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THE ROLE OF cAMP IN CARBOHYDRATE METABOLISM: AN ATTEMPT TO ELUCIDATE THE METABOLIC DEFECT AND THE INHERITANCE PATTERN OF DIABETES MELLITUS

by

ALICE JANE MICHAELS WOLF

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Abstract

THE ROLE OF cAMP IN CARBOHYDRATE METABOLISM: AN ATTEMPT TO ELUCIDATE THE METABOLIC DEFECT AND THE INHERITANCE PATTERN OF DIABETES MELLITUS

by

Alice Jane Michaels Wolf

Adviser: Professor Kurt Hirschhorn

The pattern of inheritance in diabetes mellitus appears to be polygenic, but to date the genetics of the disease as well as the basic metabolic defects are poorly understood. This is due, in part, to a varied gene expressivity which is related to ill-defined interactions with environmental factors, such as ageing, diet and neurohormonal reactions. An in vitro system could therefore be useful in delineating the basic biochemical defects of this disease. Fibroblast cultures derived from skin biopsies are eminently suitable for such a study of metabolic parameters under well-defined and controlled conditions, several generations removed from the neurohormonal influence of the body.

Goldstein et al. (1969) have shown that fibroblasts from pre-diabetic and diabetic subjects display decreased plating efficiency and recently it has been demonstrated that cyclic 3',5'-adenosine monophosphate (cAMP) can inhibit cell division. Therefore, it may be that the decreased plating efficiency of diabetic fibroblasts is due to abnormal cAMP levels. It has been demonstrated that there are increased levels of

cAMP in adipose tissue and hepatic cells derived from diabetic subjects. A number of investigators have shown the importance of cAMP in epinephrine stimulated lipolysis and other aspects of carbohydrate and lipid metabolism. More recently Solomon et al. (1970) have shown divergent effects of cAMP and its derivative dibutyryl cAMP (DBcAMP) on glucose oxidation of fat cells. I have studied the effects of these compounds on adipocytes and have shown that their actions can be modified by the variations in adipose cell size.

It seemed appropriate therefore, to investigate possible differences in the cAMP system of diabetics, and control subjects utilizing in vitro studies of fibroblasts derived from both groups. Direct assay of cAMP and adenyl cyclase in the fibroblasts revealed quantitative differences in the cAMP content of normal and diabetic cells when treated with epinephrine-insulin. Attempts were made to discern differences in metabolic function of the cells derived from these subjects. The effects of additions of exogenous cAMP and DBcAMP was also determined.

Since differences in cAMP had already been demonstrated in fat cells derived from normal and diabetic subjects, the studies on fibroblasts have been correlated with parallel studies on adipose tissue.

The following methods were used for these studies: cAMP and adenyl cyclase determinations were made by the radioimmunoassay technique of Steiner et al. (1969); the cells were challenged with epinephrine and a mixture of epinephrine and insulin. Studies of stimulated metabolic activity included $^{14}CO_2$ production from glucose-1-$^{14}CO_2$ and glycerol production. The fibroblasts were obtained without trypsinization and incubated for 48 hours in Eagle's minimum essential medium with

penicillin and streptomycin. Some flasks contained cAMP (5×10^{-3}M) or DBcAMP (1×10^{-3}M). The fat cells were first isolated by the method of Rodbell (1964), and their cell size determined as described by Hirsch and Gallian (1968). The cells were incubated in Krebs-Ringer bicarbonate buffer.

It was found in this investigation that the cAMP level of fibroblasts obtained from diabetic subjects was much less responsive to a defined insulin challenge than the cAMP level of fibroblasts obtained from normal (control) subjects. It is hoped that further study of this system may provide much needed additional information on the basic defects of diabetes mellitus which in turn may permit a more precise delineation of the inheritance pattern of the disease.

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List of Abbreviations

ACTH	adrenocorticotrophin
ATP	adenosine triphosphate
cAMP	adenosine 3',5'-monophosphate
cGMP	guanosine 3',5'-monophosphate
DBcAMP	N^6 $O^{2'}$ dibutyryl cAMP
DNA	deoxyribonucleic acid
GH	growth hormone
HGH	human growth hormone
IgG	immunoglobin G
MBcAMP	N^6 monobutyryl cAMP
MEM	minimum essential medium
MSG	monosodium glutamate
NaF	sodium fluoride
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide reduced
PDE	cyclic 3'5' nucleotide phosphodiesterases
PGE	prostaglandin
RNA	ribonucleic acid
ScAMP-TME	succinyl cAMP tyrosine methyl ester
TCA	trichloroacetic acid

INTRODUCTION

For the clinician, diabetes mellitus is probably the most frequently encountered inherited disease, manifesting itself as elevated levels of glucose in serum or urine. Although the disease and its symptoms have been known for many years, its cause remains unknown. The most widely held theory is that of an insufficiency in the secretion of effective insulin from the beta cells of the pancreatic islets. Recently Cerasi and Luft (1970) found that insulin secretion was biphasic, consisting of an initial rapidly released portion stimulated by a glycemic substance, followed by a sustained release phase. In the diabetic this initial response is defective.

Unfortunately, elevated blood glucose and insufficient effective insulin are not the only clinical manifestations of the disease. With time, vascular disease, especially microangiopathy of the eyes and kidneys, develops (Marble, 1967) which in its earlier stages can be demonstrated in the thickening of the basement membranes of small blood vessels. Interestingly, more patients with a family history of diabetes have small vessel complications than those without (Reinheimer et al., 1967), suggesting a genetic basis. In obese diabetic patients, who often demonstrate hyperinsulinism and insulin resistance, on the other hand, there is a high percentage of large vessel complications.

In primitive people diabetes mellitus was possibly not a handicap since they existed on a low mean caloric intake and were more physically active. In their feast or famine existence rapid insulin secretion may have provided them with additional adipose reserve during

times of starvation (Neel, 1962). Furthermore, Neel hypothesized that earlier somatic cell maturation of diabetic women causes earlier menarche. Also, the higher birth weights of their children could be advantageous when adequate medical care is lacking. Studies have shown that infants of diabetic mothers can better handle glucose loads than infants of normals (Neel, 1962). These mechanisms may create a slight increase in the fertility among diabetic women both compensating for any increased prenatal and perinatal mortality as well as maintaining and increasing the gene frequency(ies).

In certain populations today changes in eating patterns have markedly increased the prevalence of the diabetic phenotype. It may be such changes in dietary intake, along with improved detection methods and prolonged lifespan that have resulted in the increased frequency of clinical diabetes mellitus in the world's population.

While the ability to clinically recognize diabetes is well known, its inheritance patterns remain unestablished. For many years it was considered that diabetes was determined by a single autosomal recessive gene (Pincus and White, 1934). When it became evident that all children of two diabetic parents did not themselves become diabetic, the theory was modified to include incomplete penetrance. Using sibships containing juvenile diabetics Barrai and Cann (1965) found support for recessive transmission by segregation analyses. They demonstrated segregation frequency for backcross matings about twice that found in intercross matings, penetrance as low as 25% in both mating groups, and absence of sporadic cases, all in human families. However, on the basis of consanguinity studies, marriages between two diabetics, the

appearance of diabetes in multiple successive generations, and the theory of anteposition, Pavel and Pieptea (1968) proposed a dominant pattern of inheritance. In addition Harris (1950) proposed the theory that juvenile diabetics were homozygous for a mutant gene and maturity onset diabetics were heterozygous for the gene.

It is apparent that rather than being inherited in any single manner, diabetes represents a genetically heterogenous group of disorders. It is thus difficult to conceive of it as being caused by a single gene. The distribution of blood glucose levels do not show a bimodality between the general population and diabetics as would be expected for a single gene (Neel, 1967). As mentioned previously, not all children of conjugal diabetics become diabetic. And while it is clear that the predisposition to develop diabetes is greater among the sibs of diabetics than among the general population, the predisposition frequencies do not fit any single gene hypothesis, unless modifying genes are considered (Simpson, 1962). There is a marked excess of diabetics among the first degree relatives of diabetics, suggesting an accumulation of "high risk" factors (Simpson, 1964), and using Edwards' equation for relative frequency for multifactorial inheritance, the data support this hypothesis. Indeed, at the present time many investigators are favoring multifactorial inheritance as the most likely hypothesis.

Further evidence for the multifactorial theory is obtained from the observation that the manifestations of diabetes are influenced by genetic and environmental factors (Carter, 1969). Recessive inheritance is ruled out because the incidence is as high in sibs as in children.

Dominance can be ruled out because the incidence in sibs is increased two fold when one parent is also diabetic. Additional support for a multifactorial pattern of inheritance is derived by utilizing a method developed in quantitative genetics for dealing with threshold characters termed heritability (h^2). h^2 is estimated from the degree of resemblance between relatives, expressing the extent to which phenotypes exhibited by parents are genetically transmitted to offspring as a correlation or regression coefficient (Falconer, 1965). If the heritability of a disease is high environmental forces are unimportant. Differences due to sex and age can be incorporated into these equations. Finally, if there is even one major gene contributing to the disease, the analysis breaks down and can be observed.

When the thresholds were calculated for the control population the affected population, and its first degree relatives, the threshold for relatives was equidistant from the other two thresholds (Edwards, 1970), supporting multifactorial inheritance. In additional analyses of diabetics and their first degree relatives, diabetics were divided into age groups (Falconer, 1967). h^2 was very high in young people (70-80%) and decreased in people over 55 (30-40%), suggesting that older people are subject to environmental sources of variation that do not affect members of a family equally. When the data was calculated by sex, h^2 for brothers did not change with age, but h^2 for sisters decreased with age (Simpson, 1969). This may have occurred because there is an increase in the frequency of diabetes in the general female population with age. Most recently, when certain modifications were applied to this and new data, correlation coefficients demonstrated that diabetics of all ages have some genetic factors in common (Smith

et al., 1972).

Furthermore, it has been demonstrated that the risk of a relative of a diabetic developing diabetes increases with age to age 70 (Falconer et al., 1971). Also, the age of onset and sex of the proband have been implicated as playing a role in the risks of developing this disease (Simpson, 1969).

Finally, it must be pointed out that the majority of studies have been based on patient completed questionnaires, and of necessity reflect the inaccuracies inherent in this method of data collection (Klimt et al., 1967; Keen and Track, 1968).

In clinical studies of diabetes, certain definitions have become generally accepted. The people who are most likely to develop diabetes but are clinically normal are termed prediabetics. This classification includes the identical nondiabetic twin of a diabetic; children of conjugal diabetics; women with certain abnormal obstetrical histories; obese individuals; and subjects with diabetes-like vascular manifestations (Camerini-Davalos, 1965). The obstetrical abnormalities include tendency to have large babies, perinatal mortality, repeated miscarriages or toxemia of pregnancy. Various abnormalities have been described in prediabetics such as elevated serum levels of insulin, free fatty acids, sialic acid and synalbumin and vascular lesions and abnormalities of the basement membranes (Rimoin, 1967). The plasma insulin response to glucose loading in prediabetics is delayed but ultimately attains higher levels than in normals, and it has been suggested that another diabetogenic factor must be added to the abnormally delayed insulin secretion in prediabetics before diabetes becomes clinically manifest (Cerasi and Luft, 1967).

Another subgroup is the chemical diabetic who has a normal fasting blood glucose but whose tolerance to glucose is impaired, a first clinical sign of diabetics.

Attempts have been made to associate diabetes with better defined inherited properties and other disease states. Early reports found a possible association between ABO, Rhesus and MN blood groups (Buckwalter, 1964). One study reported an increased frequency of blood type B and a decreased frequency of AB in diabetics. The statistical significance was borderline and could be due to sampling error. A later study (Berg et al., 1967) showed no association with the ABO group system, but a possible relationship with the Hp and Gm systems. A possible relationship was also suggested between diabetes and gout, pernicious anemia and hyperthyroidism (Pyke, 1968[b]). Higher birth weights are found in the offspring of diabetic mothers (Hsia and Gellis, 1957). Normal homeostatic mechanisms in diabetic mothers are disrupted so that maternal blood glucose levels may interfere with fetal cell metabolism resulting in the development of congenital defects (Reid, 1970). Kessler (1970) suggests that there may even be a relationship between diabetes mellitus and carcinoma. The hexose monophosphate shunt which is depressed in diabetes may be activated in carcinogenesis and he feels this may present an explanation for decreased frequency of cancer in certain populations with a high frequency of diabetes.

The basic defect in diabetes mellitus, however, still remains undetermined. Experimental matings and *in vivo* tests cannot easily be performed in humans. Attempts have been made to simulate diabetes in animals by various means such as surgical pancreatectomy, injections

of alloxan, dehydroascorbic acid, chelating agents, insulin antiserum and fluoroacetic acid (Taylor, 1968[b]). Diabetic models are found in some laboratory animal populations. In the mouse diabetes is caused by a single recessive gene (Coleman and Hummel, 1967). Many of the metabolic and functional changes observed resemble those seen in man. Diabetes in the chinese hamster exhibits an inheritance similar to man, that is, a polygenic system with genes for predisposition in the presence of environmental stress (Butler, 1967).

However, to define both the pattern of inheritance and the primary defect in diabetes mellitus, in vitro studies must be carried out on human tissue and animal models. Tests can be performed which are not possible in in vivo situations. Cultured human skin fibroblasts are assumed to contain the same genetic material as other cells, but can be maintained under carefully controlled environmental conditions so that their responses to normal and abnormal physical or chemical situations can be studied. With proper storage techniques the cells are available over extended periods of time for comparative studies and, as every cell contains the same genetic material, fibroblasts should reveal basic biochemical defects in inherited diseases. Many of the experimental designs developed for other cell types can be adapted for fibroblasts.

Since the mid-fifties when it was demonstrated that fat cells were metabolically active, they have become a popular cell type for study of metabolic and enzymatic activity. These studies became even more meaningful after Rodbell (1964) devised a technique for isolating fat cells from whole tissue and made it possible to confine experiments to the single cell type

present. Unlike fibroblasts, however, fat cells must be used immediately after removal from an organism, while they are still influenced by its neurohormonal environments immediately prior to the experiment. There is no way to completely compensate for these influences. Where possible parallel studies between fat cells and fibroblasts were made to see if the two cell types reacted similarly. A frequently examined parameter is lipolysis and its sensitivity to hormonal stimulation. $^{14}CO_2$ production from glucose-1-^{14}C has been used in a similar manner, and has been compared with lipolytic activity to help determine the mechanisms of hormonal action.

In 1956 Sutherland and his co-workers discovered a compound, adenosine 3'5' monophosphate (cAMP) while investigating the hepatic glycogenolytic action of the hormones glucagon and epinephrine (Sutherland and Robison, 1969). It was found that cAMP was the substance that mediated activation of phosphorylase by epinephrine and glucagon. Further studies demonstrated that these two hormones were not the only ones whose actions were mediated by cAMP. Insulin secretion and action are believed to be under the control of the cAMP system. Also cAMP has been found in all cell types investigated, except mature erythrocytes of some mammals, as well as in cells of higher plants.

Adenylate cyclase is a membrane bound enzyme which catalyzes the conversion of ATP to cAMP and inorganic pyrophosphate in the presence of magnesium (Butcher et al., 1965). This is a reversible reaction. Under physiological conditions, cyclic 3'5' nucleotide phosphodiesterase (PDE) catalyzes the hydrolysis of cAMP to 5' adenosine monophosphate

(Cheung, 1967). This is the only known mechanism for this conversion. In cells, PDE is present in a much higher concentration than adenyl cyclase and its activity is critically related to the tissue level of cAMP. PDE is inhibited by the methylxanthines, especially caffeine and theophylline. Another link in the series of events was the discovery of a cAMP-dependent protein kinase which catalyzes the phosphorylation of phosphorylase kinase (Langan, 1969; Greengard and Kuo, 1970). This type of kinase also catalyzes the phosphorylation of histones. Such protein kinases have been isolated from many tissues.

When cAMP stimulates protein kinase, which then phosphorylates histones, the genetic material may be uncovered, regulating RNA synthesis. A defect in this system could cause serious defects, which may even be incompatible with life. In higher organisms cAMP has definitely been linked with RNA synthesis (Averner et al., 1972). In E. coli where the system is much less complex, cAMP can reverse catabolite repression of induced enzyme synthesis. By using inducers and inhibitors, cAMP appeared to act on transcription (Jacquet and Kepes, 1969). The lac system, which is known in detail, has a promoter site at which RNA polymerase binds to the DNA and m-RNA synthesis is initiated. Only mutants which have deletions for most of the promoter are unresponsive to cAMP (Pastan and Perlman, 1970). An intact promoter locus is required for the stimulatory effect of the cAMP receptor-cAMP complex. This complex promotes the formation of a pre-initiation complex between RNA polymerase and DNA (Nissley et al., 1972).

Adenyl cyclase consists of two subunits, not unlike aspartate transcarbamylase. The regulatory subunit faces the extracellular space and interactions with it are transferred to the catalytic

subunit on the inside. Phospholipids are believed to regulate the activity of the enzyme (Pastan and Perlman, 1971). Phospholipase C, depending on its concentration, will increase or decrease adenyl cyclase activity.

The following criteria must be satisfied to support adenyl cyclase's role in hormonal action (Sutherland et al., 1968).

I. Adenyl cyclase in broken cell preparations should respond to the same hormones which are effective in the intact tissue. Analogues should show the same order of potency and competitive antagonists should behave similarly.

II. The level of cAMP in intact tissues should change appropriately in response to hormonal stimulation. If possible, the physiological response should be simultaneously monitored and the change in the cAMP level should precede the physiological response.

III. Hormones which stimulate adenyl cyclase should be potentiated by drugs which inhibit PDE activity, when the concentration of the hormone does not produce the maximal response.

IV. When possible, attempts should be made to mimic the effects of the hormone with exogenous cAMP or its derivatives.

To establish a relationship of cAMP to a disease state, the preceding criteria should be satisfied and preparations from normal and diseased tissues should be compared. If differences are present then the biochemical lesion in the disease may involve cAMP.

cAMP has been referred to as the "second messenger" (Butcher et al., 1968[a]). The first messenger, the hormone, travels from the cells of

origin to the target tissue to cause an alteration in the intracellular level of cAMP which then sets off the chain of events leading to the cell's final response. Most rapidly-acting hormones stimulate adenyl cyclase activity, thus increasing the intracellular level of cAMP. Insulin, however, decreases the intracellular level of cAMP.

cAMP has been implicated in diabetes mellitus (Sutherland and Robison, 1969). cAMP enhances the release of insulin from pancreatic beta cells. Insulin travels to the liver and adipose tissue to suppress the accumulation of cAMP and may also antagonize the action of cAMP in muscle. The mechanism by which insulin affects cAMP concentration is unknown. The cAMP-forming mechanism may be defective in diabetics, manifested as a defective adenyl cyclase system or as excessive PDE activity. Or if the adenyl cyclase mechanism is normal, amyloid deposits found within the pancreatic islets might act as a barrier delaying the transfer of insulin from the beta cells to the bloodstream. This barrier might impede glucagon from gaining access to the adenyl cyclase of the beta cells. In both cases, if the formation of cAMP could be increased, the defect would be overcome. Another possibility may be a defective basement membrane which impedes the release of insulin by reverse pinocytosis. In the following studies attempts were made to demonstrate a role for the adenyl cyclase system in normals and diabetics.

Investigators have shown that cell size can be a determining factor in the cell's metabolic response (Zinder and Shapiro, 1971). Larger fat cells have higher basal (Hartman et al., 1971) and epinephrine stimulated lipolysis (Jacobsson and Smith, 1972). Basal $^{14}CO_2$ production is unaffected by cell size, but insulin stimulation

decreases as cell size increases (Salans and Dougherty, 1971; Salans et al., 1968). These phenomena are true for both rat and human adipose cells (Smith, 1971; Goldrick and McLoughlin, 1970). Thus, a valid comparison could be made between the properties of rat and human fat cells and human diabetic cells. When fat cells from nondiabetics and diabetics are compared, diabetic cells are less sensitive to insulin stimulation of $^{14}CO_2$ production (Bjorntrop, 1966).

In order to satisfy the fourth criterion established by Sutherland, rat adipocytes of varying sizes were incubated with cAMP or its derivative, N^6, $O^{2'}$ dibutyryl cAMP (DBcAMP) and their glycerol release and $^{14}CO_2$ production were examined. Both cAMP and DBcAMP were used because many investigators reported that cAMP is without effect on lipolysis either because of rapid destruction by PDE or failure to penetrate the cell readily (Goodman, 1969). Substituting DBcAMP may not always be valid, however, since Solomon et al. (1970) found that in $^{14}CO_2$ production, cAMP and DBcAMP have divergent actions.

