exist. The variable operation of these systems, whose expression would in turn be regulated by genetic factors, could also contribute to modulation of cytoskeletal activity.

Thus, the hypothesis predicts that impairment of biomechanical functions would channel energy to storage in chemical form. In these studies, this possibility was

investigated using agents that suppress or inhibit the function of microtubules or microfilaments in a characterized system of cultured rat and human preadipocytes. Since adipose cells are endowed with specialized biochemical mechanisms for triacylglycerol accretion, they provide an ideal model to search for the presence of the energy relationships proposed by the hypothesis. The mode of inhibition by the agents will now be described briefly.

Cytochalasin D is one of a series of naturally occurring secondary metabolites of fungi (Sheterline, 1983). The chemical structure of this compound is given below:



It was the agent of choice because, unlike other members of this family, it minimally influences the glucose transport system (Sheterline, 1983). The major site of action on F-actin polymers permitted focus on the agent's effect on cellular biomechanical functions. Low concentrations of cytochalasin D retard actin assembly (Flanagan & Lin, 1980; MacLean-Fletcher & Pollard, 1980) by binding with high

affinity to F-actin with a stoichiometry of approximately one cytochalasin molecule per filament or one site per 500-10,000 actin monomers (Brown & Spudich, 1981). This binding inhibits monomer addition to the barbed end of the actin filament, preventing elongation (MacLean-Fletcher & Pollard, 1980). In addition, intercalation of cytochalasin D within contractile networks may increase filament breakage (Sheterline, 1983). Some investigators (Sheterline, 1983) also believe cytochalasins can displace anchored actin filaments from their barbed end capping proteins, free or attached to the plasma membrane.

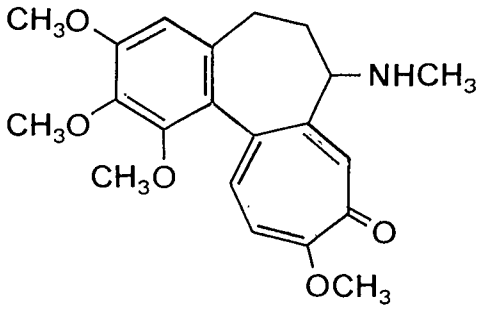
Haynes and Weller (1978) examined the ultrastructure of fibroblasts as a measure of the degree of toxic changes occurring as a result of chemical treatment, as well as giving an indication as to the mode of action of the agents. They found that cytochalasin B (Carter, 1967), at a concentration sufficient to cause a large decrease in cell motility, resulted in only moderate changes in ultrastructure. These findings would suggest that, at the levels also used in the studies described here with the fibroblast-like preadipocytes, cytochalasin probably resulted in only a low degree of toxicity. One of the most noticeable features found by Haynes and Weller (1978) upon treatment of fibroblasts with cytochalasin B was an increase in the number of lipid containing globules. This is in agreement with my finding of lipid accretion upon treatment of rat and human adipocyte precursors with cytochalasin D.

Lin et al. (1978) reported that cytochalasin B affected the morphology of cultured fibroblasts and inhibited motile processes such as membrane ruffling, cytokinesis and axon growth cone activity. Within 20 minutes following addition of 5-20 μM cytochalasin B to Balb/c 3T3 cells (mouse fibroblasts) they noted a rapid retraction of the cytoplasm towards the centre of the cell, leaving an arborized pattern of cellular processes attached to the substrate, in addition to a cessation of membrane ruffling activity. Lin et al. (1978) moreover, obtained similar morphological findings with cytochalasin D. This agent, however, was found to be 5 to 8 times more potent than cytochalasin B in affecting 3T3 cells. In addition, Croop and Holzer (1975) described the dendritic condition of fibroblast-like cells exposed to cytochalasin B as largely due to the unequal retraction of cytoplasm and plasma membrane from the former boundaries of the spread out cells. This sequence resulted in gradual rounding of the central regions of the cells, leaving behind arms and processes of varying shapes, lengths and widths. Changes similar to these have been described by Miranda et al. (1974) using HeLa, Bero, HEp^2 and MDBK cells and cytochalasin D.