The possibility was considered that large cell size may be the major factor in the cell's response and therefore rats were injected with monosodium glutamate (MSG) and/or rat growth hormone (GH). MSG is known to increase the animal's fat cell size, while the effects of chronic injections of GH in rats is not established. In humans, treatment with human growth hormone (HGH) enhances the insulin response to glucose (Sutherland and Robison, 1969). In some animals injections of GH are diabetogenic (Taylor, 1968[a]). The cells from these rats were tested with cAMP, DBcAMP, insulin and growth hormone. Growth hormone was used since it demonstrates many insulin-like activities. Both of these hormones increase transport of glucose into adipose tissue,

increase the uptake and utilization of a variety of monosaccharrides and increase the uptake and degradation of leucine. Also, they increase the incorporation of amino acids into adipose tissue protein. In addition, like insulin, GH interferes with some aspect of the formation of cAMP because it does not decrease the lipolytic stimulation produced by DBcAMP (Goodman, 1970). The known effects of insulin in large cells can be compared to those of GH, cAMP and DBcAMP. It can then be demonstrated whether these cells respond like large cells or whether the injections changed their expected sensitivities.

Fat cells are known to contain cAMP (Butcher et al., 1965). In order to satisfy Sutherland's second criterion, fat cells of varying sizes were studied with epinephrine and epinephrine-insulin. Insulin was not used alone because others have shown that it was not possible to show significant depression of basal levels of cAMP (Butcher et al., 1966). Since metabolic responses to epinephrine and to insulin are affected by cell size it is possible that cAMP levels might reflect changes of sensitivity to these substances. A diminished effect of insulin on cAMP levels in larger cells may be suggestive of the responsiveness of diabetic cells.

Human fat cells studies of $^{14}CO_2$ production were also performed with cAMP and DBcAMP. These studies were performed on tissues from normals, obese patients, and growth hormone deficient children. Obese patients were used because of their large fat cell size (Salans et al., 1968). GH deficient children often have abnormal glucose tolerance tests and show low insulin levels with fasting (Raiti and Blizzard, 1970) or upon glucose and arginine administration. The results from

humans and rats can be compared (Kahlenberg et al., 1966; Gries and Steinke, 1967; James and Burns, 1969).

Studies were also performed on human skin fibroblasts. Goldstein et al. (1969) demonstrated that there was a decreased plating efficiency in prediabetic fibroblasts as compared to normal fibroblasts. As in fat cells, $^{14}CO_2$ production in fibroblasts is stimulated by insulin (Goldstein and Littlefield, 1969; Wolf et al., 1971). It is possible that cAMP and DBcAMP would produce different responses in cell strains derived from normal and diabetic or diabetic-like persons.

All human diploid fibroblasts have a finite life-span and proceed through three phases (Hayflick and Moorhead, 1961). During the last phase the cells degenerate and a longer period of time is required to form a confluent monolayer (Hayflick, 1965). Diabetic cultures arrive at this stage within a few subdivisions. They have a shorter in vitro replicative life-span than normal cells. It was hypothesized that they may have already gone through more divisions in vivo (Martin et al., 1970).

Recently, DBcAMP has been added to malignant cultures and their growth rate has decreased (Johnson et al., 1971[a]; Johnson et al., 1971[b]; Hsie and Puck, 1971; Heidrick and Ryan, 1970). cAMP levels in these cell lines are lower than in normal cells (Sheppard, 1972; Otten et al., 1971). cAMP concentration probably is related to growth rate. There may be a correlation between the growth rate in normal fibroblasts and basal cAMP levels. If a parallel can be drawn from the malignant cells, the slower growing diabetic cells may have a higher cAMP content than normal cells. Diabetic and growth hormone deficient cells were compared to see if this correlation is actually obtained.

Changes in metabolic activity should be reflected by changes in cAMP levels. Insulin should decrease cAMP levels. cAMP stimulated levels may differ between diabetic and normal cells. The cells may also show a difference to stimulation by other substances, such as epinephrine.

To fulfill the first criterion, adenyl cyclase activity can be measured in broken cell preparations. This will determine whether adenyl cyclase is being acted on directly by the hormone. It may also indicate if it is the enzyme which may be different in different cells.

To establish other metabolic differences between cell types a biological type of assay can be used. Cells that differ metabolically may remove different amounts of substances from the medium in which they are incubating. To detect these changes biologically, studies can be done in which one type of cell is incubated in medium from different cell types. In this case the medium from fibroblast cultures was added to fat cell incubation medium. Fat cells are quite sensitive metabolically and they may be able to detect any differences in the medium itself or the presence of any inhibitors added to the medium by the fibroblasts.

From the above studies it is possible that a biochemical defect may be found in diabetic cells which may then be useful in establishing the patterns of inheritance in diabetes mellitus.

METHODS AND MATERIALS

I. Tissue Preparation

Free Fat Cells:

Sprague Dawley rats were decapitated and their epididymal or parametrial pads were removed, washed in warm saline solution and weighed. Isolated fat cells were prepared by Rodbell's method (1964). The tissue was incubated in 6 ml Krebs Ringer bicarbonate buffer, pH 7.4, and collagenase, 3.3 mg/ml (Worthington Biochemicals Corp., Freehold, N. J.) for one (1) hour at 37°C in a metabolic shaker with gentle shaking. After a short period of vigorous shaking, the cells were poured through a nylon filter to remove the interstitial tissue. The fat cells were collected by low speed centrifugation and the infranate was removed. The cells were washed three times with buffer to remove the collagenase. The concentrated cell fraction was used directly in the experiments.

Human adipose tissue was obtained by needle aspiration from the patient's buttock (Hirsch et al, 1960) or from surgical specimens from the abdominal wall. The tissue was washed in Krebs Ringer bicarbonate buffer pH 7.4 at 37°C before use. Minced tissue was used in the experiments due to the small quantity available.

Fat Cell Size:

Adipose cell size (µg lipid/cell) was determined as described by Hirsch and Gallian (1968). The number of fixed cells was compared with the total amount of lipid present. The cells were fixed by incubating them at 37°C for 48 hours in a solution of osmium tetroxide in 1 M collidine buffer. Duplicate samples were taken from each specimen.

These cells were electronically counted in a Coulter Electronic Counter (Coulter Electronics, Inc., Hialeah, Fla.).

From the same specimen, a sample was taken for lipid extraction. The sample was placed in a Folch solution (2 chloroform: 1 methanol) and extracted overnight (Folch et al,, 1957). After rectification with distilled water, the amount of lipid in the chloroform layer was determined by measurement of carboxyl esters present as described by Rapport and Alonzo (1955).

Cell size of the rat free cells was calculated by pipetting a known volume of cells into each solution. The minced tissue was weighed before placing it into each solution. All fat cell calculations were done on a per cell basis.

Fibroblasts:

Skin biopsies obtained from normal, diabetic, and growth hormone deficient persons were used to initiate human fibroblast cultures. The skin explants, and later the fibroblasts were maintained in McCoy's Medium 5A (BBL, Los Angeles, Calif.) with 15% fetal calf serum, penicillin (100 units/ml), streptomycin (100 mg/ml) and glutamine (2mM/ml) (Grand Island Biological Company, Grand Island, New York). Cultures were grown in 25 cm^2 Falcon flasks (Bioquest, Los Angeles, Calif.). The media was changed once a week. The pH was stabilized at 7.2 by gassing with 90% air:10% carbon dioxide when necessary.

After approximately 4 weeks enough fibroblasts had migrated out of the explants to start propagating the culture. The cells were dispersed with 0.25% trypsin (Grand Island Biological Co.). Subsequently, when a confluent monolayer was reached, the cells were split 1:3, each split representing 1 trypsinization number.

The quantity of tissue used in each experiment was determined by DNA measurements as described by Tedesco and Mellman (1967). After the addition of 5% perchloric acid, the samples were incubated at 70°C for 20 minutes. Diphenylamine reagent (1.5gm. in 100ml glacial acetic acid, 1.5 ml concentrated sulfuric acid, 0.5 ml aqueous acetaldehyde) was added and the tubes incubated for 20 hours at 30°C. The tubes were read at 600 μm in a Beckman spectrophotometer (Beckman Instruments, Inc., Palo Alto, Calif.).

II. Metabolic Studies

Lipolytic activity:

Lipolytic activity was measured in isolated rat adipocytes by pipetting adipocytes into siliconized 25ml Erlenmeyer flasks containing 2ml Krebs Ringer bicarbonate buffer with 0.5 mg/ml glucose and 5% fat free albumin (Armour fraction V, Chicago, Ill.). Each flask was sealed by a rubber cap, equilibrated at pH 7.4 by gassing with 95% O_2: 5% CO_2 for 5 minutes, and incubated for 2 hours at 37°C with gentle shaking. All incubations were done in triplicate. The amount of glycerol released into the medium was determined by the enzymatic method of Wieland (1957). From each sample a duplicate glycerol determination was made.

For the above incubations the flasks were divided into at least three (3) groups: those containing 5 mM cAMP (Sigma, St. Louis, Missouri); 1 mM DBcAMP (Sigma); and no additional compounds. In some experiments the cells were also tested with glucagon free insulin (Eli Lilly, Indianapolis, Ind.) 500 μU/ml, and rat growth hormone (1 μg/ml) (Sigma).

The glycerol reaction mixture contained adenosine triphosphate, nicotinamide adenine dinucleotide (NAD) (Sigma), glycerol dehyrogenase and glycerokinase (Boehringer Mannheim, N.Y., N.Y.) in hydrazine hydrate buffer. After 40 minutes the conversion of NAD to nicotinamide adenine dinucleotide reduced (NADH) was measured in a Gilford micro-spectrometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at 342 μm.

$^{14}CO_2$ Production:

$^{14}CO_2$ production was measured in 25 ml siliconized Erlenmeyer flasks containing 2 ml of buffer with 0.5 μC/ml glucose-1-^{14}C (Schwarz Mann, Orangeburg, N.Y.). The flasks were sealed by rubber stoppers from which plastic wells were suspended and the contents were equilibrated at pH 7.4 by gassing with 95% O_2: 5% CO_2 for 5 minutes. They were incubated at 37°C with gentle shaking. At the end of the incubation period, 0.25ml hydroxyl hyamine (New England Nuclear Corp., Boston, Mass.) was injected into the well and 1 ml 6N H_2SO_4 added to the medium. $^{14}CO_2$ was captured by the hydroxyl hyamine during an additional hour of shaking and was counted in a Packard TriCarb Scintillation Counter (Hewlett-Packard Corp., Avondale, Pa.) at 80% efficiency in a liquofluor (New England Nuclear Corp., Boston, Mass.) -toluene mixture of 1:20. Corrections for background and isotope impurities were made by incubating flasks with medium without tissue. All incubations were done in triplicate. The same additional compounds were added as in the glycerol incubations.

The rat adipocytes and human adipose tissue was incubated in Krebs Ringer bicarbonate buffer with 1 mg/ml glucose and 2 mg/ml gelatin. An equal volume of rat adipocytes was pipetted into each

flask and the flasks were incubated for 2 hours. The amount of lipid added to each flask was calculated from a given volume of cells extracted in Folch solution. The sample of minced human tissue was approximately equally divided among the flasks. The flasks were incubated for 4 hours. The tissue was caught in small Nitex filters and the lipid was extracted in M solution (20 ml of 4 isopropanol: 1 heptane: 0.1 1N sulfuric acid). To determine the amount of ^{14}C incorporated into triglyceride an aliquot of the rectified heptane layer was counted in liquorfluor-toluene solution in the Packard scintillation counter.

The fibroblasts were prepared for this experiment by incubating them overnight in Minimum Essential Medium (MEM) for suspension cultures (Grand Island Biological Co.) with glutamine (2 mM) penicillin (100 units/ml) and streptomycin (100 mg/ml). The cells were scraped from the sides of the flasks with a rubber policeman and washed with MEM for monolayer cultures (Grand Island Biological Co.). The cells from several flasks were combined. The fibroblasts were incubated in MEM for monolayer cultures for 24 or 48 hours.

Eluates:

$^{14}CO_2$ production from glucose-1-^{14}C in fat cells was measured following the above procedure but in an altered incubation medium. Two days before the experiment, the McCoy's medium was removed from the fibroblast cultures which were in monolayer and fresh medium, later used for the experiment, was added. Simultaneously, a flask with just medium was also incubated for 2 days at 37°C, under an atmosphere of 90% air:10% CO_2. The fat cell incubation medium was composed of incubated McCoy's medium and Krebs Ringer bicarbonate buffer (2:5).

The effect of the McCoy's medium from each cell strain on fat cell CO_2 production was studied with and without glucagon-free insulin (1000 μU/ml).

III. Cyclic AMP studies

cAMP content:

The basal cAMP content of fat cells and fibroblasts was measured. This level was compared with the amount of cAMP present after the cells had been stimulated with epinephrine (1 μg/ml) (Parke Davis, Detroit, Mich.) or with a combination of glucagon-free insulin (50 μU/ml) and epinephrine (1 μg/ml).

Measurement of cAMP content of human skin fibroblasts was performed in 25 cm^2 Falcon flasks. Flasks of the same trypsinization number were chosen and were trypsinized three days before the experiment. The night before the experiment those flasks which contained the fibroblasts in monolayer, or nearly in monolayer were refed with 15% McCoy's medium. Cells from each flask represented a single cAMP determination.

The cAMP content of human skin fibroblasts was determined after the cells had been washed free of fetal calf serum and antibiotics by rinsing them three times with McCoy's 5A medium with no additives. Cells were incubated in 3 ml McCoy's 5A medium with or without hormones at 37°C for 5 minutes after being equilibrated at pH 7.2 by gassing with 90% air:10% CO_2 for 30 seconds. One (1) ml 6% cold trichloroacetic acid (TCA) was added, and the cells were scraped from the flasks and transferred to test tubes.

Isolated rat fat cell cAMP content was determined by adding 0.03 ml of cells to 0.5 ml of Krebs Ringer bicarbonate buffer with 5% fat free albumin with or without added hormones. The cells were

incubated in a metabolic shaker with gentle shaking at 37°C for 5 minutes and equilibrated at pH 7.4 by gassing with 95% O_2:5% CO_2 for 30 seconds. The reaction was stopped by adding 0.1 ml ice-cold 50% TCA. The cells in TCA solution were sonicated at high speed for 30 seconds (Sonifer, Brinkman, Westbury, N.Y.) at 4°C and centrifuged at 17,000 rpm for 20 minutes in a Superspeed refrigerated centrifuge (Sorval, Norwalk, Conn.). The supernate was removed and extracted 3 times with 5 ml water saturated petroleum ether. The aqueous fraction was heated at 70°C for 3 minutes and lyophilized (Virtis, Gardner, N.Y.). The pellet from the fibroblasts was kept for DNA measurements; the pellet from the fat cells was discarded and fat cell number was determined from an aliquot of fat cells.

cAMP was measured by the radioimmunoassay of Steiner et al. (1969, 1972). The reagents, rabbit anti-cAMP antibody, succinyl cAMP-methyl tyrosine ester I^{125} (ScAMP-MTE I^{125}), and sheep anti-rabbit-immunoglobulin G (IgG) were purchased from Collaborative Research Inc. (Waltham, Mass.) and from Schwarz Mann.

The lyophilized samples were diluted with cold 0.05 M sodium acetate buffer, pH 6.4 so that their cAMP concentration would be in the range measured by the radioimmunoassay. 0.3 ml of the cAMP unknown was first pipetted into the test tube. 0.1 ml rabbit anti-cAMP antibody in 0.05 M sodium acetate buffer, pH 6.4, and 0.1 ml ScAMP-MTE I^{125} were added. The solution of ScAMP-MTE I^{125} from Schwarz Mann contained sheep anti-rabbit IgG. These tubes were left to incubate overnight at 4°C. With reagents from Collaborative Research, sheep anti-rabbit IgG and normal rabbit serum (Grand Island Biological Co.) were added after the samples had been incubating for 3 hours at

$4^{o}C$. The samples were incubated overnight.

To obtain the precipitated bound ScAMP-MTE I^{125}, 2.5 ml 60% saturated ammonium sulfate was added to each tube. The tubes were centrifuged at 5000 rpm in a Superspeed refrigerated centrifuge at $4^{o}C$ for 30 minutes. The supernate was discarded. In a few experiments, the precipitates were separated by 45 μ millipore filters (Millipore, Bedford, Mass.). The precipitates were counted in a Nuclear Chicago gamma spectrophotometer (G.D. Searle and Co., Des Plaines, Ill.). All incubations and cAMP determinations were done in triplicate.

Adenyl cyclase:

Adenyl cyclase activity was measured in fibroblasts that had been homogenized in 50 mM Tris-HCl buffer (Sigma) pH 7.6 at $4^{o}C$ and centrifuged at 600 x g for 10 minutes at $4^{o}C$. This procedure was repeated three times. The membranes were incubated at $30^{o}C$ in a reaction mixture containing 50 mM Tris HCl, pH 7.6, 4 mM ATP (Sigma), 3.0 mM $MgCl_2$, 10 mM theophylline, 10 μg pyruvate kinase (Sigma) and 2.6 mM phosphoenolpyruvic acid (Sigma) for 15 minutes. The samples were boiled for 3 minutes and centrifuged at 2500 x g for 10 minutes. The supernate was removed for assay and was diluted with 250 μl sodium acetate buffer pH 6.4. The samples were divided into those containing 1 μg/ml epinephrine; 1 μg/ml epinephrine and 50 μU insulin; and 10 mM NaF. Corrections for cAMP present initially were made by adding boiled tissue to the incubation medium, incubating for 15 minutes, and adding enzyme to the incubation medium but not incubating it. The cAMP formed was measured with the radioimmunoassay for cAMP as described above.

RESULTS

I. Fat Cell Studies

Glycerol release with cAMP and DBcAMP in fat cells of varying sizes:

Rat weights and fat cell sizes used are shown in Table 1. As reported by others, the addition of cAMP produced little stimulation above basal lipolysis. DBcAMP, however, caused significant increases in glycerol release. These effects were observed in cells of different sizes (Table 2). Cell size appeared to be a determining factor in the magnitude of the lipolytic response of the cell. Cell size and basal glycerol release were positively correlated ($r = 0.7867$). Since cAMP in itself did not markedly change lipolysis, increases in activity of cAMP stimulated cells were simply reflections of increases in basal lipolytic activity. DBcAMP stimulated activity was much higher than basal; thus lipolytic activity can change independently of basal levels. In the smaller cells DBcAMP stimulated activity was high and it didnot increase significantly with size change (Graph 1). Consequently, as cell size increased the per cent change decreased, indicating that there is a limit to the amount of stimulation possible. Per cent change due to the addition of cAMP was low and did not change significantly (Graph 2).

CO_2 production in rat adipocytes of varying sizes:

Fat cell size plays a role in CO_2 production. The cell sizes tested ranged from 0.02 to 0.7171 µg lipid/cell (Table 3). Basal $^{14}CO_2$ production was not affected by cell size (Table 4). However, stimulation due to the addition of cAMP decreased as cell size increased. Inhibition which was observed in the response to DBcAMP

decreased as cell size increased, this substance becoming slightly stimulatory in the larger cells (Graph 3). Indeed, in the largest cells the effects produced by cAMP and DBcAMP were about equal. In the smaller cells per cent change was negative for DBcAMP and became positive with increasing cell size. In the largest cells cAMP had little effect and this caused per cent change to decrease with changes in cell size (Graph 4).

Cell size may be altered by chronically injecting animals with certain compounds; such as, monosodium glutamate (MSG), growth hormone (GH) and MSG-GH. Rats injected with these substances were used in a series of experiments. These chemicals seemed to produce increased fat cell sizes (Table 5). If the compounds basically affect the cell by speeding up the processes which normally increase cell size, then the cell's responses to exogenous cAMP and hormones should parallel those for normally occurring large cells. Chronic injections of rat GH may change the cell's responses and its adenyl cyclase system.

The injected rats had above normal fat cell sizes. The largest cells were found in the MSG-GH male injected animals. When the results for glycerol release were compared with the results of the preceding glycerol experiment, it was seen that most of the results were within acceptable per cent confidence limits for their fat cell sizes (Table 6). The only exception was the growth hormone injected males. Thus, the injected chemicals did not appear to significantly affect the fat cells' basal response or their responses to cAMP and DBcAMP. The effects of growth hormone and insulin on lipolysis were small (both negative and positive) and no significant differences could be detected among the groups.

In general $^{14}CO_2$ production in the injected rats was quantitatively low (Table 7). The per cent change over basal when cAMP, DBcAMP, insulin or GH was added to the medium was also low. Although insulin is known to stimulate CO_2 production, only in the MSG injected males and the GH injected males was the per cent stimulation significant. Many of these results demonstrating low response may be attributable to the extremely large fat cell size of the animals. When compared with the preceding experiment on $^{14}CO_2$ production, some of the results were below the acceptable per cent confidence limits for that cell size. Fat cells from males and females that received the same treatment did not always produce the same CO_2 response. Comparison of the results from the $^{14}CO_2$ production and glycerol release experiments suggest that the chronic injections had a greater effect on fat cell $^{14}CO_2$ production.

The entire series of injections and in vitro incubations must be repeated a number of times before any definite conclusions can be made as to the effects of chronic injections of MSG, GH and MSG-GH on in vitro studies with cAMP, DBcAMP, insulin and GH.

CO_2 production in human fat cells:

In a manner similar to rat fat cells there was no correlation between fat cell size and $^{14}CO_2$ production from glucose-1-^{14}C (Table 8, Graph 5). This relationship held true when all patients were considered as one group and when they were divided into their respective groups: normal, GH deficient and obese (Table 9). Unlike rat fat cells, there was no significant correlation between cell size and $^{14}CO_2$ production when the cells were stimulated by cAMP or DBcAMP.

However, if the responses from the fat from GH deficient children were analyzed separately there was a significant positive correlation of cAMP stimulated $^{14}CO_2$ production with fat cell size. If per cent change due to cAMP or DBcAMP over basal is calculated, the only correlation with cell size was for cAMP stimulation of GH deficient fat cells (Table 10, Graph 6).

The fat cell sizes of the patients from which fat was sampled form a continuous spectrum of cell sizes. However, on the basis of clinical diagnosis they were easily separable into three groups. The fat cell sizes of the growth hormone deficient and the obese patients were significantly different from the normals (Table 11). There were no statistically significant differences between their basal $^{14}CO_2$ production or their cAMP or DBcAMP stimulated responses, either absolute or per cent change.

With ^{14}C-glucose present in the medium in which fat cells are incubating, the incorporation of ^{14}C into triglycerides can also be measured (Table 12). Fat cell size had no significant effect upon basal or DBcAMP stimulated incorporation, either absolute values or per cent change (Graph 7, 8). When the cell sizes are treated as one distribution cAMP stimulation was positively correlated with increasing cell size ($r = 0.5316$). However, when the cells were considered in groups, only the growth hormone deficient cells showed a significant correlation ($r = 0.9225$), although the correlation coefficient was also high for the obese cells ($r = 0.7141$) (Table 13). No significant relationship existed between the per cent stimulation due to cAMP and cell size.

Although there were no significant differences between the per cent changes attributed to cAMP or DBcAMP when normal cells are compared to growth hormone deficient cells, there were significant differences between the two groups for basal incorporation and cAMP and DBcAMP stimulated activity (Table 14). This may indicate that the higher cAMP and DBcAMP levels are more a function of the basal levels than of the increased sensitivity to cAMP and DBcAMP, since both groups were stimulated to approximately the same degree.

The differences that were evident between the normal and obese groups were basal and cAMP stimulated ^{14}C incorporation. Again there was no difference in the per cent stimulation due to cAMP.

cAMP levels in rat fat cells:

The cAMP levels in fat cells of various sizes are shown in Table 15. As demonstrated by regression equation analyses, there were positive correlations between cell size and basal cAMP levels (r = 0.9051), epinephrine (r = 0.7125) and epinephrine-insulin (r = 0.922) treated levels (Graph 9) with larger fat cells containing more cAMP. In the range of cell size investigated there does not appear to be a limit to the amount of cAMP contained. Per cent change produced by epinephrine and epinephrine-insulin as compared to basal levels showed no correlation with cell size. Epinephrine-insulin stimulation compared to epinephrine alone also showed no correlation (Graph 10).

II. Fibroblast studies

CO_2 production in fibroblasts:

$^{14}CO_2$ production from glucose-1-$^{14}CO_2$ by fibroblasts was stimulated by insulin. Insulin tended to stimulate the cells, except in one

experiment where it inhibited $^{14}CO_2$ production for all strains tested (Table 16). The insulin may have been old or the solution incorrectly made. Not enough strains of each type were tested to make any valid comparisons among normals, diabetics, and GH deficient cells, and the variation in per cent stimulation was very large.

Since cAMP is a labile compound at 37°C, and since rapid changes in intracellular cAMP levels produce large changes in other parameters, attempts were made to localize the time of its action. After incubating the cells for 24 hours, cAMP was added and the cells were further incubated for 4 hours. This produced slight inhibition of CO_2 production. When the cells were incubated for 24 hours with cAMP present all the time, the results were inconsistent. Cells incubated with cAMP for 48 hours usually showed a stimulation of CO_2 production, but the per cent variation was large. It appears that cAMP may inhibit $^{14}CO_2$ production during the initial periods, only later becoming stimulatory. Also, in the four hour incubations the cells may not have had sufficient time to recover from the initial shock of the addition of the cAMP or DBcAMP. At 48 hours the responses to DBcAMP were very similar to those produced by cAMP, being inconsistent at 24 hours, but stimulatory at 48 hours. However, there seemed to be no definite relationship between the per cent stimulation produced by the two compounds.

cAMP levels in human cultured fibroblasts:

Intracellular cAMP content was measured in cultured human skin fibroblasts whose donors had been normal, diabetic or GH deficient. In each experiment at least one normal strain was compared to at least one abnormal strain.

All cell strains were sensitive to epinephrine stimulation (Table 17). The basal cAMP levels of the three groups were not significantly different, and epinephrine stimulation did not produce any significant differences between the groups (Table 19). The per cent change in the presence of epinephrine also was not significantly different (Graph 11).

Most cell strains were at least slightly sensitive to insulin depression of epinephrine stimulated cAMP levels. In normals, basal and epinephrine-insulin treated levels of cAMP were not significantly different (Table 18). But, insulin had lower or no effects on these levels in diabetic and GH deficient cells (Table 17). The analyses of variance have been calculated with and without cell strain #606 because in these experiments #606 appeared to behave differently in comparison to the other normals. When #606 was included with the normals there were no statistically significant differences between normals and diabetics, or normals and growth hormone deficient cells for their response to epinephrine-insulin, either in absolute values or per cent change over basal (Table 19). However, if #606 is not included there were significant differences between normals and diabetics and normals and growth hormone deficient. The per cent change produced by epinephrine-insulin over epinephrine was significantly different from the normals in diabetics and growth hormone deficient cells, even when #606 was included. There was no overlap among the normals and the other two groups with the exception of #606. Insulin alone was not tested because others have demonstrated the need for adenyl cyclase stimulation to show inhibitory effects.

Adenylate cyclase in fibroblasts:

Epinephrine and NaF stimulated adenyl cyclase levels in all cell strains (Table 20). NaF produced extremely high stimulation. However, insulin treatment depressed epinephrine stimulated cAMP levels below basal levels in most strains, but two cell strains (45T8 and 679T9) had epinephrine-insulin levels which were insignificantly above basal levels. The per cent change produced by the presence of insulin with epinephrine compared with epinephrine alone was always between 46 and 96 per cent (Graph 12).

In normal cell strains there were statistically significant differences between basal and epinephrine, and basal and NaF stimulated cAMP levels, but no difference between basal and epinephrine-insulin treated levels (Table 21). To parallel the procedure followed for cAMP levels, the calculations were done with and without #606. In these cases omission of #606 produced no differences.

When the three groups of cultured strains were compared, no significant differences were seen among them for basal activity, epinephrine stimulated activity or epinephrine-insulin treated activity (Table 22). Similarly, there were no statistically significant differences between the strains for per cent changes of epinephrine or epinephrine-insulin over basal levels, or of epinephrine-insulin over epinephrine. Omission of #606 from the normal group still did not result in significant differences.

There was no significant difference between normal and diabetic strains for NaF stimulated levels or for per cent change of NaF over basal levels. However, a significant difference existed between normal and growth hormone deficient cells for per cent change of NaF over

basal levels and a suggestive difference ($P<0.10$) between NaF stimulated and basal levels.

Influence of fibroblast media on $^{14}CO_2$ production in rat adiopocytes:

Incubation of isolated rat adipocytes in McCoy's medium diminished the stimulation of $^{14}CO_2$ production normally produced by insulin. The greatest inhibition was observed with McCoy's medium which had been maintained at 37°C for 2 days without cells (Table 23). Incubation with fibroblasts altered the medium in some manner so that it was less inhibitory. There were no significant differences between the cell free media from normal and growth hormone deficient cells. Unfortunately, on different days $^{14}CO_2$ production with McCoy's medium incubated without cells varied, making it difficult to make valid comparisons among strains tested at different times.

DISCUSSION

I. Metabolic studies

For metabolic studies isolated fat cells are preferred to whole tissue, due to their homogeneity. The most commonly used method designed to free cells is trypsinization. However, tryspin has adverse effects on fat cells, decreasing their glucose metabolism and causing insensitivity to the antilipolytic action and stimulatory effect on glucose oxidation of insulin (Fain and Loken, 1969). The collagenase technique (Rodbell, 1964) is believed to leave all the metabolic properties intact and is the most widely utilized in fat cells. This was the method applied in my studies. Recently, McKeel and Jarett (1970) have proposed another method for isolating cells.

One of the first activities attributed to cAMP mediation was lipolysis. It was observed that agents which raised intracellular cAMP levels increased lipolytic activity and those that lowered intracellular cAMP, lowered lipolytic activity. Based on the assumption that cAMP was causing these changes, either directly or indirectly, the addition of exogenous cAMP to the incubation medium should produce a similar effect to that produced by an agent which raised intracellular cAMP levels. Many investigators have found that cAMP was ineffective, or was required in very high concentrations to elicit a response. Therefore, they used its dibutyryl analogue, DBcAMP. In order to compare DBcAMP and cAMP, both compounds were used in all experiments performed here.

Lipolysis:

All four of Sutherland's criteria (Sutherland et al,, 1968) have

been applied to lipolysis and have been met. In rat fat cells, epinephrine, norepinephrine, glucagon, adrenocorticotrophin (ACTH), thyroid stimulating hormone, and DBcAMP are lipolytic. However, insulin, the prostaglandins (PGE) and growth hormone (GH) are antilipolytic (Appelman and Sevilla, 1970). Theophylline and caffeine, inhibitors of phosphodiesterase (PDE) act synergistically with the lipolytic compounds. Nicotinic acid, a stimulator of PDE, inhibits lipolytic action and beta adrenergic blocking agents which block the formation of cAMP also inhibit lipolytic activity (Robison et al., 1968).

Glycerol release is a saturable process. Changes in cAMP level above 50% stimulation (with epinephrine) do not cause any further increases in free fatty acid release (Butcher, 1966). There is a correlation between cAMP levels and lipolysis when intracellular cAMP concentration is between 180-300 picomoles/gm wet weight. Lipolytic changes due to epinephrine stimulation are seen within five minutes, but significant alterations in cAMP levels are seen within 30 seconds (Butcher et al., 1968[a]). This delayed response is probably due to the chain of events which leads to the activation of triglyceride lipase. A cascade mechanism involving protein kinase, glycogen phosphorylase and glycogen synthetase is probably set into motion (Appelman and Sevilla, 1970). Beside increased levels of cAMP, this reaction requires adenosine triphosphate (ATP) and magnesium ions (Mg^{+2}) (Robison et al., 1971).

The adenyl cyclase system and lipolysis are extremely sensitive to alteration in pH. This may be related to changes in the biological effect of drugs or to the ionization of the receptor site at different

pH. At low pH, less free fatty acids dissociate, causing them to accumulate in the cell, resulting in decreased lipolysis (Nahas et al., 1971).

Numerous investigators (Blecher et al., 1968) have found no lipolytic effect with cAMP, and thus confined most of their studies to the lipolytic effect of DBcAMP. One reason may have been their choice of buffer. The ionic composition of the medium is very critical for the cells' response. Mosinger and Vaughan (1967) demonstrated that cAMP stimulated lipolysis in phosphate-saline (with sodium) buffer, but was inhibitory when basal lipolysis was high in phosphate-saline (with potassium) buffer. In Krebs-Ringer phosphate buffer cAMP had no effect.

Another explanation may be the concentration of cAMP added. With 10^{-3}M cAMP, the lipolytic response with theophylline was not increased. However, when 5×10^{-3}M or 1×10^{-2}M was used, this response was greatly stimulated (Goodman, 1969). Theophylline may decrease the breakdown of the exogenous cAMP. My studies, using 5×10^{-3}M cAMP showed a small stimulatory lipolytic effect of cAMP in the same buffer used by Goodman, but without the added theophylline. Changing the buffer from Krebs-Ringer bicarbonate to phosphate-saline (with sodium) may have increased the response (Mosinger and Vaughan, 1967). Also, in that buffer the effects of cAMP and DBcAMP are qualitatively and quantitatively similar (Braun et al., 1969). Even in Krebs-Ringer bicarbonate buffer in my studies the DBcAMP response is highly significant and shows the rate limiting effect on lipolysis.

Since DBcAMP stimulation is so great, alterations of this stimulatory activity by other compounds can be used as an indication of their effects on the adenyl cyclase system. Insulin alone is without effect

on DBcAMP lipolysis. However, it reverses the synergistic effect of theophylline or caffeine with low levels of DBcAMP. Nicotinic acid inhibitied DBcAMP stimulated lipolysis (Blecher et al., 1968). Unlike insulin, tolbutamide and phenformin, which non-competitively inhibit lipolysis are able to inhibit stimulation produced by all concentrations of DBcAMP and theophylline (Brown et al., 1969). Amitriptyline, another antilipolytic agent, inhibits lipolysis stimulated by DBcAMP and theophylline (Lovrien et al., 1972).

DBcAMP as a substitute for cAMP:

To strengthen arguments for the substitution of DBcAMP for cAMP, studies on its physiological and chemical properties have been made. Blecher (1971) found that PDE does not attack DBcAMP because of the steric hinderance of the substituent groups on the N^6 and $O^{2'}$ positions. During metabolic studies in adipocytes, these groups are not removed enzymatically, thus allowing the entry of DBcAMP into the cells. The non-enzymatic formation of butyric acid amounted to not more than 4% over 2 hours of incubation. Other investigators, however, have urged caution in the use of DBcAMP. Commercial preparations of DBcAMP are not pure, containing monobutyryl cAMP (MBcAMP) and butyric acid and cAMP. This required that the compound be purified before use (Johnson et al., 1971[a]). However, even when the compound is purified, a 3 hour incubation in Krebs-Ringer bicarbonate buffer will cause up to 60% of DBcAMP to decompose to MBcAMP and other products. When DBcAMP is stored dry or in ethanol for 4 months, no changes are noticed by chromatography. If stored as an aqueous solution DBcAMP decomposed at -15^{o} to $+5^{o}$ C (Swislocki, 1970). After storage and use of an opened

bottle of dry DBcAMP for more than 6 months, I noted changes in biological activity as compared to a new unopened bottle. The odor of butyric acid was also apparent. In addition, in a number of cases DBcAMP and cAMP have demonstrated opposite effects in skeletal muscle, isolated fat cells, HeLa cells, melanophores and intestinal smooth muscle (Robison, et al., 1971). The problem of decomposition may have resulted in incubation with compounds other than DBcAMP which may themselves have been responsible for the effects attributed to DBcAMP. Recently, investigators have run parallel experiments with the decomposition products. This must cast some doubt on the validity of many previous studies using DBcAMP as a substitute for cAMP. Since the DBcAMP available commercially is not 100% pure, at no time do I attribute actions to cAMP solely on the basis of results observed with DBcAMP. However, I continued to study DBcAMP to compare my results with previous reports.

Effects of cell size:

In my studies, basal glycerol and cAMP stimulated release in rat fat cells was positively correlated with increasing cell size. However, the response to DBcAMP is unaffected by cell size. This may illustrate the maximal response of the lipolytic system and thus its saturation point. If maximally stimulated by cAMP or a derivative, the lipase system is unaffected by cell size. When the system is unsaturated, cell size plays an important role in lipolysis and lipase activity (Netel et al., 1969). In rat cells enlarged by high fat, low carbohydrate diet, there is a decrease in epinephrine and glucagon stimulated lipolysis calculated by tissue weights (Gorman et al., 1972). Norepinephrine stimulation on a

per cell basis, like DBcAMP stimulation is unaffected by changes in cell size (Hartman et al., 1971).

$^{14}CO_2$ production:

Unlike lipolysis, basal $^{14}CO_2$ production is unrelated to cell size. However, stimulation by insulin is negatively correlated with increasing cell size (Salans and Dougherty, 1971). This same pattern was observed in my studies of basal and cAMP stimulation. The similarity between the responses to insulin and cAMP was not expected because insulin action is believed to be mediated by decreased cAMP levels. The cyclic nucleotides of cytidine, inosine, thymidine and uridine also stimulate $^{14}CO_2$ production. However, DBcAMP and cyclic guanosine 3'5' nucleotide and caffeine and theophylline inhibit $^{14}CO_2$ production (Kitabchi et al., 1970; Bray, 1967). The divergent effects of cAMP and DBcAMP become greater with increasing concentrations (Solomon et al., 1970). This divergent effect was observed only when both butyryl groups were present and when glucose-1-^{14}C or glucose-U-^{14}C was used. DBcAMP was stimulatory with glucose-6-^{14}C (Solomon and Kitabchi, 1972). At constant concentrations, I found that the effects of cAMP and DBcAMP on $^{14}CO_2$ production from glucose-1-^{14}C became more similar as fat cell size increased. At the present time, it is difficult to explain the inconsistency between the two actions of cAMP and insulin, unless cAMP is being altered in some manner or cAMP is not involved in glucose oxidation. The divergence of cAMP and DBcAMP may reflect differences in their structure or action.

Effects of GH and/or MSG injections:

Insulin exerts its action by decreasing cAMP levels in cells by an unknown mechanism. GH has many insulin-like properties. Incubation

with it enhances submaximal lipolytic activity stimulated by catecholamines and theophylline. Stimulation by DBcAMP is unaffected, suggesting that GH stimulated the synthesis of a protein involved in the formation but not in the action of cAMP (Fain, 1968). The different response may be explained by GH's biphasic effects. There is an initial inhibition of lipolysis followed by an acceleration of the lipolytic response to epinephrine in the presence of glucocorticoids or of theophylline. Its effects on glucose metabolism are also biphasic with early acceleration followed later by inhibition (Goodman, 1970). The results from my study demonstrated antilipolytic or insignificantly lipolytic effect on cells from GH and/or MSG injected animals. In all groups the insulin response is very similar to the GH effects, thus agreeing with the insulin-like properties of GH's first phase of action. However, GH effect on $^{14}CO_2$ production is slightly negative or positive, not following a definite pattern. If a parallel is drawn with insulin, then larger cells would not be expected to show a significant response. Insulin response in these cells was generally poor and may have been affected by the previous injections.

In general my studies indicate that except for the significant increase in fat cell size, the GH and/or MSG injected rats' fat did not differ significantly in its lipolytic response from what would be expected for cells of equal size. It is possible that the cells were so large that any differences were obscured by cell size. $^{14}CO_2$ production, however, is more affected. The males who had smaller cells, are less responsive than expected for their cell size. Since they were injected with GH, which is sometimes diabetogenic, their lack of

response may be correlated with the poor responsiveness to insulin of human diabetics. The female's extremely large cell sizes might have obscured any differences.

Comparisons between lipolysis and $^{14}CO_2$ production:

Although two metabolic parameters may be mediated by cAMP, the effects of a particular substance can be very different on each parameter. PGE and nicotinic acid, which are antilipolytic with epinephrine, have no effect on its stimulation of glucose oxidation. Both effects of epinephrine seem to involve beta adrenergic receptors, as beta blockers inhibit both actions. In contrast, beta blockers interfered only with the lipolytic activity of ACTH and glucagon. The effects of these hormones on glucose utilization are not accompanied by an increase in production and release of free fatty acids. These two metabolic parameters operate independently. Glucose oxidation may involve a transport mechanism, rather than cAMP (Blecher et al., 1969). This may explain a lack of correlation between lipolytic activity and glucose oxidation stimulated by cAMP and DBcAMP.