The findings of Croop and Holzer (1975) and Lin et al. (1978) are consistent with my observations of rounding of the human and rat fibroblast-like preadipocytes and the arborized cell processes remaining, marking the original extent of the pretreated cell. Their uneven distribution

probably reflects the fact that only at certain points do the margins of the cells adhere tightly to the substratum (Croop & Holtzer, 1975).

Demecolcine, a plant alkaloid, was another agent chosen to inhibit microtubule requiring motility processes of cells. The chemical structure of demecolcine (N-methyl-N-desacetylcolchicine is given below:



The basis for the action of demecolcine, an agent similar in action and structure to colchicine (but lacking one carbonyl group), is its ability to bind to αβ tubulin dimers at high affinity sites. Many microtubule-containing structures of the cell represent a "steady state" of polymerization of tubulin at the net assembly or A-end and an equal depolymerization or loss of subunits from the disassembly or D-end (Wilson, 1986). Demecolcine inhibits the net uptake of tubulin at the A-end and, therefore, net tubular flux from one end of the microtubule to the D-end. At high concentrations, this drug exhibits a "capping" effect on the

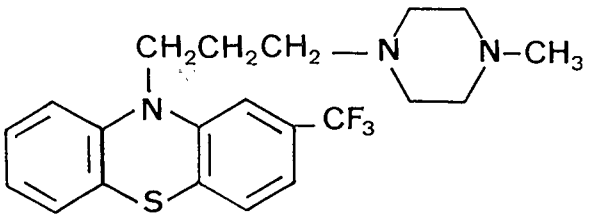
microtubule A-end almost abolishing tubulin addition and loss at steady state (Wilson, 1986).

Various types of actin-based effects on local surface activity, such as membrane ruffling, microspike formation, and phagocytosis often occur in regions of the cell close to the end of microtubules. The site of these movements in the cell is markedly changed upon treatment with colchicine (Sheterline, 1983). For example, in the presence of colchicine, membrane ruffling occurs around the entire periphery of the cell, resulting in a disorganized, wandering movement rather than the normal directed linear motion of fibroblast-like cells in culture (Sheterline, 1983).

Microtubules influence the distribution of intermediate filaments in most cells in culture. In fibroblasts, intermediate filaments spread out in a radial pattern similar to that of cytoplasmic microtubules, extending from a region near the cell nucleus towards the cell periphery. Treatment with low doses of colchicine results in gross distortion of morphology, with changes in ultrastructure characterized by a loss of cytoplasmic microtubules and aggregation of the 10 nm filaments into a dense filamentous cap lying adjacent to the nucleus (Haynes & Weller, 1978; Sheterline, 1983). My observations are in agreement with those of Croop and Holtzer (1975) who found that Colcemid (demecolcine) induced elongated, spindle-shaped fibroblasts to assume a more isodiametric configuration.

A well known clinical observation relates to the development of obesity in patients placed on psychotropic agents, particularly phenothiazines and butyrophenones (i.e. haloperidol) (Rockwell et al., 1983; Klett & Caffey, 1960). Although this effect has been ascribed in part to increased appetite and decreased physical activity, the fact that phenothiazines inhibit the action of the Ca^{2+}-calmodulin complex (Sheterline, 1983) which mediates a number of cytoskeletal functions, would suggest that these agents could diminish energy transduction for biomechanical motions leaving, according to the hypothesis, more energy for triacylglycerol storage.

To explore this possibility, the cultured human and rat preadipocytes were also treated with trifluoperazine. The chemical structure of this drug is given below:



This phenothiazine binds at the Ca^{2+}-calmodulin hydrophobic site which normally interacts with target enzymes. The contractile activities dependent on calmodulin and thereby

inhibited by trifluoperazine include, phosphorylation of myosin light chains by myosin light chain kinase and dissociation of caldesmon from F-actin. The effect of this drug used over a concentration range 10 μM - 25 μM consistent with binding to calmodulin (ki 1.0 x 10^{-6} M) produced less inhibition of motility than did cytochalasin D and demecolcine, however, did result in significant lipid accumulation supporting the proposed hypothesis.