Human adipose tissue compared with rat adipose tissue:

In general most fat cell studies were performed on rats for convenience, and their results extrapolated to possible mechanisms of action in man. The rat may not be the best model. Unlike the rat, human adipose tissue has a poor capacity to synthesize fatty acids de novo. The citrate cleavage enzyme is missing and many other enzymes are in low concentration. In humans the rate of incorporation of labeled citrate into fatty acids is much lower than in rats. The majority of the carbons found in human lipids are metabolized through alpha-glycerol phosphate, so that most of the labels appear in the

glycerol molecule. Only 0.6% glucose-^{14}C is recovered as fatty acids. The rest is incorporated into glyceride-glycerol (Galton, 1968). The glycerol moiety in older and diabetic rats also contains most of the label (Shrago et al., 1971). Thus, a good model for human fat tissue would be older larger rats, as were used in my studies. Another animal which would provide a good comparison is the dog (Carlson et al., 1970).

Perhaps the most basic difference between human and rat adipose cells is that human cells have both alpha and beta adrenergic receptors while rat cells have only beta receptors (Robison et al., 1972). Glycerol release in rats is sensitive to glucagon, GH and ACTH. Human cells are not sensitive to those three hormones. Both adipose cells are similarly affected by the combinations of norepinephrine and PGE, DBcAMP and theophylline (James and Burns, 1969), and insulin and theophylline. Insulin increased glucose oxidation and incorporation of glucose into glycerol in both human and rat cells, but the insulin responsiveness was much lower in humans than in rats (Kahlenberg et al., 1966). This may be a function of cell size because as rat weight increases, the responsiveness decreases. In man, age also seemed to influence the response. In rats, isolated adipocytes demonstrate higher metabolic activity than whole tissue both basally and with insulin. Tissue segments are more active than isolated cells in man (Gries and Steinke, 1967). The decreased responsiveness may be due to increased cell breakage during isolation. The quantity of tissue required to perform the experiments is much greater than can be obtained by needle biopsy. Most published reports relied on surgical specimens which may raise the question of the effect of the anesthesia

on the cells' responses. Human cell responses are more variable than rat responses. In my studies due to the size of the specimens, segments of tissue were used.

Lipolysis in human adipose tissue:

As the majority of studies with human adipose tissue are concerned with lipolytic activity some comments must be included. The anti-lipolytic effect of insulin is slight with no effect on epinephrine lipolytic activity. Norepinephrine is a strong lipolytic agent whose action can be potentiated by theophylline (Galton and Bray, 1967; Possa, 1970). Epinephrine at 10^{-3} µg/ml produces a response which is maximal at 1 µg/ml. Lipolytic stimulation by DEAE II, a lipolytic peptide derived from human pituitary powder, can be reduced by insulin (Burns and Langley, 1968[a]). Similar to rat cells, human cells responded to DBcAMP, but not to cAMP (Burns and Langley, 1968[b]). Lipolytic stimulation by DBcAMP can be potentiated by theophylline and insulin can inhibit this increase. cAMP and theophylline produce a lower response than theophylline alone. Neither phentolamine (an alpha adrenergic blocking agent) nor propranolol (a beta adrenergic blocking agent) influence the effect of DBcAMP plus theophylline. Phentolamine increases the effect of epinephrine, but has no effect in rats. Propranolol causes epinephrine stimulated lipolysis to fall below basal values (Burns and Langley, 1970[a]). Alterations in epinephrine stimulated lipolysis are paralleled by changes in cAMP levels (Burns and Langley, 1970[b]). PGE interferes with norepinephrine plus theophylline stimulation, but not with DBcAMP stimulation (Moskowitz and Fain, 1969; Efendić, 1970[a]).

Formation of cAMP is the rate limiting factor in the lipolytic response. cAMP must be stimulating a lipase (Ostman et al., 1969). Once stimulated, glycerol release continues at an increased rate for at least four hours (Robison et al., 1972). The increased concentration of cAMP in the buffer can be detected during the first hour (Burns et al., 1971).

$^{14}CO_2$ production:

Like rat cells, human adipose cells are sensitive to insulin stimulation of $^{14}CO_2$ production. The magnitude of the response is dependent on the glucose concentration present (Goldrick, 1967). In general, the human cells tested in my studies were less responsive to cAMP and DBcAMP than rat cells, thus paralleling the pattern of response to insulin.

Cell size also probably plays a determining role in a human cell's response. Larger cells from the same specimen have a greater rate of lipid synthesis and are less sensitive to the stimulating effects of insulin (Smith, 1971). In some studies subcutaneous and omental fat from the same patient were compared because subcutaneous fat cells are larger than omental cells. Again the larger cells had a higher rate of basal lipolysis, but the insulin and theophylline stimulated lipolysis does not differ (Goldrick and McLoughlin, 1970). There is no difference in DBcAMP stimulated activity, suggesting the maximal rate may have been achieved as observed in rat cells (Efendić, 1970[b]). However, PGE inhibits basal lipolysis more in subcutaneous cells (Carlson and Hallberg, 1968). Norepinephrine, epinephrine and isoproterenol stimulated lipolysis is greater in larger cells (Jacobsson

and Smith, 1972). Thus, depending on the individual hormone or compound, cell size may be important. The range of human cell sizes which can be studied is not as great as that in rats, and changes which would be seen over a wider range are not significant over a narrower range. This may explain the difference in human and rat correlation with cell size. In my studies there were no significant correlations between cell size and response. The human cell sizes were in a range which did not show great changes in the rats. The human cells were also in the range where there was no inhibition with DBcAMP and the amount of difference between cAMP and DBcAMP was becoming smaller.

^{14}C incorporation into triglycerides:

As mentioned, the major portion of lipid synthesis in humans is into glyceride-glycerol. The incorporation of ^{14}C into triglycerides provides a measure of this production. Although cAMP stimulated $^{14}CO_2$ production more than DBcAMP, I found that DBcAMP stimulation of ^{14}C incorporation is generally higher than cAMP stimulation. cAMP stimulated levels are positively correlated with cell size when all the results are considered as one distribution, but only the growth hormone deficient cells are significantly correlated when each group is analyzed separately. Upon separation, the other groups may not have produced a wide enough range of sizes to demonstrate a difference. Since DBcAMP stimulation is not correlated with cell size, it is suggested that DBcAMP is maximally stimulating the pathway. cAMP stimulation, although lower, would still be considered effective.

$^{14}CO_2$ production in human skin fibroblasts:

Normal serially cultured fibroblasts degrade glucose to CO_2 or lactate, with the later predominating. The metabolism of these cells will differ qualitatively and quantitatively depending on the method of cultivation, pH and species of serum used (Cristofalo and Kritchevsky, 1965). There exists a direct correlation between rates of glucose uptake and rates of proliferation and population density (Kruse and Miedema, 1965). Thus, studies of rates of glucose oxidation should be conducted under carefully controlled conditions. When glucose-1-^{14}C is added to the medium, only a small but sufficient proportion of its oxidation is represented by $^{14}CO_2$ production.

Attempting to find the basic genetic defect(s) in diabetes, Goldstein and Littlefield (1969) studied glucose oxidation in normal and diabetic fibroblasts. They were looking for the cause of decreased plating efficiency observed in prediabetic cells (Goldstein et al., 1969). Basal rates showed no differences. Insulin produced a slight but significant stimulation. Again, no differences were observed between the two cell types. It was hoped that the diabetic fibroblasts would show insulin resistance, like diabetic fat cells.

Insulin is a requirement for normal growth and metabolism of cultured cells (Schwartz and Amos, 1968). It facilitates glucose uptake and stimulates protein synthesis, DNA and RNA synthesis, glycolysis and pinocytosis in cultured cells (Griffiths, 1970). In fat cells these activities may not require insulin because in vivo conditions may be stimulating enough. In my studies, when only glucose-1-^{14}C is used, insulin significantly stimulates $^{14}CO_2$ production

during 48 hour incubations of fibroblasts from normal patients, with the exception of one experiment. It inhibits $^{14}CO_2$ production in cells from growth hormone deficient children. This may indicate insulin resistance in those cells. Goldstein and Littlefield (1969) may not have detected this difference because of their choice of buffer, position of label in the glucose molecule and use of trypsin. Also, their incubation period was much shorter.

In general, incubation with cAMP and DBcAMP produced significant stimulation of $^{14}CO_2$ production, with the stimulation due to cAMP being greater. Inherent in this experiment was the degradation of DBcAMP during the 48 hour incubation period. Shorter incubations produced more inconsistant results. The greater stimulation by cAMP might be explained by the recent findings of Ryan and Durick (1972) that cAMP may be actively transported into the cell and DBcAMP is not.

Effect of fibroblast media on fat cells:

Unfortunately, the eluate experiments did not produce the results that were hoped for. Very precise standardizing of all parts of the experiments would be required before highly reliable conclusions could be obtained. The fibroblasts should have always been fed with the same lot numbers of McCoy's medium, antibiotics and especially, fetal calf serum. These lots should be standard throughout the cell strains compared. Attempts to standardize the number of cells per flask from which the medium was taken should be made by trypsinizing the cells from different strains on the same day, keeping the number of days between trypsinization and use constant, and at trypsinization the flasks should always be inoculated with the same number of cells. For

the rat portion of the experiment, the same strain of rats, purchased from the same supplier, at the same age and in the same nutritional state should be used. These precautions may eliminate the basic dissimilarities present in the experiment.

II. cAMP system

The mechanisms of action of adenyl cyclase are only now becoming apparent. This information transfer unit is composed of a discriminator, a transducer, and an amplifier. The discriminator selects the input signals and thus imparts specificity to the system. The transducer converts signals coming from the discriminator so that they are recognized as instructions by the amplifier and accelerate its catalytic function. This system can amplify the input signal (the hormone) by a factor of 10^4 to 10^5. The amplifier is most likely Mg^{+2}-ATP and its activity is enhanced by the binding of Mg^{+2} to a site distinct from the catalytic site. Each hormone interacts with a distinct and specific discriminator that is located on the outer surface of the plasma membranes (Birnbaumer et al., 1970).

Only one adenyl cyclase molecule has been identified, and it gains its specificity by the receptor site. Receptors for the hormones and NaF are believed to bind at different points because of the differences in their characteristics of activation. Fluoride and ACTH stimulated activities are affected differently in the presence of potassium and manganous ions, and ACTH concentration curves are hyperbolic while fluoride concentration curves are sigmoid. Also, the Km for fluoride activation is much more temperature sensitive than that of ACTH (Birnbaumer et al., 1969). ATP can protect against the

effect of urea on the NaF stimulation of adenyl cyclase, but cannot prevent urea's effect on hormonal stimulation (Rodbell et al., 1970). NaF is found to stimulate all broken cell preparations without specificity, suggesting that it may act at some point beyond the discriminator.

Evidence for one adenyl cyclase is derived from the studies in which more than one hormone was added simultaneously without observing any additive responses. The specificity of the receptors can be demonstrated by the calcium requirement of ACTH, the trypsin sensitivity of the insulin, glucagon, secretin and ACTH receptors, but the resistance of epinephrine and NaF receptors (Rodbell et al., 1970). When two activators are competing for the same binding site, their effect when combined at concentrations near their Km is approximately equal to the arithmetic mean of their effects tested separately at twice that concentration (Birnbaumer and Rodbell, 1969). All activators of adenyl cyclase have no effect upon the Km of the reaction, but increase the Vmax of the enzyme (Drummond et al., 1971).

Adenyl cyclase prepared from broken cell preparations is a very labile compound. It is rapidly degraded even at $0^{o}C$. However, preparations can be stored at $-80^{o}C$ for at least two weeks and still be sensitive to epinephrine. Enzymatic activity remains constant for at least 10 minutes of incubation and is maximal at pH 8 (Vaughan and Murad, 1969).

A major concern in using any assay for cAMP or adenyl cyclase is the degradation of cAMP by PDE. PDE is inhibited by the methylxanthines, inorganic polyphosphates and nucleoside triphosphates

(Cheung, 1967). It requires Mg^{+2} and is stimulated by imidazole (Butcher and Sutherland, 1962). PDE requires a protein activator which is removed during the purification process (Cheung, 1970). Unlike adenyl cyclase, PDE has been found in multiple molecular forms. No one tissue contains more than four components (Monn and Christiansen, 1971). Most tissues contain two forms, a high molecular weight fraction in which the enzymatic affinity for cyclic guanosine 3',5'-monophosphate (cGMP) is greater than that for cAMP and a lower molecular weight fraction that has a high affinity for cAMP and is negatively cooperative (Thompson and Appleman, 1971). Further involvement of cGMP with PDE is an observed increase in the hydrolysis rate of cAMP in the presence of low concentrations of cGMP (Beavo et al., 1971). It has been suggested that a pool of unlabelled cAMP, instead of theophylline which can be inhibitory on adenyl cyclase activity, be used to protect labelled cAMP produced during an assay from destruction by PDE (Sheppard, 1970).

Of great importance in the cascade of enzymes in the adenyl cyclase system is cAMP-dependent protein kinase. In vivo, this enzyme catalyzes the phosphorylation of histone by ATP. In the presence of cAMP, its activity is stimulated twenty fold, associated with a decrease in Km of the enzyme for ATP, but with no effect on the Km for the histone (Miyamota, et al., 1969). There is evidence that a protein associated with the 80s ribosomal fraction is the substrate in vivo and that the serine and threonine residues are phosphorylated by the kinase (Walton et al., 1971). From in vivo studies in liver, kinase phosphorylates specific serine residues in the lysine rich (f 1) histone (Langan, 1969). cAMP activation of protein kinase results in

the induction of RNA and protein synthesis. This phosphorylation is not blocked by actinomycin D or cycloheximide which indicates that this reaction occurs on intact histone molecules and that it is not dependent on the synthesis of new enzyme molecules. Induction of RNA synthesis might be brought about by a change in DNA-histone interaction resulting from histone phosphorylation (Langan, 1971). Thus, a role for cAMP in regulating transcription or translation is implied. The mechanism by which hormones can control RNA and protein synthesis probably acts through the effect of hormones on adenyl cyclase (Langan, 1970).

The number of assays for cAMP and adenyl cyclase appearing in the literature seems to multiply at an incredible rate, with each new method claiming advantages over previous methods. Considerations in choosing a method must include the quantity of tissue available, the time involved, the specificity desired, the number of samples to be run, and the difficulty involved in separating the final product. The first system devised was based upon phosphorylase activation with a number of intervening enzymatic steps (Butcher et al., 1965). Other methods involve the conversion of labeled ATP to cAMP with purification by zinc barium precipitation (Krishna et al., 1968) or separation by chromotagraphy (Bar and Hechter, 1969[a]; Makman, 1970; Rao et al., 1971). Many other methods have been proposed (Breckenridge, 1964; Turtle and Kipnis, 1967; Aurbach and Houston, 1968; Brooker, 1971; Brooker et al., 1968; Breckenridge, 1971; Gilman, 1970; Kuo and Greengard, 1970; Wastila et al., 1971). My attempts to use the assays involving the conversion of ATP-αP^{32} or ATP-^{14}C to labeled cAMP were not very successful. The amount of tissue that was required minimally was

enormous in terms of the number of flasks of fibroblasts necessary. After running the assay it became apparent that even more tissue was required to distinguish any differences above blank values, due to the low adenyl cyclase activity of the cells.

In my studies a radioimmunoassay for cAMP was used to study the cAMP content and adenyl cyclase activity. The assay is based on the competition between a labeled and unlabeled antigen for binding on a specific antibody. For development of the assay, an antibody of high specificity for the antigen to be measured and a highly specific radioactive derivative of the antigen to be used as a marker are required (Steiner et al., 1970). The antibody to cAMP was obtained by immunizing rabbits with an antigen prepared by conjugating succinyl cAMP with human serum albumin. The highly specific radioactive derivative was prepared by synthesizing succinyl cAMP tyrosine methyl ester and iodinating the phenol hydroxyl group of the tyrosine with ^{125}I. The original description suggested 5-10 mg of tissue and found that displacement of ^{125}I-ScAMP-TME by unlabeled cAMP, when plotted as a semilogarithmetic function was linear over a concentration range of 2-100 picomoles cAMP (Steiner et al., 1969). The free and antibody bound ^{125}I labeled cyclic nucleotide could be separated by a second antibody precipitation or by ammonium sulfate fractionation. Further work with the assay showed that binding equilibrium was reached in 24 hours, but sensitive and reproducible assays were obtained after 4 to 6 hours of incubation. By choosing the appropriate antiserum and decreasing the volume of the assay the sensitivity could be increased to the femtomolar range (10^{-15}). In general, most antisera used were

able to measure 0.01 pmoles of cAMP per tube and the results obtained were in close agreement with those from other assays (Steiner et al., 1972[a]). To measure adenyl cyclase activity the same incubation procedure utilized by Bar and Hechter (1969[a]) was used but in smaller volumes and without a radioactive label. At the end of the incubation the supernate which contains cAMP could be added directly to the radioimmunoassay. Again the results were in close agreement with previous studies (Steiner et al., 1972[b]). I found this assay reproducible and reliable, requiring less than one μg of DNA per tube. It was also convenient for running over 100 samples simultaneously. Due to the high specificity of reactivity with only cAMP no very precise time-consuming purification was necessary before the unknown cAMP was added to the reagents. Use of this assay for measurement of adenyl cyclase was advantageous because the assay could detect smaller changes in cAMP concentration than other assays.

Fat cell cAMP content:

Many of the assays for cAMP and adenyl cyclase were originally designed for rat fat cells, which are a convenient source of material and respond to a wide variety of hormones. One report found that isolated rat fat cells contain 6.3±0.75 pmoles cAMP/mg protein which can be stimulated to 29.9±5.6 pmoles/mg protein by 1.25 μg/ml epinephrine (Steiner et al., 1972[b]). The cell size of these animals was not reported and any differences between those studies and mine may be due to cell size and manner of calculating data. My studies showed a range of 0.01 to 0.321 pmoles per cell for basal cAMP levels. I also showed that there was a positive correlation with increasing cell size.

Epinephrine (1μg/ml) stimulated cAMP levels were varied from 0.042 to 0.371 pmoles per cell, and again increases in cAMP levels correlated with larger cell size. Cell size is an important factor in cAMP concentration of fat cells.

In attempting to measure any changes in cAMP levels, the time period after the addition of the hormone or chemical is very critical. Reports have appeared in which no effects were demonstrated while later studies, which "caught" the activity of the tissue more quickly, demonstrated a pronounced effect. Epinephrine produces a detectable change in fat cell cAMP within 30 seconds after its addition. The maximal effect appeared at six minutes and then declined (Butcher et al., 1968[b]). My studies were performed after incubations of 5 minutes, the time which produced the best and most consistent response, without imposing impossible conditions on the experiments.

cAMP mediates the effects of insulin, but the mechanism is unestablished. Insulin lowers cAMP levels. In an early study, Jungas (1966) reported an inhibitory effect of insulin on epinephrine stimulated cAMP. He stated that this resulted from insulin's inhibition of adenyl cyclase. Insulin can also decrease cAMP levels in cells exposed to ACTH and glucagon (Butcher et al., 1968[a]). There may also be a relation between cAMP levels and the stimulatory effects of cAMP on the glucose entry mechanism (Blecher, 1967). Some investigators have failed to detect changes in adenyl cyclase activity with insulin. This may be explained by the insulin concentration used. Cuatrecasas (1972) observed an inhibitory effect with 5 μU/ml insulin. This effect was no longer apparent with more than 200 μU/ml insulin. He was

unable to detect an effect of insulin on cAMP breakdown or recovery. In my studies, 50 μU/ml insulin was always capable of lowering epinephrine stimulated cAMP levels to near basal levels.

Insulin receptors:

Recently, the entire phenomenon of insulin sensitivity has been investigated in detail. Basic to these experiments was the development of an assay which measured the specific binding of ^{125}I-insulin to intact cells and membrane fractions. The binding was time- and temperature-dependent, saturable with respect to insulin, and involved no chemical alterations or formation of stable covalent bonds (Cuatrecasas, 1971[a]). This receptor is localized on the cell membrane and is a protein. The disappearance of the insulin response in trypsin treated cells can be attributed to the destruction of the receptor by trypsin (Cuatrecasas, 1972). Trypsin, which does not penetrate the cell membrane, may be removing certain sialic acid residues from the membrane and in this manner interfere with the transmission of information from the insulin receptor complex (Cuatrecasas, 1971[b]). Treatment with phospholipase increases the affinity of insulin to its receptors, because the enzyme probably alters the accessibility and exposure of the receptor sites (Cuatrecasas, 1971[c]). Definite evidence that insulin does not enter the cell, but interacts with the surface, is derived from studies in which insulin is covalently bound to Sepharose beads and still mimicked native insulin (Cuatrecasas, 1969).

In my studies, attempts were made to correlate diabetic insulin resistance with large fat cell resistance. cAMP levels in the larger cells are higher and their epinephrine-insulin levels are higher, but

the per cent inhibition produced by insulin on epinephrine stimulated cAMP levels does not change significantly with cell size. Insulin resistant rat fat cells do not show a decrease in the quantity of insulin receptors or their affinity for insulin. The resistance probably occurs after the initial interaction of insulin and the cell membrane (Cuatrecasas, 1972). The primary defect may involve an abnormality in the transmission of signals which may be related to the dilution of insulin receptors over the surface of large cells. The total insulin binding capacity remains similar in all cells, but the increased surface area of the large cells results in a diminished number of insulin receptors per unit of membrane area. The signal transmission from insulin receptor to transport system is partially disrupted by the increase in intervening membrane space or structures, resulting in insulin resistance. The fat cell may have the capacity to expand without affecting insulin sensitivity until a critical size is reached (Livingston et al., 1972).

Older rats, which normally would have larger fat cells than younger rats, demonstrate a decrease in epinephrine, norepinephrine and NaF stimulated adenyl cyclase compared to younger rats. However, their PDE activity is higher (Forn et al., 1970). In rats made diabetic by a variety of methods, no change in PDE was found by Muller-Oerlinghausen et al. (1968). In contrast, Senft et al. (1968) reported a decrease in PDE activity in rats made diabetic by alloxan, which could be reversed by insulin. Lower PDE activity is also detected in adipose tissue of spontaneously diabetic mice (Kupiecki, 1969). A possible explanation for the positive correlation with

increasing cell size would be lower PDE activity in larger cells, allowing the accumulation of more cAMP.

Adenyl cyclase has been found in Chang's liver cells, 3T6 fibroblasts, HeLa cells, malignant mouse cells and rat and hamster fibroblasts (Klein and Makman, 1971; Peery et al., 1971). Adenyl cyclase is present in human cultured skin fibroblasts and it can be measured. The cells have very low adenyl cyclase activity (Makman, 1970). Rao et al. (1971) found that the formation of cAMP is linear from 5-20 minutes, after which it declined due to PDE activity. Adenyl cyclase activity is maximal with 10 mM NaF. The specific activities fall in a narrow range and do not appear to be affected by the number of culture passages or the cell strain used. However, in rat embryo fibroblasts and SV40 transformed 3T3 cells, fluoride stimulated, but not basal levels, fell with successive passages (Peery et al., 1971). Since extensive studies on the effects of passage number on adenyl cyclase activities have not been performed, I tried to limit the range of passage numbers used. I found no significant differences among the strains and that 10 mM NaF produced the greatest stimulation.

cAMP levels:

cAMP content has been measured in a number of mouse fibroblast lines. Steady-state levels of cAMP in normal density-dependent fibroblasts are twice as high as in the corresponding viral and spontaneous transformants. During logarithmic growth, both cells have similar cAMP levels, but at confluency the transformed cells contain less cAMP (Burger et al., 1972). Agents which stimulate confluent normal cells to resume division decrease cellular cAMP levels

temporarily; the depression of cellular cAMP levels may be a signal for continued division. Otten et al. (1971) agree that cAMP levels are lower in more rapidly growing cell lines. However, they found that levels of cAMP rise during contact inhibition of growth, whereas in cultures of non-contact inhibited cell lines, the cAMP levels fell when the cells became confluent. Catecholamine-induced cAMP levels, at least in astrocytoma cells, are dependent on the cell density, being greater in cells in the log phase of growth than in cells near terminal density (Clark and Perkins, 1971). The addition of trypsin decreases cAMP levels within 5 minutes. Serum, which contains PDE activity, depresses cAMP levels. Insulin without a cAMP stimulator is found to decrease cAMP levels. The concentration used, 80 mU ml^{-1} is extremely high (Sheppard, 1972). Cuatrecasas (1972) finds that only low concentrations of insulin are effective.

Otten et al. (1972) found that cAMP levels rose as human diploid fibroblasts become confluent. Like Sheppard (1972) they found that serum and trypsin decreased cAMP levels. However, they found that insulin alone does not alter basal cAMP levels, even at 125 mU/ml. With PGE present, which increased cAMP levels, insulin lowered cAMP levels.

Since all my studies were performed with contact inhibited human diploid fibroblasts, it was important to standardize their stage of growth at the time of assay. All assays were performed three days after trypsinization when the cells had reached confluency. The cells were in phase II of their growth cycle and in general were in passage eight and nine. Some of the diabetic cells were in earlier passage

numbers but because of their shorter in vitro life span, at later passage numbers they may already be degenerating. Unfortunately, due to differences in age and disease state it is difficult to precisely standaradize cell "age".

Human skin fibroblasts are sensitive to epinephrine and, like fat cells, they are sensitive to the inhibitory effects of low concentrations of insulin on epinephrine stimulated cAMP levels. This insulin effect is related to the presence of insulin receptors in fibroblasts, which are similar in properties to fat cell receptors (Gavin et al., 1972). In agreement with the established properties of fat cell receptors, my use of 50 μU/ml insulin to produce an effect seems to be within the expected range. Sheppard (1972) and Otten et al. (1972) were both using enormous concentrations which were well beyond physiological range. Their results may not have been the direct result of the insulin on the receptors, but the result of molecular alterations produced by the huge insulin concentrations.

Analysis of the combined results from all normal fibroblast strains shows a statistically significant difference between basal levels and epinephrine stimulated levels ($P<0.025$), but no difference between basal and epinephrine-insulin treated levels ($P<0.25$). However, there is only a questionable difference between epinephrine and epinephrine-insulin responses ($P<0.1$). Careful examination of the data reveals that strain #606 appears to have high epinephrine-insulin treated cAMP levels as compared to the other normal strains. In the other normal cell strains insulin significantly depressed epinephrine stimulated levels.

The normal cells, excluding #606, show between 60.5 and 80 per cent differences between epinephrine and epinephrine-insulin values. The per cent change for #606 is 28.1 which falls in the same range as that of the diabetic and growth hormone deficient cells (-22 to 60%). Even when #606 is included in the analysis of this calculation, there is a significant difference between normal and diabetic cells ($P<0.005$), and normal and growth hormone deficient cells ($P<0.05$). Because of this discrepancy all data for cAMP levels has been calculated with and without #606 included with the normal strains.

Comparisons of normal cells with diabetic cells or growth hormone deficient cells demonstrate no differences in basal or epinephrine stimulated levels. Although cells from growth hormone deficient children were used because the children had some diabetic-like characteristics, I did not feel justified in including them within the diabetic group, since little is known about their in vitro metabolic responses. They are younger than the diabetic patients and may not have all the same characteristics as the diabetics. However, whenever there was a difference demonstrated in the diabetic cells compared to normals, there was also a similar difference in the growth hormone deficient cells.

Although the cAMP responses to epinephrine-insulin of normal cells compared to diabetic or growth hormone deficient cells do not show significant differences when #606 was included in the calculations, these differences were statistically significant when #606 was omitted ($P<0.025$ diabetic cells; $P<0.001$ growth hormone deficient cells).

To insure that no error had been made in assaying #606, it was retested, yielding similar results. Unfortunately I was unable to obtain a complete history on the individual from whom strain #606 was developed, as this strain was obtained from the cell bank at Camden, New Jersey. The file on this donor simply states that he is a chromosomally normal male in his twenties. It is quite possible that he may have a family history of diabetes, be a prediabetic, or have abnormal carbohydrate metabolism.

Adenyl cyclase activity:

This data presents preliminary evidence that there may be a defect in the adenyl cyclase system of diabetics. Consequently adenyl cyclase activity in the same lines was measured. The fibroblasts demonstrate sensitivity to epinephrine, epinephrine-insulin and NaF. The stimulation produced by epinephrine and NaF elevated enzyme activities significantly, while the response to epinephrine-insulin was not significantly different from basal activities. The differences which appeared in cAMP levels after exposure to epinephrine-insulin are not reflected in differences in adenyl cyclase activities.

The discrepancy between the results for cAMP and adenyl cyclase does not rule out a defect in the adenyl cyclase system of diabetics. In broken cell preparations previously unexposed receptors may become accessible. This study did not include assays for PDE activity and it is possible that differences may be present at that level, which are not evident when the cells are broken.

Suspicion of the adenyl cyclase system for the possible biochemical defect in diabetes mellitus draws support from the regulation of

insulin secretion by adenyl cyclase. In intact mice, intravenous administration of cAMP or DBcAMP stimulates insulin release, which can be blocked by beta adrenergic blockers (Bressler et al., 1969). In vitro studies with perfused isolated rat pancreas also demonstrate cAMP stimulation of insulin release (Sussman and Vaughan, 1967). The defective insulin release observed in prediabetics and diabetics may be due to an alteration of specific receptors of the beta cells for glucose. During hyperglycemia there is diminished cAMP formation because the beta cell is unable to recognize the hyperglycemic state and activate adenyl cyclase. Under basal conditions, adenyl cyclase behaves normally (Luft and Cerasi, 1970). This abnormality may also be present in nonpancreatic cells (Luft and Cerasi, 1970). It is interesting that administration of aminophylline to prediabetics will normalize or improve their insulin response to glucose infusion. This PDE inhibitor has no effect on diabetics or normals. In prediabetics, aminophylline may be increasing cAMP levels to those required for normal insulin secretion. Normals already have the required levels; diabetics may require a greater stimulus (Cerasi and Luft, 1969).

Suggestions from in vitro studies of a relationship between diabetes and the adenyl cyclase system may be derived from a comparison of the growth behavior of diabetic cells and the addition of DBcAMP to the medium of malignant cells. As mentioned, diabetic cells begin to degenerate after a short in vitro life. They enlarge and grow very slowly. Their growth and appearance very closely resembles that seen in DBcAMP treated cultures of malignant cells (Johnson et al., 1971[a]; Hsie et al., 1971; Hsie and Puck, 1971; Johnson et al., 1971[b]; Sheppard, 1971; Ryan and Heidreck, 1968). On a purely observational

level, diabetic cells seem to adhere to the substratum more tightly than normal cells. This has also been observed in DBcAMP treated cells (Johnson and Pastan, 1972). Unfortunately, in the strains used, there was no evidence for the accumulation of cAMP. It is possible that this occurs at higher passage numbers and may be a phenomenon common to all fibroblasts with aging.

From my investigations it appears that the adenyl cyclase system may play an important role in diabetes mellitus. Fat cells which demonstrate insulin resistance are also less sensitive to nonsaturating cAMP and BDcAMP stimulation of metabolic activities. There may be an error or alteration in the transmission signals, producing decreased sensitivity. Human fibroblasts provide even more convincing evidence for an alteration in this membrane bound system. Since their environment before incubation is standardized, the changes observed in cAMP levels are more meaningful. The insulin resistance of diabetic adipose cells might be logically explained by the decreased sensitivity to insulin of cAMP content in fibroblasts. Many more cell strains, both normal and diabetic, must be examined before any definite conclusions can be made. Then family studies may help to define the pattern of inheritance of diabetes mellitus, and predict the development of the disease in suspected prediabetics.

Summary

Although the pattern of inheritance of diabetes mellitus remains undetermined, the basic defect is unquestionably metabolic. My studies identified certain in vitro biochemical characteristics of human skin fibroblasts and fat tissue from rats and humans. Fibroblasts are shown to be metabolically active, sensitive to hormones and to have measureable quantities of adenyl cyclase and cAMP

The established involvement of cAMP in lipolysis prompted the study of this parameter in fat cells. Since larger rat adipocytes are more similar to human cells than smaller rat cells, the effects of cell size were studied. Basal levels of lipolysis are positively correlated with cell size, as are cAMP stimulated levels. However, the per cent change is low and statistically insignificant suggesting that increases in lipolysis observed with cAMP stimulation are reflections of increased basal levels. One (1) mM DBcAMP stimulated lipolytic activity is unaffected by cell size.

This investigation demonstrates that under resting conditions intracellular cAMP levels increase as cell size increases. These increasing levels may explain the increased rate of lipolysis in large rat adipocytes and perhaps in human cells. The cAMP level is only rate limiting for lipolysis within a range of 180-300 pmoles cAMP/gm wet weight. It is demonstrated that epinephrine stimulated intracellular cAMP levels are also positively correlated with cell size. This would explain the positive correlation between epinephrine stimulated lipolysis and cell size observed by some investigators. The effects of other hormones may be explained similarly. Normal human fat cells may follow the patterns observed in large rat cells.

Since diabetes is expressed as abnormal glucose metabolism, glucose oxidation may be expected to differ in diabetics. The exact role of cAMP in this parameter is not established, but the known effects of insulin and epinephrine on CO_2 production suggest that the adenyl cyclase system may be involved. It is demonstrated that basal $^{14}CO_2$ production is unaffected by cell size in both rats and humans. The addition of cAMP causes changes in $^{14}CO_2$ production to be negatively correlated with cell size in rats, but not significantly correlated in humans, except in growth hormone deficient children. DBcAMP changes are positively correlated with cell size in rats, but not in humans. The range of cell sizes used in humans may not have been great enough to show those differences. Also, the larger human cells were obtained from people not in the normal group. Finally, the cells' neurohormonal environment immediately prior to the experiment may have altered their responses. Both the growth hormone deficient children and the obese patients have larger than normal fat cell sizes, but both groups may also have some diabetic characteristics. Unfortunately, no clear differences could be detected between the normals and the other two groups. To parallel the fat cell studies $^{14}CO_2$ production was studied in fibroblasts. No significant differences could be detected between groups for sensitivity to cAMP or DBcAMP.

Direct study of cAMP levels and adenyl cyclase activities in fibroblasts can detect differences not reflected in the end products of the metabolic pathways measured. It is demonstrated that fibroblasts in the same phase of growth and life cycle appear to have approximately the same intracellular cAMP levels and react similarly to epinephrine. The cells are sensitive to epinephrine and the combination

of epinephrine-insulin, this combination depressing the epinephrine stimulated levels of cAMP. However, in cells derived from biopsies from normal persons, this depression is much greater than in cells derived from diabetic patients and growth hormone deficient children. This suggests an alteration in the response of the cAMP system to insulin, possibly a basic defect in diabetes. Although the significant differences between normals and diabetics were also observed between normals and growth hormone deficient children, who often demonstrate diabetic-like characteristics, enlargement of the sample may uncover specific differences for diabetes mellitus which may be useful in the detection of this disease.

Significantly, measurement of adenyl cyclase activities in the same patients did not reveal any differences in basal activity, or activity in the presence of epinephrine, epinephrine-insulin or NaF. The differences observed in cAMP levels could also be caused by differences in PDE activities of the abnormal cells. There may also be alterations in the insulin receptor sites which may be responsible for the differences observed. Both PDE activity and the insulin receptor sites require further study.

My studies suggest that the adenyl cyclase-cAMP system may be involved in diabetes mellitus. It is also shown that fibroblasts, easily obtainable from diabetics and their families, are a feasible and reliable tool for investigation of this disease.

Table 1: Animal sizes used for lipolysis

body weight (gms)	sex	pad weight (gms)[a]	cell size µg lipid/cell	no. animals
386.0±31.4	M	2.003±0.124	0.2130	5
266.7± 3.3	F	1.301±0.197	0.2812	3
350.0±20.0	F	3.955±0.173	0.4758	2
560.0	M	4.760±0.139	0.5846	1
550.0	M	3.124±0.057	0.7171	1
360.0	F	16.760	1.1180	1

Average weights ± S.E.M.

[a] epididymal pads in M and parametrial pads in F.

Table 2: Glycerol production in rat adipocytes of varying sizes

	μmoles glycerol x 10^{-6}/cell		
cell size	Basal	cAMP	DBcAMP
0.2130	0.621±0.033	0.838±0.000	3.562±0.453
0.2812	0.163±0.005	0.243±0.173	0.932±0.080
0.4758	0.726±0.001	0.903±0.011	3.999±0.018
0.5846	2.472±0.084	3.383±0.136	10.544±0.337
0.7171	1.034±0.048	1.284±0.030	3.643±0.039
1.1180	4.120±0.469	3.580±0.315	4.504±0.182

Average values ± S.E.M.

Table 3: Animal sizes used for $^{14}CO_2$ production

body weight (gm)	sex	epididymal pad weight (gm)	cell size (µg lipid/cell)	no. of animals
256.0±2.70	M	0.795±0.053	0.0200	4
260.0±0.00	M	0.670±0.030	0.2577	2
267.6±1.47	M	1.411±0.076	0.2816	5
560.0	M	4.760±0.129	0.5846	1
520.0	M	2.447±0.211	0.6000	1
550.0	M	3.124±0.057	0.7171	1

Average values ± S.E.M.

Table 4: $^{14}CO_2$ production in rat adipocytes of varying sizes

	cpm/cell		
cell size	Basal	cAMP	DBcAMP
0.0200	87.29± 5.440	193.50± 5.725	25.51± 5.896
0.2577	145.76± 0.280	211.10±22.000	156.70±14.070
0.2816	53.68± 1.290	148.71±16.920	83.32± 2.630
0.5846	67.98± 3.327	95.32± 4.860	92.80±18.050
0.6000	58.12± 5.770	150.30±23.257	120.10± 5.007
0.7171	85.51±17.320	106.52± 2.069	105.22±10.106

Average values ± S.E.M.

Table 5: Injected animal sizes

treatment	sex	body weight (gm)	pad[a] weight (gm)	cell size	no. of animals
NaCl	M	386.00±31.40	2.0030±0.124	0.2130	5
NaCl	F	310.00± 7.07[b]	3.5704±0.850	0.5311	2
NaCl	F	360[b]	16.7600	1.1181	1
GH-NaCl	M	371.00± 9.87	2.4500±0.461	0.4531	3
GH-NaCl	F	346.70±30.31[b]	6.7900±1.520	1.2080	3
MSG-NaCl	M	346.00±16.48	1.6580±0.414	0.5490	4
MSG-NaCl	F	not done			
MSG-GH	M	423.33±21.74	4.0830±2.070	2.0645	3
MSG-GH	F	287.50±11.93	6.3680±1.770	0.7440	4

[a]M epididymal pad, F parametrial pad

[b]Animals were born at the same time as the others but were killed at least 4 months later

Average values ± S.E.M.

GH growth hormone

MSG monosodium glutamate

Table 6: Lipolysis in injected rats

μmoles glycerol x 10^{-6}/cell

treatment-sex	NaCl M	NaCl F	GH M	GH F	MSG M	MSG-GH M	MSG-GH F
Basal	0.62±0.03	5.12±0.54	0.84±0.00	1.21±0.02	1.96±0.01	3.86±0.09	0.98±0.01
expected			1.0044	3.7637	1.3834	8.1609	2.1843
L_1			2.8365[a]	6.4478[b]	2.7686[a]	14.3180[a]	3.5167[a]
L_2			-0.8279[a]	1.0796[b]	-0.0018[a]	2.0038[a]	0.8519[a]
cAMP	0.84±0.00	4.55±0.44	0.85±0.04	1.61±0.02	3.14±0.13	4.76±0.07	1.17±0.24
% change	34.70	-11.07	19.0000	39.9600	60.1000	23.3000	19.0200
expected			1.2554	3.5902	1.5522	6.2389	2.1553
L_1			2.8932[a]	6.4641[b]	2.7958[a]	11.7631[a]	3.3489[a]
L_2			-0.3824[a]	0.7163[b]	0.3086[a]	0.7147[a]	0.9617[a]
DBcAMP	3.56±0.45	4.22±0.26	1.27±0.78	0.96±0.10	4.71±0.71	6.05±0.14	1.48±0.39
% change	497.20	-17.51	51.1900	-23.4500	140.6000	56.9000	50.5500
expected			4.3142	5.7727	4.4995	7.4275	4.8763

Table 6: continued

treatment-sex	NaCl M	NaCl F	GH M	GH F	MSG M	MSG-GH M	MSG-GH F
L_1			6.4002[c]	13.0680[a]	8.7578[a]	25.7213[a]	8.9570[a]
L_2			2.3282[c]	-1.5234[a]	0.2412[a]	-10.8663[a]	0.7956[a]
growth hormone	0.63±0.01	3.35±0.24	3.16±0.06	1.29±0.22	2.19±0.50	3.89±0.20	1.02±0.11
% change	0.50	-34.64	-62.3800	6.6100	11.7000	-0.5000	3.7600
insulin	0.54±0.15	2.65±0.98	0.27±0.05	1.26±0.14		4.05±0.11	0.82±0.07
% change	-17.30	-48.24	-68.0900	4.1300		4.9000	-16.5800

[a] 95% confidence limits

[b] 98% confidence limits

[c] 99% confidence limits

Average values ± S.E.M.