Thus, all agents used in these studies, namely, cytochalasin D, demecolcine and trifluoperazine, inhibit the function of microtubules, microfilaments, or associated structures of the cytoskeletal matrix. According to our hypothesis, such inhibition would lead to increased channeling of energy to chemical storage. That such diversion of energy might indeed occur, was suggested by the accretion of triacylglycerol in human and rat preadipocytes treated with the inhibitors.

Studies were conducted using human and rat preadipocytes in subculture. A sufficient number of new cell generations are produced under these in vitro conditions to exclude any influence of such in vivo principles as circulating hormones or dietary constituents. Thus, the preadipocytes in culture are valuable for studies of genetic factors. It has been previously established that the minuscule quantities of estrogens in fetal bovine serum do not influence cell growth, and that added 17-beta-estradiol stimulates equally the replication of subcultured cells from

both male and female human subjects (Roncari & Van, 1978). Thus, the results obtained in my studies are most probably applicable to both genders.

Unlike hepatocytes, which accumulate triacylglycerol upon exposure to certain toxic agents, adipose cells do not deposit triacylglycerol under similar conditions. In fact, certain infections and malignancies characterized by weight loss or cachexia are associated with the mobilization of triacylglycerol from adipose tissue. This process often persists even with an adequate energy intake (Torti et al., 1985).

The differentiation of 3T3-F442A preadipocytes, a sub-line of Swiss mouse 3T3 cells, is characterized by numerous enzymatic events and by a programmed change in cell morphology from a flat fibroblastic form to a nearly spherical shape (Spiegelman & Farmer, 1982). Accompanying the morphological changes, are large and specific decreases in biosynthetic rates for β and y actin, vimentin, as well as α and β tubulin, as reported by Spiegelman and Farmer in 1982. In cells undergoing adipose differentiation, these investigators found that the synthesis of actin decreased by 90%, and that of tubulin by more than 95%. Translation in vitro of mRNA isolated from differentiated cells indicates that the decrease in biosynthetic rates for cytoskeletal proteins results from altered levels of active mRNA (Spiegelman & Farmer, 1982). Cloned cDNA probes for β-actin and tubulin revealed that the changes in mRNA

activity corresponded to a specific, quantitatively equal loss of these sequences during cellular differentiation. Spiegelman and Farmer (1982) have concluded that these biosynthetic changes in the biosynthesis of cytoskeletal elements, are very early events in differentiation and participate in the development of the adipocyte morphology.

The suppression of major elements involved in cellular biomechanical functions during differentiation described by Spiegelman and Farmer (1982) may well be related to our hypothesis, and may be a critical determinant for cell rounding and triacylglycerol accretion during adipose differentiation. Indeed, alterations in the cytoskeleton are believed to precede the subsequent biosynthetic events specific for adipocyte differentiation (Spiegelman & Farmer, 1982). It may be proposed that the inhibition of cytoskeletal function induced in my studies partly mimicked certain early events of adipose differentiation. Thus, instead of decreased biosynthesis of cytoskeletal elements, my experimental approach was directed at inhibition of their function, an effect that may have accounted for the observed triacylglycerol accretion and cell rounding.

The next part of the Discussion will refer to "endogenous" compounds which are known inhibitors of adipose differentiation, and will propose a relationship between the suppressive effect and our hypothesis.

Studies examining the ability of type β transforming growth factor (TGF-β) to modulate cell development have been

conducted on differentiating mouse 3T3-L1 preadipocytes, another sub-line of 3T3 cells with a special susceptibility to differentiation. Ignotz and Massagué (1985) found that TGF-β inhibits potently (ID_{50} ≈ 25 pM) the adipogenic conversion of 3T3-L1 cells if exposed to the agent before they become committed to differentiation. Inhibition of this process was monitored by decreasing glycerol phosphate dehydrogenase activity and ATP-citrate lyase activity. The characteristics of that inhibition suggest the possibility that TGF-β interferes directly with the mechanism that controls the co-ordinate expression of differentiation-specific mRNA's including glycolytic and lipogenic enzymes. It is conceivable, but of course requires direct testing that the inhibitory effect of TGF-β is primarily mediated by maintenance or stimulation of cytoskeletal activity, possibly through an influence at the level of DNA transcription, with a secondary suppressive effect on the processes leading to triacylglycerol synthesis and storage. Similar possibilities apply to cachectin, as follows.