GH - growth hormone

MSG - monosodium glutamate

cell sizes as in Table 5; NaCl F = 1.1181 µg lipid/cell

Table 7: $^{14}CO_2$ production in injected rats

			cpm/cell				
treatment-sex	NaCl M	NaCl F	GH M	GH F	MSG M	MSG-GH M	MSG-GH F
Basal	24.90±1.52	15.99±1.02	14.75±2.15	23.67±1.90	29.47	68.08±2.30	14.86±1.26
expected			81.4081	52.4696	77.7320	19.6362	71.0012
L_1			149.1329[c]	141.5681[a]	127.8669[b]	145.7727[a]	128.6587[a]
L_2			13.6833[c]	-36.5289[a]	27.5971[b]	-106.5075[a]	13.3437[a]
cAMP	21.25±5.58	16.12±0.75	25.64±1.62	29.20±1.69	24.88±1.50	58.20±3.15	8.86±1.28
% change	-14.00	0.86	75.10	23.46	15.60	-14.40	-44.10
expected			145.0260	41.7585	131.9073	-75.4075	106.3320
L_1			204.0032[c]	119.2366[a]	185.5439[c]	109.6272[c]	189.4502[c]
L_2			87.0488[c]	-36.7196[a]	78.2707[c]	-260.4422[c]	23.2138[c]
DBcAMP	30.53±6.73	12.68±0.46	9.09±1.99	32.10±3.71	4.99±1.77	90.80±4.63	20.32±0.85
% change	22.61	-20.66	-38.30	35.62	-83.00	33.40	28.00
expected			105.8834	161.8033	109.0319	230.3901	124.6471

Table 7: continued

treatment-sex	NaCl M	NaCL F	GH M	GH F	MSG M	MSG-GH M	MSG-GH F
L_1			187.6964[c]	306.8496[b]	183.3865[c]	385.1521[a]	239.9773[c]
L_2			24.0704[c]	16.7570[b]	34.6773[c]	75.6281[a]	9.3169[c]
growth hormone	31.57±2.14	13.04±2.62	6.62±1.48	26.85±0.10	15.68±4.95	92.80±6.17	19.23±1.61
% change	26.00	-18.43	-55.10	13.41	-46.30	36.30	21.10
insulin	39.46±9.32	8.74±0.41	29.91±1.28	15.20±0.47	100.25±9.70	89.26±0.91	8.90±2.01
% change	58.46	-45.30	192.80	-35.70	240.10	31.10	-25.00

[a] 95% confidence limits

[b] 98% confidence limits

[c] 99% confidence limits

Average values ± S.E.M.

GH - growth hormone

MSG - monosodium glutamate

Cell sizes as in Table 5; NaCl F = 0.5311 µg lipid/cell

Table 8: $^{14}CO_2$ production in human fat cells

patient	cell size	diagnosis	sex	Basal	cAMP	DBcAMP
E.R.	0.2096	normal	M	79.99±11.81	213.45±44.57	120.40±14.24
M.F.	0.2324	"	M	24.60± 6.59	33.21± 6.49	14.70± 7.53
R.F.	0.2661	"	M	49.94± 4.74	55.04± 1.95	11.65± 2.60
J.O.	0.2784	"	M	22.50± 6.50	54.34± 0.44	41.22± 2.12
L.G.	0.3134	"	M	74.85± 3.09	137.48± 3.85	44.71± 4.92
R.O.	0.3357	growth hormone deficient	M	59.0 ± 1.53	66.00±11.00	55.30± 3.93
D.R.	0.3524	"	F	65.70± 4.10	108.30± 2.33	78.70± 9.21
C.H.	0.3972	"	F	46.22± 2.69	127.82±24.61	50.76±13.10
R.C.	0.4056	"	M	74.30±11.87	172.30± 3.38	97.00± 8.00
R.M.	0.4125	"	M	89.42± 8.65	163.43±19.06	86.29± 6.67
M.C.	0.4712	obese	M	16.83± 4.60	23.23± 4.27	8.03± 1.59
J.C.	0.6025	"	F	98.3 ±16.84	170.00±66.53	127.54±14.03

Table 8: continued

patient	cell size	diagnosis	sex	Basal	cAMP	DBcAMP
D.G.	0.6074	obese	F	35.21± 2.89	34.42±14.31	49.17± 1.32
V.C.	0.6505	"	F	80.70± 7.26	112.08± 6.08	89.64± 5.26
A.P.	0.7500	"	F	56.35± 0.56	76.48± 3.76	30.13± 4.16

Average values ± S.E.M.

Table 9: $^{14}CO_2$ production

in human fat cells as a function of cell size

Group	regression equation (y=)	correlation coefficient (r)	
Normals			
Basal	1.4852x-49.5	0.0022	not significant
cAMP	-473.485x+221.935	-0.2590	not significant
DBcAMP	-478.394x+170.845	-0.4490	not significant
Growth Hormone deficient			
Basal	215.614x-15.135	0.4410	not significant
cAMP	1266.841x-354.59	0.9670	significant $P<0.01$
DBcAMP	292.818x-35.84	0.4860	not significant
Obese			
Basal	144.185x-31.384	0.4503	not significant
cAMP	187.2x-32.139	0.3225	not significant
DBcAMP	86.665x+7.592	0.1872	not significant

Table 10: Per cent change $^{14}CO_2$ production as a function of human fat cell size

Group	regression equation (y=)	correlation coefficient (r)	
All			
cAMP	107.537x+35.85	0.2713	not significant
DBcAMP	55.89x-30.039	0.1585	not significant
Normals			
cAMP	325.69x+3.128	0.2020	not significant
DBcAMP	-328.94x+79.96	-0.1980	not significant
Growth Hormone deficient			
cAMP	219.98x-721.48	0.9850	significant P<0.01
DBcAMP	120.25x-35.727	0.3570	not significant
Obese			
cAMP	51.644x+9.929	0.1436	not significant
DBcAMP	-259.96x+158.087	-0.5620	not significant

Table 11: Comparison of $^{14}CO_2$ production in human fat cells

	df	SS	MS	F_s
Cell size				
Normal vs growth hormone deficient				
Between groups	1	0.0367	0.0367	28.2307
Within groups	8	0.0108	0.0014	sign. $P<0.001$
Normal vs obese				
Between groups	1	0.2975	0.2975	34.1954
Within groups	8	0.0695	0.0087	sign. $P<0.001$
Basal activity				
Normals vs growth hormone deficient				
Between groups	1	684.9218	684.9218	1.3794
Within groups	8	3972.0483	496.5060	not sign.
Normals vs obese				
Between groups	1	126.0959	126.0959	0.1386
Within groups	8	7273.7225	909.2153	not sign.
cAMP stimulation				
Normals vs growth hormone deficient				
Between groups	1	2253.0337	2253.0337	0.6017
Within groups	8	30196.2577	3774.5322	not sign.
Normals vs obese				
Between groups	1	587.6835	587.6835	0.1259
Within groups	8	37334.7299	4666.8412	not sign.

Table 11: continued

	df	SS	MS	F_s
DBcAMP stimulation				
Normals vs growth hormone deficient				
Between groups	1	1842.5036	1842.5036	1.5850
Within groups	8	9299.2097	1162.4012	not sign.
Normals vs obese				
Between groups	1	516.8558	516.8558	0.2451
Within groups	8	16863.7445	2107.9681	not sign.
Per cent change cAMP				
Normals vs growth hormone deficient				
Between groups	1	1640.0994	1640.0994	0.3190
Within groups	8	41121.1955	5140.1494	not sign.
Normals vs obese				
Between groups	1	5285.8185	5285.8185	1.9961
Within groups	8	21184.2227	2648.0278	not sign.
Per cent change DBcAMP				
Normals vs growth hormone deficient				
Between groups	1	148.9715	148.9715	0.0602
Within groups	8	19780.5851	2472.5731	not sign.
Normals vs obese				
Between groups	1	28.9680	28.9680	0.0090
Within groups	8	25611.1966	3201.3996	not sign.

Table 12: ^{14}C incorporation into triglycerides in human fat cells

cell size	basal	cAMP	DBcAMP
0.2096	31.27± 9.61	49.30± 7.18	41.17± 9.40
0.2324	22.47± 2.53	26.20± 3.09	18.64± 1.21
0.2661	41.66± 0.54	23.60± 4.24	25.75± 2.20
0.2784	16.50± 3.18	22.76± 9.18	85.72± 3.49
0.3134	43.47± 2.38	52.66± 2.39	93.66± 7.87
0.3357	78.29± 1.45	76.93± 7.97	137.55± 9.36
0.3524	55.70± 4.71	51.00± 9.41	122.30± 4.82
0.3972	45.15± 1.86	112.98± 0.17	94.94± 9.38
0.4056	110.44± 4.09	137.76± 9.36	244.92±20.84
0.4125	72.89± 5.11	126.99±14.35	184.20± 2.62
0.4712	41.39± 3.85	44.99±14.94	41.29± 3.11
0.6025	97.67±10.08	113.55±15.18	163.05±11.23
0.6074	51.73± 9.15	61.55± 2.30	70.55±17.31
0.6505	91.48± 3.35	111.72± 1.86	174.04± 4.41
0.7500	52.93± 7.01	91.14± 6.20	58.94± 8.66

Average values ± S.E.M.

Table 13: ^{14}C incorporation into triglycerides in human fat cells as a function of cell size

Group	regression equation (y=)	correlation coefficient (r)	
Normal			
Basal	-138.5x+68.3221	-0.4709	not significant
cAMP	25.0147x+28.3987	0.0697	not significant
% stim.	-264.8823x+86.8329	-0.0403	not significant
DBcAMP	610.5x-105.6789	0.7270	not significant
% stim.	1838.1764x-377.052	0.4021	not significant
Growth Hormone deficient			
Basal	163.727x+10.227	0.2668	not significant
cAMP	1108.0909x-319.9445	0.9225	significant $P<0.05$
% stim.	1311.9545x-451.5258	0.6727	not significant
DBcAMP	883.4772x-179.4694	0.4970	not significant
% stim.	666.3863x-137.6266	0.7989	not significant
Obese			
Basal	-114.035x+135.4976	-0.3734	not significant
cAMP	226.4637x-55.5735	0.7141	not significant
% stim.	204.2341x-97.1227	0.8227	not significant
DBcAMP	264.5831x-59.6625	0.3890	not significant
% stim.	137.288x-43.1421	0.2892	not significant
All % stim.			
cAMP	63.4492x+6.0961	0.2332	not significant
DBcAMP	-94.3752x+126.3256	0.1416	not significant

Table 14: Comparisons of ^{14}C incorporation into triglycerides in normal, growth hormone deficient and obese fat cells

	df	SS	MS	F_s
Basal				
Normals vs growth hormone deficient				
Between groups	1	4289.0408	4289.0408	8.5850
Within groups	8	3996.5752	499.5719	sign. $P<0.025$
Normals vs obese				
Between groups	1	3233.8828	3233.8828	8.1378
Within groups	8	3179.1142	397.3893	sign. $P<0.025$
cAMP stimulation				
Normals vs growth hormone deficient				
Between groups	1	10965.3700	10965.3700	14.3265
Within groups	8	6123.1130	765.3891	sign. $P<0.01$
Normals vs obese				
Between groups	1	6171.7404	6171.7404	10.7564
Within groups	8	4590.1658	573.7707	sign. $P<0.025$
DBcAMP stimulation				
Normals vs growth hormone deficient				
Between groups	1	26932.9860	26932.9860	11.5281
Within groups	8	18690.3192	2336.2899	sign. $P<0.01$
Normals vs obese				
Between groups	1	5901.4914	5901.4914	2.3335
Within groups	8	20231.8883	2528.9860	not sign.

Table 14: continued

	df	SS	MS	F_s
Per cent change cAMP				
Normals vs growth hormone deficient				
Between groups	1	3222.1865	3222.1865	1.1158
Within groups	8	22101.1032	2887.6379	not sign.
Normals vs obese				
Between groups	1	236.4870	236.4870	0.1189
Within groups	8	15874.1080	1984.2645	not sign.
Per cent change DBcAMP				
Normals vs growth hormone deficient				
Between groups	1	452.2562	452.2562	0.0620
Within groups	8	579615.0184	72451.8773	not sign.
Normals vs obese				
Between groups	1	8335.9238	8335.9238	1.3164
Within groups	8	50655.8576	6331.9822	not sign.

Table 15: cAMP levels in isolated rat adipose cells

	cAMP pmoles/cell		
		stimulant	
cell size	basal	epinephrine	epi.-insulin
0.1500	0.025	0.045	0.028
0.1726	0.030	0.062	0.047
0.1766	0.010	0.042	0.033
0.2000	0.095	0.291	0.072
0.2380	0.003	0.063	0.006
0.3636	0.072	0.417	0.138
0.4033	0.111	0.190	0.086
0.6180	0.321	0.371	0.188

Table 16: $^{14}CO_2$ production in cultured human fibroblasts[a]

strain	diagnosis	date	cAMP-B / B	DBcAMP-B / B	insulin-B / B
		48 hour incubation			
27	normal	6/10	347.34	97.29	-87.36
11	"	5/25	80.39	501.89	138.51
13	"	5/25	86.36	21.41	60.45
25	"	7/9	952.40	299.71	602.46
25	"	7/28	-4.29	3.86	N.D.
45	"	2/15	1285.48	N.D.	257.66
27	"	7/7	172.66	95.29	N.D.
27	"	8/12	-75.80	84.52	N.D.
21	growth hormone deficient	6/10	342.85	-58.33	-28.51
21	"	7/7	-13.76	33.91	N.D.
12	"	5/25	-65.73	-17.41	13.59
41	"	2/15	-39.20	N.D.	-8.13
P.S.	diabetic	7/9	203.82	168.45	245.46
		24 hour incubation			
45	normal	2/15	-14.00	N.D.	159.27
27	"	6/8	49.61	97.3	N.D.
27	"	8/12	244.19	9556.23	N.D.
41	growth hormone deficient	2/15	-15.12	N.D.	-3.29

Table 16: continued

strain	diagnosis	date	cAMP-B / B	DBcAMP-B / B	insulin-B / B
	24 hour incubation -- 4 hours with compound				
27	normal	7/15	-16.52	24.53	N.D.
25	"	7/28	-21.35	-4.53	N.D.
41	growth hormone deficient	8/12	-16.16	254.36	N.D.

B basal

N.D. not done

[a] in per cent stimulation

Concentrations used:

5mM cAMP

1mM DBcAMP

40,000 μU insulin/ml

Table 17: cAMP levels in human cultured skin fibroblasts

		pmoles cAMP/µg DNA		
			stimulant	
strain	diagnosis	basal	epinephrine	epi.-insulin
45T8	normal	3.320	50.750	12.540
44T9	"	5.262	20.320	5.830
592T9	"	7.435	21.160	8.376
679T9	"	4.683	14.018	3.810
606T9	"	8.948	47.714	34.307
42T7	diabetic	2.100	18.233	13.725
54T5	"	5.010	26.387	14.000
55T7	"	4.643	19.113	15.081
57T5	"	15.261	44.368	35.207
58T8	"	9.478	23.669	22.339
20T8	growth hormone deficient	0.916	12.922	13.564
39T8	"	2.836	13.640	13.090
40T9	"	5.089	18.605	22.970
41T9	"	9.793	27.060	16.924

Table 18: Comparisons of cAMP levels in normal fibroblasts with and without stimulation

	df	SS	MS	F_s
Basal vs epinephrine				
Between groups	1	1545.3970	1545.3970	10.4008
Within groups	8	1188.6670	148.5834	sign. P<0.025
Basal vs epi.-insulin				
Between groups	1	124.0100	124.0100	1.5707
Within groups	8	631.6290	78.9540	not sign.
Epi. vs epi.-insulin				
Between groups	1	793.8633	793.8633	3.5680
Within groups	8	1779.9238	222.4905	sign. P<0.1
Basal vs epinephrine (without #606)[a]				
Between groups	1	914.7901	914.7901	6.7807
Within groups	6	809.4536	134.9089	sign. P<0.05
Basal vs epi.-insulin (without #606)				
Between groups	1	12.1377	12.1377	1.4805
Within groups	6	49.1872	8.1980	not sign.
Epi. vs epi.-insulin (without #606)				
Between groups	1	716.5970	716.5970	5.0401
Within groups	6	853.0522	142.1770	sign. P<0.07

[a] For explanation of calculations without #606 see discussion

Table 19: Comparisons of cAMP levels in normal, diabetic and growth hormone deficient fibroblasts

	df	SS	MS	F_s
Basal				
Normals vs diabetics				
Between groups	1	4.7069	4.7069	0.2949
Within groups	8	127.6798	15.9599	not sign.
Normals vs growth hormone deficient				
Between groups	1	3.5910	3.5910	0.3924
Within groups	7	64.0660	9.1523	not sign.
Normals (without #606) vs diabetics				
Between groups	1	10.0379	10.0379	0.6033
Within groups	7	116.4629	16.6376	not sign.
Normals (without #606) vs growth hormone deficient				
Between groups	1	0.5340	0.5340	0.0608
Within groups	6	52.6778	8.7796	not sign.
Epinephrine stimulation				
Normals vs diabetics				
Between groups	1	49.2498	49.2498	0.2434
Within groups	8	1618.5632	202.3204	not sign.
Normals vs growth hormone deficient				
Between groups	1	360.4371	360.4371	1.9479
Within groups	7	1295.2680	185.0380	not sign.
Normals (without #606) vs diabetics				
Between groups	1	0.0963	0.0963	0.0005
Within groups	7	1249.7525	178.5361	not sign.

Table 19: continued

	df	SS	MS	F_s
Normals (without #606) vs growth hormone deficient				
Between groups	1	144.7679	144.7679	0.9371
Within groups	6	926.8869	154.4812	not sign.
Epi.-insulin stimulation				
Normals vs diabetics				
Between groups	1	125.9256	125.9256	1.0629
Within groups	8	947.7501	118.4688	not sign.
Normals vs growth hormone deficient				
Between groups	1	29.8400	29.8400	0.2969
Within groups	7	703.4956	100.4994	not sign.
Normals (without #606) vs diabetics				
Between groups	1	343.4215	343.4215	8.6279
Within groups	7	278.6578	39.8083	sign. $P<0.025$
Normals (without #606) vs growth hormone deficient				
Between groups	1	261.9282	261.9282	275.2213
Within groups	6	5.7104	0.9517	sign. $P<0.001$
Per cent change: Epi. over basal				
Normals vs diabetics				
Between groups	1	34154.6736	34154.6736	0.1929
Within groups	8	1416261.8368	177032.7296	not sign.
Normals vs growth hormone deficient				
Between groups	1	4974.8580	4974.8580	0.0174
Within groups	7	1998227.4894	285461.0699	not sign.

Table 19: continued

	df	SS	MS	F_s
Normals (without #606) vs diabetics				
Between groups	1	37640.2502	37640.2502	0.1851
Within groups	7	1422743.9587	203249.1370	not sign.
Normals (without #606) vs growth hormone deficient				
Between groups	1	2318.4645	2318.4645	0.0069
Within groups	6	1994710.5674	332451.7612	not sign.
Per cent changes: epi.-insulin over basal				
Normals vs diabetics				
Between groups	1	41185.5897	41185.5897	1.5376
Within groups	8	214283.7471	26785.4684	not sign.
Normals vs growth hormone deficient				
Between groups	1	401352.0564	401352.0564	2.6093
Within groups	7	1076681.8970	153811.6996	not sign.
Normals (without #606) vs diabetics				
Between groups	1	64263.0281	64263.0281	2.5060
Within groups	7	179502.5910	25643.2273	not sign.
Normals (without #606) vs growth hormone deficient				
Between groups	1	435585.1096	435585.1096	2.5084
Within groups	6	1041900.3899	173650.0650	not sign.
Per cent changes: epi.-insulin over epi.				
Normals vs diabetics				
Between groups	1	3528.0108	3528.0108	17.8301
Within groups	8	1582.9390	197.8674	sign. $P<0.005$

Table 19: continued

	df	SS	MS	F_s
Normals vs growth hormone deficient				
Between groups	1	5778.0851	5778.0851	7.5920
Within groups	7	5327.4997	761.0713	sign. $P<0.05$
Normals (without #606) vs diabetics				
Between groups	1	4690.1677	4690.1677	27.8200
Within groups	7	1180.1265	168.5895	sign. $P<0.005$
Normals (without #606) vs growth hormone deficient				
Between groups	1	6937.1318	6937.1318	10.6053
Within groups	6	3924.6870	654.1145	sign. $P<0.025$

Table 20: Adenyl cyclase activity in human cultured skin fibroblasts

	pmoles cAMP/μg DNA/hr				
		stimulant			
strain	basal	epinephrine	epi.-insulin	NaF	(NaF-B)/B[a]
Normal					
45T8	10.900	39.840	12.849	175.379	1508.920
44T9	11.155	23.875	3.828	125.988	1029.430
529T9	16.379	45.169	12.182	297.287	1715.050
679T9	3.168	9.283	3.511	41.261	1202.360
606T8	13.665	26.078	1.132	160.903	1077.456
Diabetic					
42T7	15.754	62.469	8.139	269.407	1610.110
54T5	21.811	57.442	19.172	423.108	1839.880
55T7	7.106	12.186	5.153	81.988	1053.790
58T4	15.997	33.032	13.763	213.174	1243.560
Growth hormone deficient					
20T8	14.504	30.544	5.024	74.434	414.570
39T9	5.303	7.543	2.890	62.074	1062.951
40T9	5.603	8.403	4.535	46.431	728.750
41T9	9.079	13.158	4.548	31.179	243.430

[a] per cent stimulation by NaF over basal

Table 21: Comparisons of adenyl cyclase activity in normal fibroblasts

	df	SS	MS	F_s	F_s (without #606)
Basal vs epi. stimulation					
Between groups	1	791.7086	791.7086	7.0469	5.0324
Within groups	8	898.7866	112.3483	sign. $P<0.05$	sign. $P<0.070$
Basal vs epi.-insulin stimulation					
Between groups	1	47.3713	47.3713	1.7653	0.3818
Within groups	8	214.6793	26.8349	not sign.	not sign.
Basal vs NaF stimulation					
Between groups	1	55584.6200	55584.6200	12.9137	7.7997
Within groups	8	34434.5100	4304.3140	sign. $P<0.01$	sign. $P<0.050$

Table 22: Comparisons of adenyl cyclase activities in normal, diabetic and growth hormone deficient fibroblasts

	df	SS	MS	F_S	F_S (without #606)
Basal activity					
Normal vs diabetic					
Between groups	1	37.6035	37.6035	1.2682	1.3628
Within groups	7	207.5520	29.6503	not sign.	not sign.
Normal vs growth hormone deficient					
Between groups	1	13.1384	13.1384	0.6466	0.2835
Within groups	7	142.3327	20.3185	not sign.	not sign.
Epinephrine stimulated activity					
Normal vs diabetic					
Between groups	1	343.4840	343.4840	0.9906	0.6842
Within groups	7	2427.1060	346.7294	not sign.	not sign.
Normal vs growth hormone deficient					
Between groups	1	431.7849	431.7849	2.6360	2.2595
Within groups	7	1146.6286	163.8041	not sign.	not sign.

Table 22: continued

	df	SS	MS	F_s	F_s (without #606)
Epi.-insulin stimulated activity					
Normal vs diabetic					
Between groups	1	52.4092	52.4092	1.5765	0.7420
Within groups	7	232.8318	33.2617	not sign.	not sign.
Normal vs growth hormone deficient					
Between groups	1	13.3514	13.3514	0.7794	2.1843
Within groups	7	119.9017	17.1288	not sign.	not sign.
NaF stimulated activity					
Normal vs diabetic					
Between groups	1	16725.6428	16725.6428	1.2424	0.9625
Within groups	7	94229.9054	13461.4151	not sign.	not sign.
Normal vs growth hormone deficient					
Between groups	1	25268.5100	25268.5100	4.9770	3.8410
Within groups	7	35397.0500	5056.7214	sign. $P<0.10$	sign. $P<0.10$

Table 22: continued

	df	SS	MS	F_s	F_s (without #606)
Per cent change: Epi. over basal					
Normal vs diabetic					
Between groups	1	36.8361	36.8361	0.0044	0.0445
Within groups	7	57759.2410	8251.3201	not sign.	not sign.
Normal vs growth hormone deficient					
Between groups	1	19417.2272	19417.2272	4.2951	5.4579
Within groups	7	31644.9428	4520.7061	not sign.	not sign.
Per cent change: epi.-insulin over basal					
Normal vs diabetic					
Between groups	1	9.5503	9.5503	0.0081	0.2374
Within groups	7	8246.0657	1178.0094	not sign.	not sign.
Normal vs growth hormone deficient					
Between groups	1	673.3770	673.3770	0.5533	1.9183
Within groups	7	8519.5560	1217.0794	not sign.	not sign.

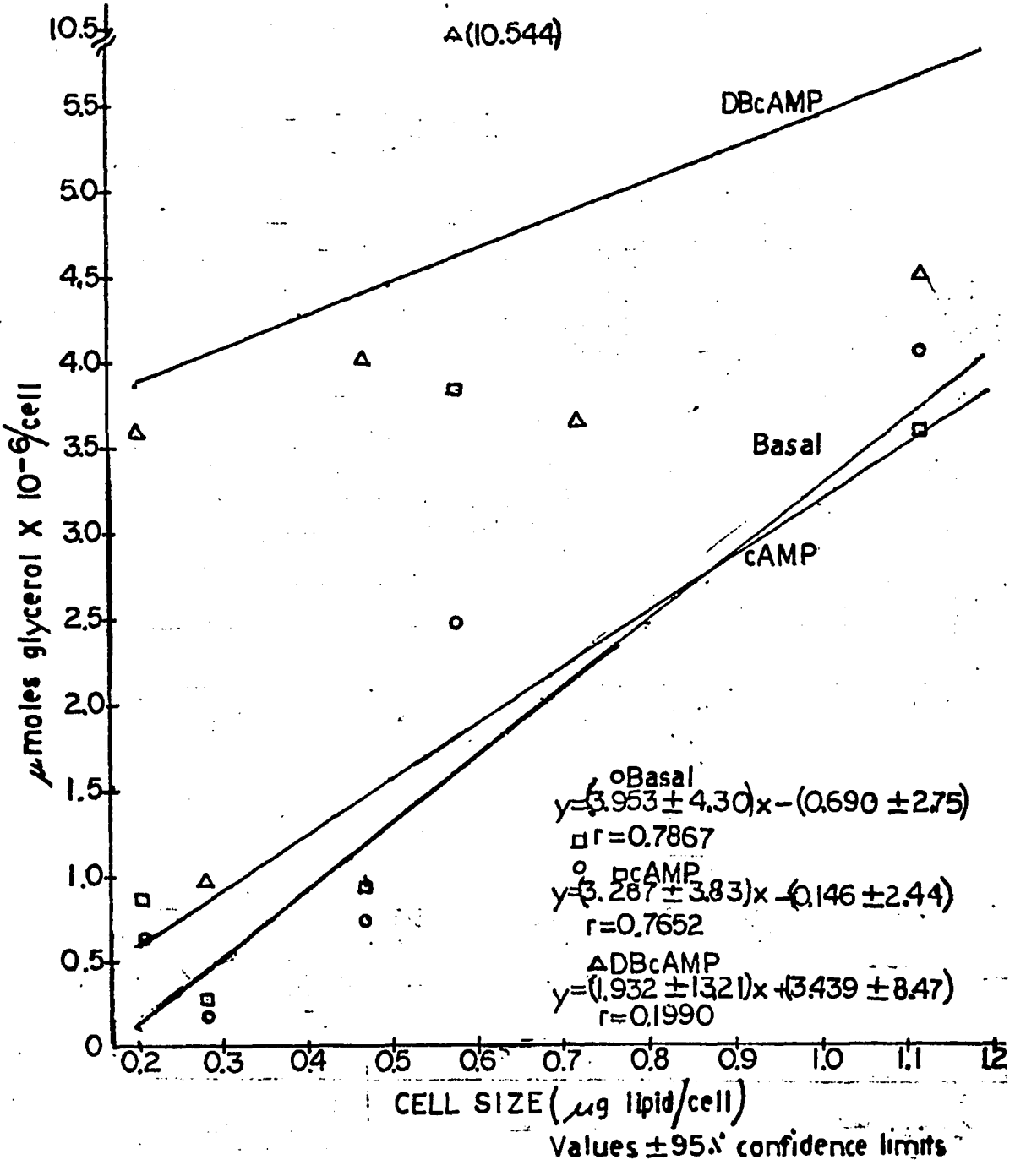
Table 22: continued

	df	SS	MS	F_s	F_s (without #606)
Per cent change: NaF over basal					
Normal vs diabetic					
Between groups	1	37666.4594	37666.4594	0.2344	0.0967
Within groups	7	1124509.1301	160647.1614	not sign.	not sign.
Normal vs growth hormone deficient					
Between groups	1	1070987.3310	1070987.3310	10.1340	10.0534
Within groups	7	739777.7468	105682.5353	sign. $P<0.025$	sign. $P<0.025$
Per cent change: epi.-insulin over epi.					
Normal vs diabetic					
Between groups	1	53.0034	53.0034	0.1985	0.0145
Within groups	7	1868.4999	266.9286	not sign.	not sign.
Normals vs growth hormone deficient					
Between groups	1	143.7980	143.7980	0.4966	0.0371
Within groups	7	2021.7220	288.8174	not sign.	not sign.

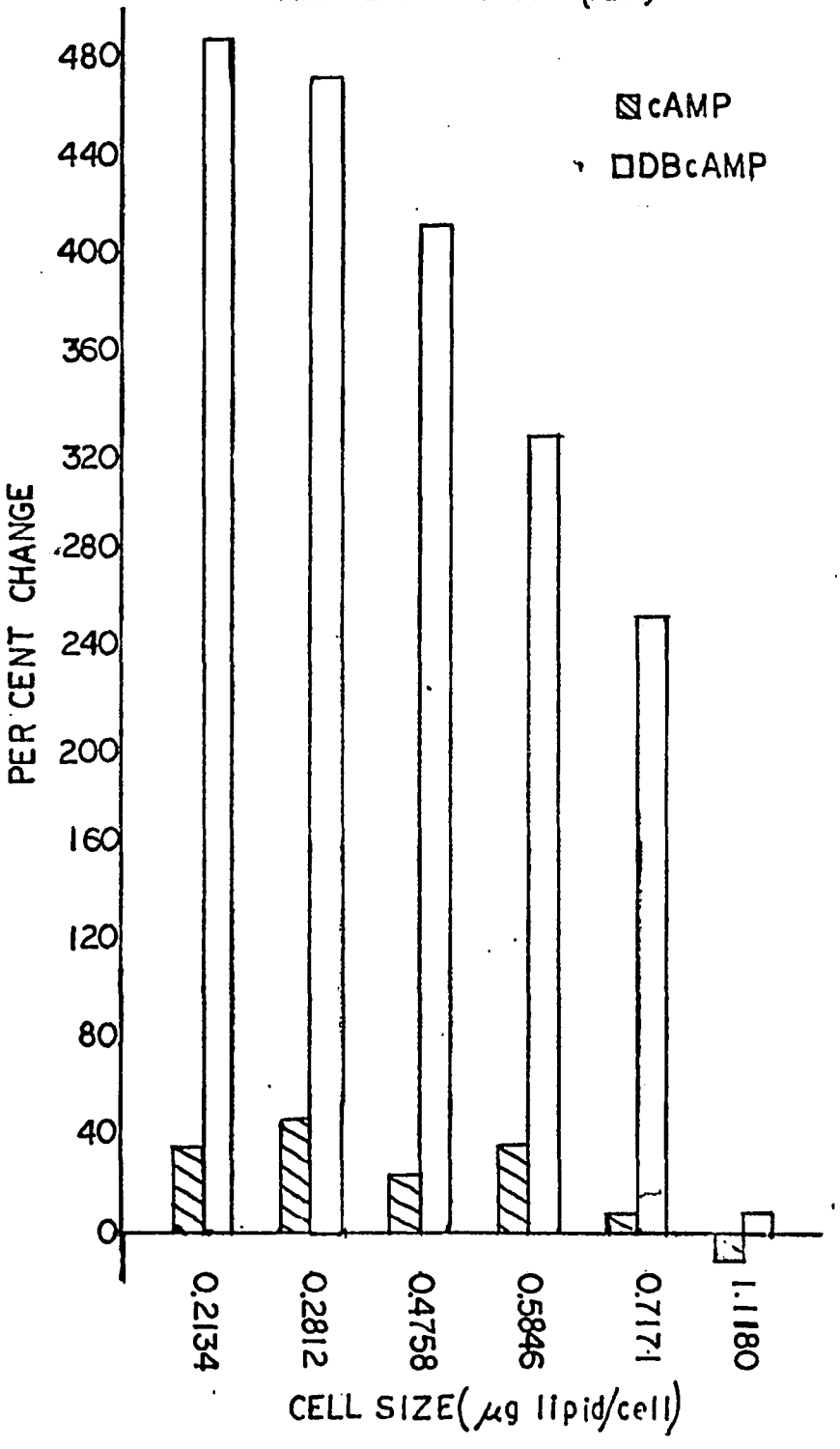
Table 23: $^{14}CO_2$ production in rat adipocytes with fibroblast medium

			cpm/cell		
date	strain	diagnosis	basal	insulin	% change
7/23	no cells	(incubated)	36.03	36.72	1.87
"	27	normal	69.75	84.37	21.00
"	31	growth hormone deficient	28.20	66.80	36.87
8/10	no cells	(incubated)	61.77	58.31	-5.6
"	30	normal	249.14	262.65	5.42
"	31	growth hormone deficient	70.11	166.57	137.60
10/4	no cells	(incubated)	40.71	10.27	131.68
"	32	normal	215.84	280.15	29.79
"	38	normal	122.72	169.53	38.14
10/12	no cells	(incubated)	6.70	6.78	1.19
"	no cells	(unincubated)	11.72	8.19	-27.00
"	25	normal	13.65	11.9	-12.82
"	31	growth hormone deficient	19.71	10.04	-49.00

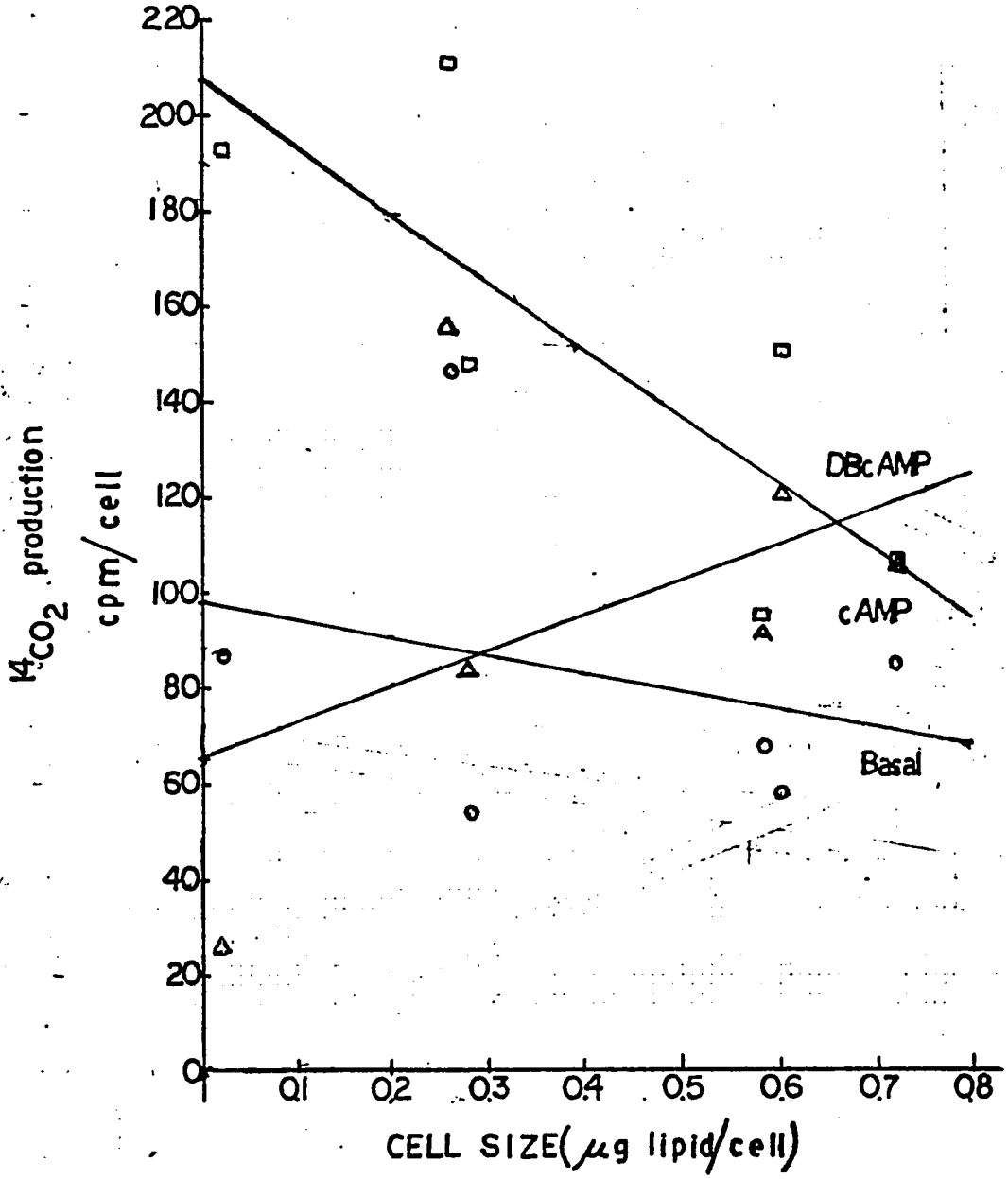
Graph 1: Regression equation for glycerol release as a function of cell size(rats)



Graph 2: Per cent change in lipolysis with cAMP and DBcAMP (rats)



Graph 3: $^{14}CO_2$ production as a function of cell size in rat adipocytes



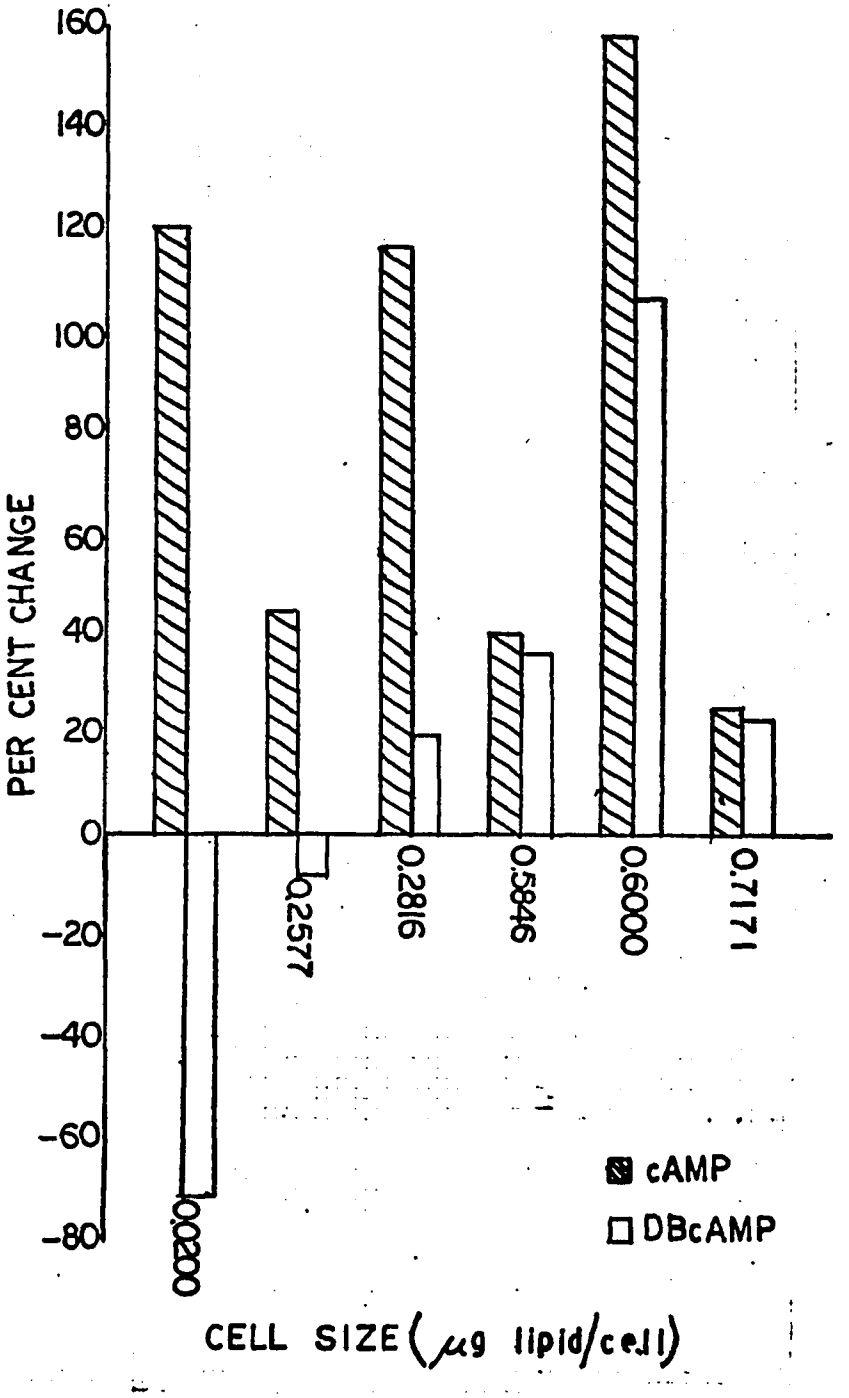
oBasal
$y=(-38.334 \pm 155.54)x+(98.778 \pm 79.87)$
$r=-0.3023$