Cachectin (tumor necrosis factor) is a recently described protein hormone secreted by macrophages (Cerami et al., 1985). Its multiple biological effects include the induction of a state of shock and of wasting or cachexia (Torti et al., 1985; Beutler & Cerami, 1986). Thus, certain infections and malignancies, conditions characterized by substantial production of cachectin, can lead to progressive weight loss and cachexia, despite consumption of adequate

nutrient energy. The weight loss has been ascribed to the influence of cachectin on adipose differentiation and metabolism of adipocytes (Beutler et al., 1986; Beutler & Cerami, 1986; Cerami et al., 1985). Indeed, cachectin prevents differentiation of preadipose cell lines, in part through inhibition of synthesis of specific adipose-related mRNA's including the messenger for glycerol phosphate dehydrogenase (Torti et al., 1985; Beutler et al., 1986; Beutler & Cerami, 1986). The latter catalyzes the synthesis of sn-glycero-3 phosphate, which provides the glycerol backbone of adipocyte triacylglycerols. Inhibition of transcription is probably also responsible for the marked inhibition of the activity of other lipogenic enzymes and of lipoprotein lipase, the enzyme responsible for assimilation of the fatty acyl chains from circulating lipoprotein-triacylglycerols into adipocyte triacylglycerols (Beutler et al., 1986; Torti et al., 1985; Beutler & Cerami, 1986). These inhibiting influences occur in both differentiating adipocytes and in mature fat cells. In the latter, exposure to cachectin leads to a decrease in specific mRNA's to the low levels present before differentiation (Torti et al., 1985). Thus, cachectin induces both inhibition of triacyl-glycerol deposition and lipid mobilization resulting in contracted adipocytes, and wasting in vivo.

It should be noted, however, that cachectin does not decrease transcriptional activity for such cytoskeletal elements as β-actin (Torti et al., 1985). It is conceivable

that cachectin might actually increase gene expression related to proteins involved in the functions of microtubules and microfilaments, resulting in secondary effects on adipocyte differentiation and triacylglycerol metabolism. (During normal adipose differentiation, suppression of gene expression related to the cytoskeleton precedes enhancement related to lipid assimilation and synthesis.) This proposal would then be compatible with our hypothesis in that increased utilization of energy for cytoskeletal activity would be associated with decreased triacylglycerol synthesis and storage.

It is intriguing and possibly relevant to the preceding discussion that human H-ras oncogene protein, when microinjected into quiescent rat embryo fibroblasts, results in a marked increase in cell surface ruffling and fluid-phase pinocytosis (Bar-Sagi & Feramisco, 1986). Observations were made by scanning electron microscopy, phase-contrast, and fluorescence microscopy, the latter quantifying the uptake of dextran tagged with a fluorescent probe. The stimulatory effect of the ras oncogene protein was associated with, possibly causally, stimulation of phospholipase A_2 activity (Bar-Sagi & Feramisco, 1986). It is thus possible that at least some of the wasting associated with certain malignancies, even before they become widespread, is due to increased energy utilization for cellular biomechanical activity. It will be of great interest to determine whether ras oncogene proteins inhibit adipose differentiation and deplete the triacylglycerol content of mature fat cells.

IX. CONCLUSION AND PROSPECTIVE

These studies indicate that cytochalasin D, demethylcolchicine, and trifluoperazine, agents that directly or indirectly inhibit cytoskeletal functions, lead to triacylglycerol deposition in cultured human and rat adipocyte precursors. Thus, at least at the level of isolated adipocyte precursors, these findings are consistent with the novel hypothesis relating decreased cytoskeletal activity to increased triacylglycerol deposition in adipose cells.

Clearly, the hypothesis will require testing by several additional approaches. In future studies, it will be critical to determine whether energy utilization for cellular biomechanical functions is depressed in obesity, whether the decrease is most profound in massive corpulence, and whether the quantity of energy made thus available, can account for the accumulation of adipocyte triacylglycerol responsible for obesity.

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