□cAMP
$y=(-136.796 \pm 147.79)x+(207.008 \pm 70.34)$
$r=-0.7930$

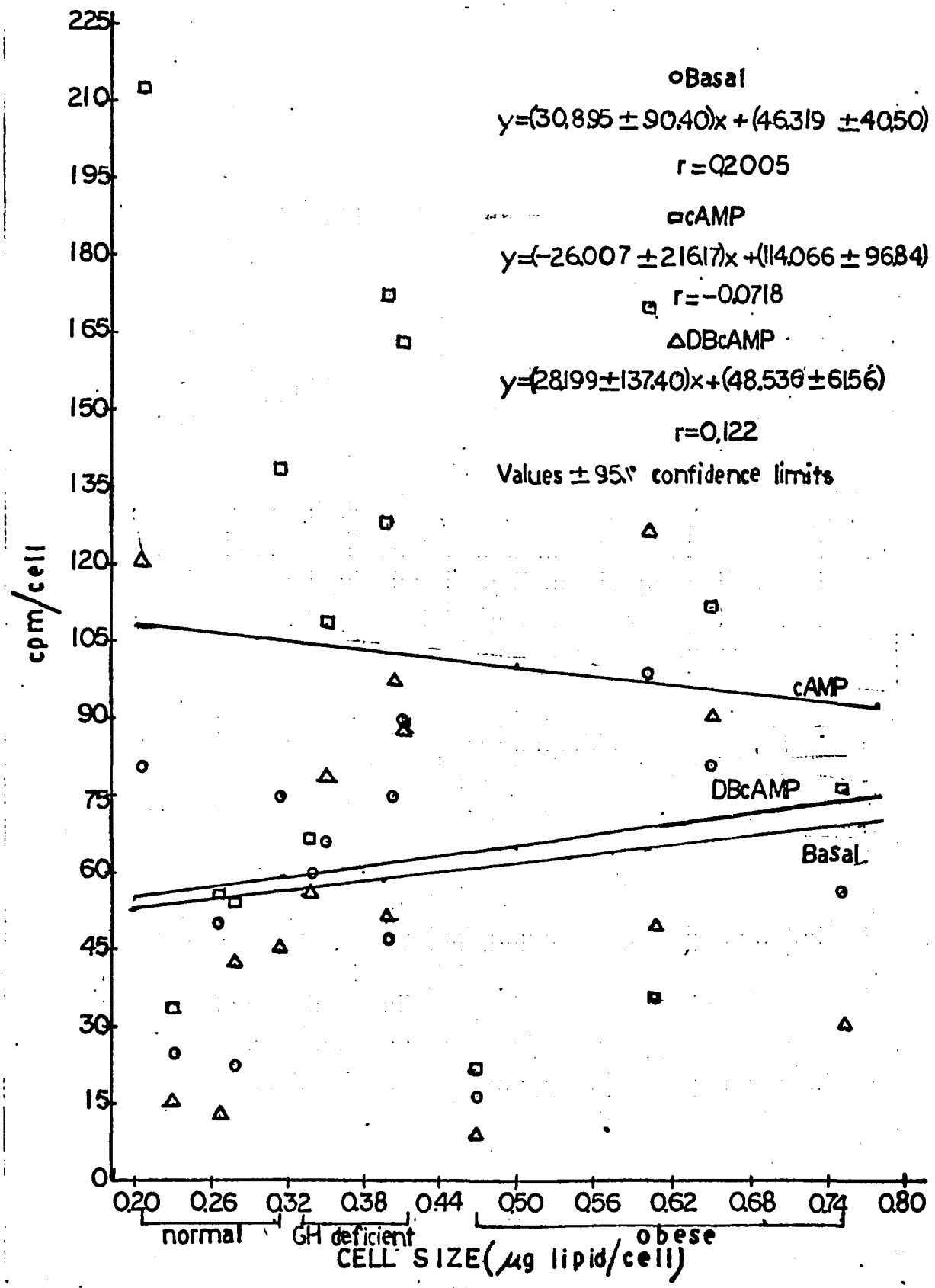
△DBcAMP
$y=(80.078 \pm 207.88)x+(65.069 \pm 96.55)$
$r=0.4808$

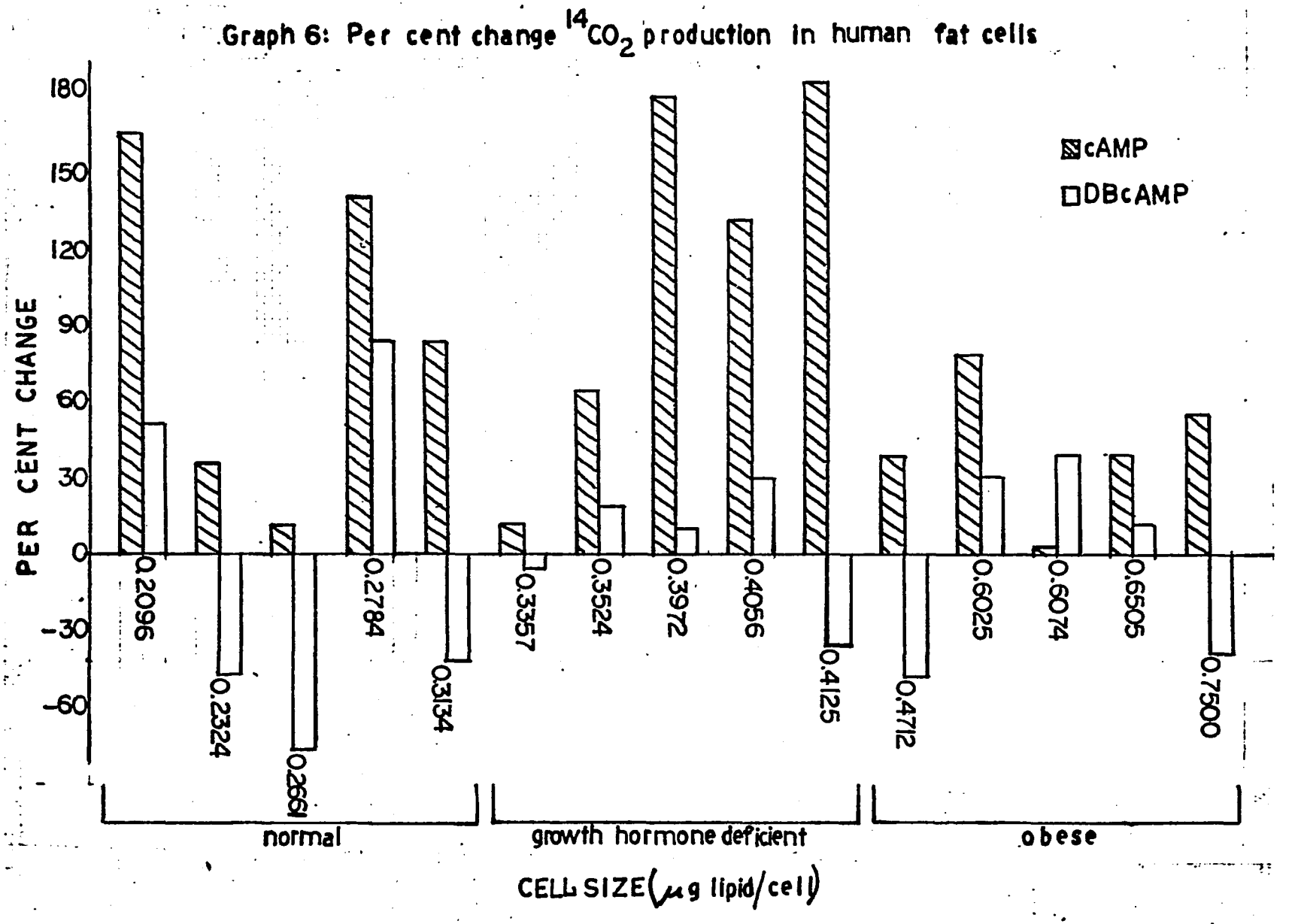
Values ± 95% confidence limits

Graph 4: Per cent change in rat adipocytes with cAMP and DBcAMP

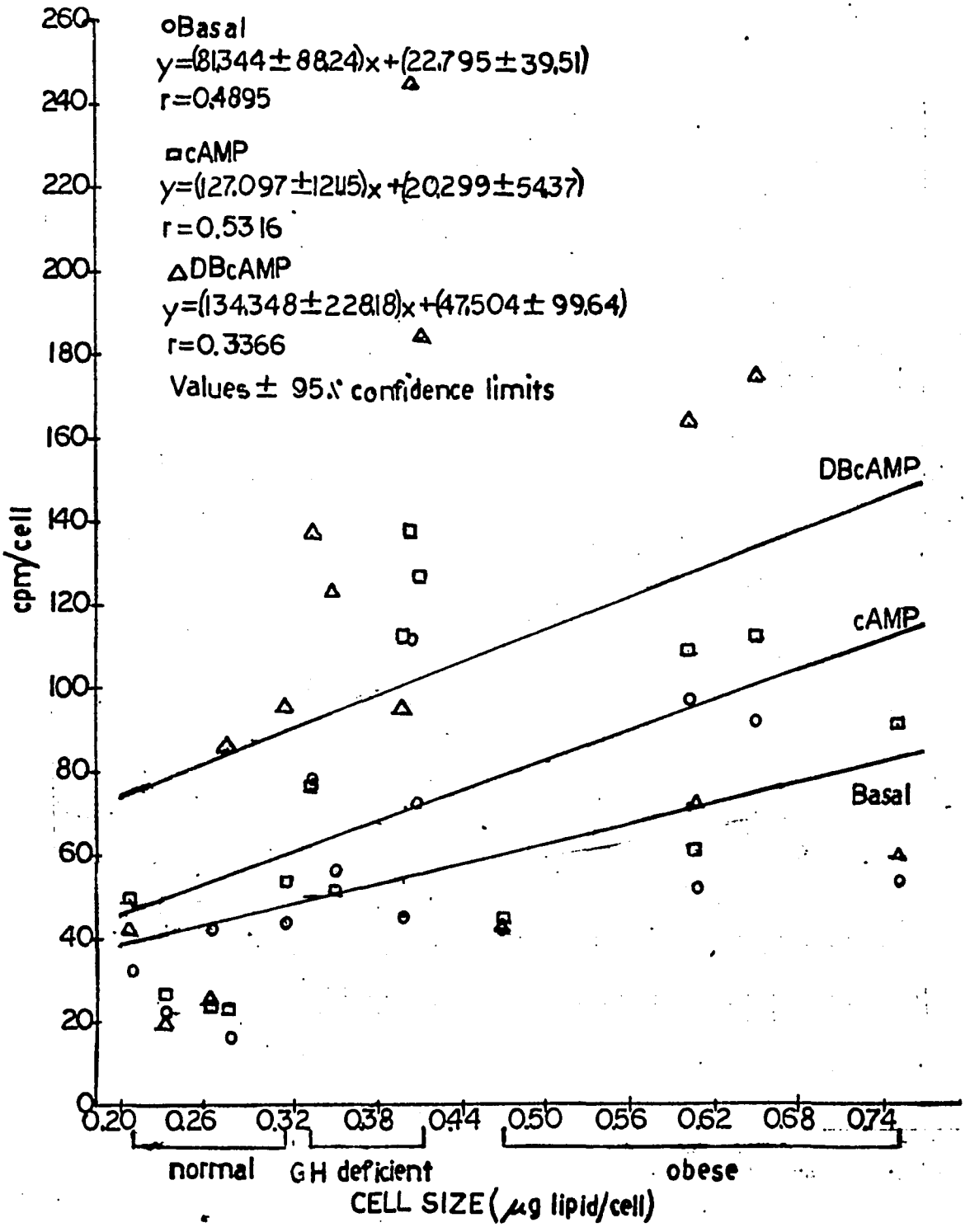


Graph 5: $^{14}CO_2$ production in human fat cells $^{14}CO_2$ production

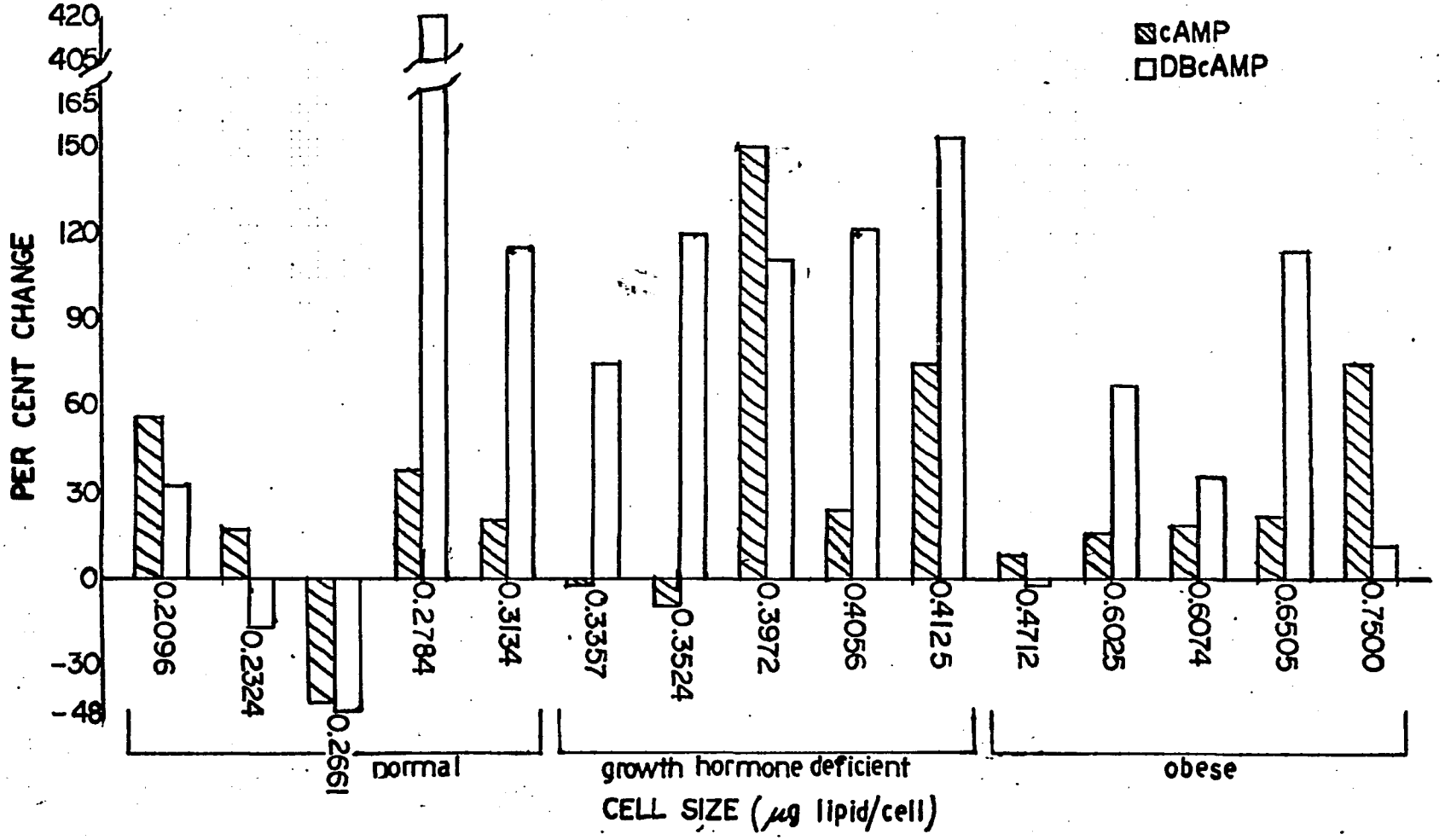



Graph 6: Per cent change $^{14}CO_2$ production in human fat cells
PER CENT CHANGE
180
150
120
90
60
30
0
-30
-60
cAMP
DBcAMP
0.2096
0.2324
0.2661
0.2784
0.3134
0.3357
0.3524
0.3972
0.4056
0.4125
0.4712
0.6025
0.6074
0.6505
0.7500
normal
growth hormone deficient
obese
CELL SIZE (μg lipid/cell)

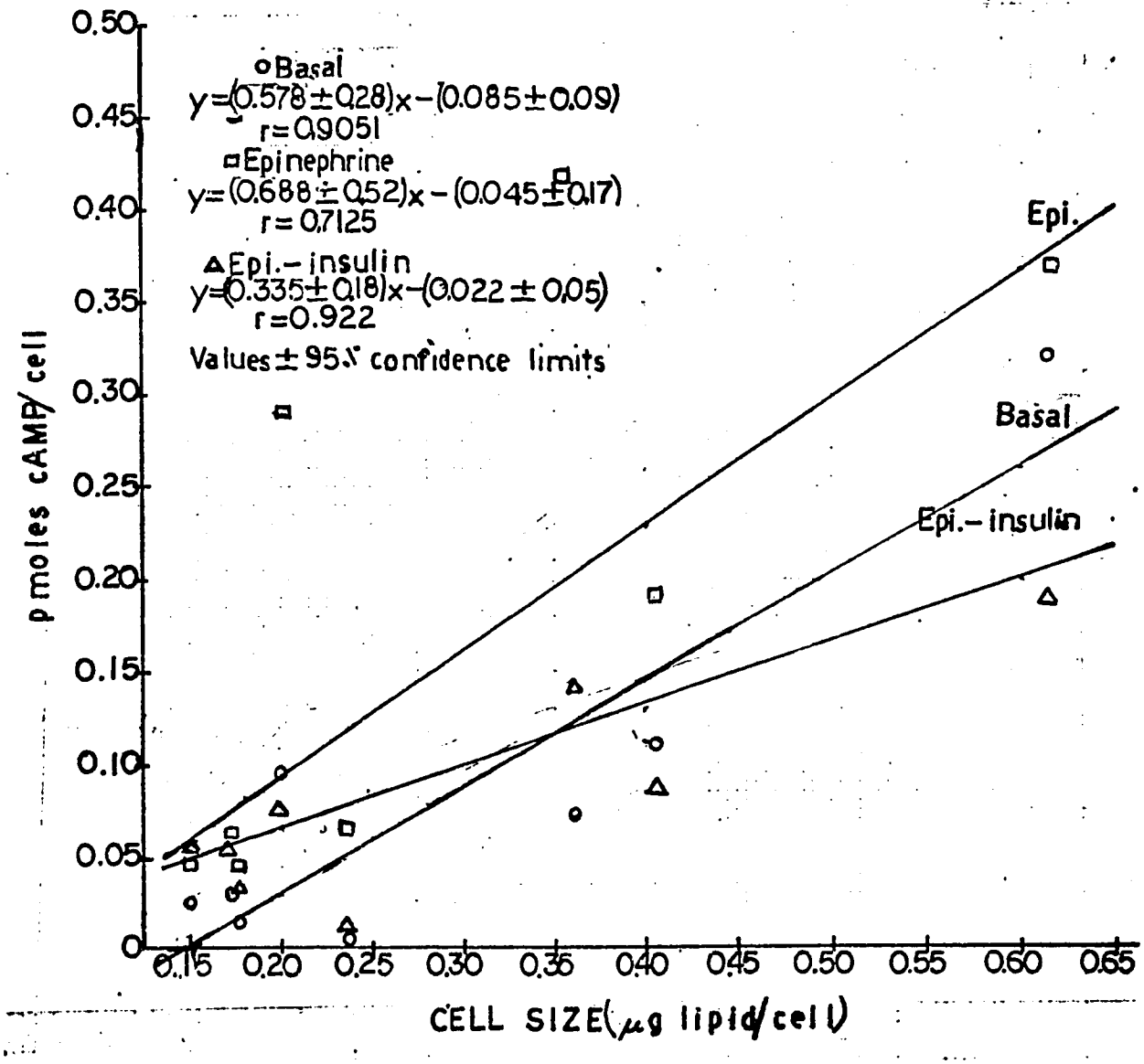
Graph 7: Incorporation of ^{14}C into triglycerides in human fat cells



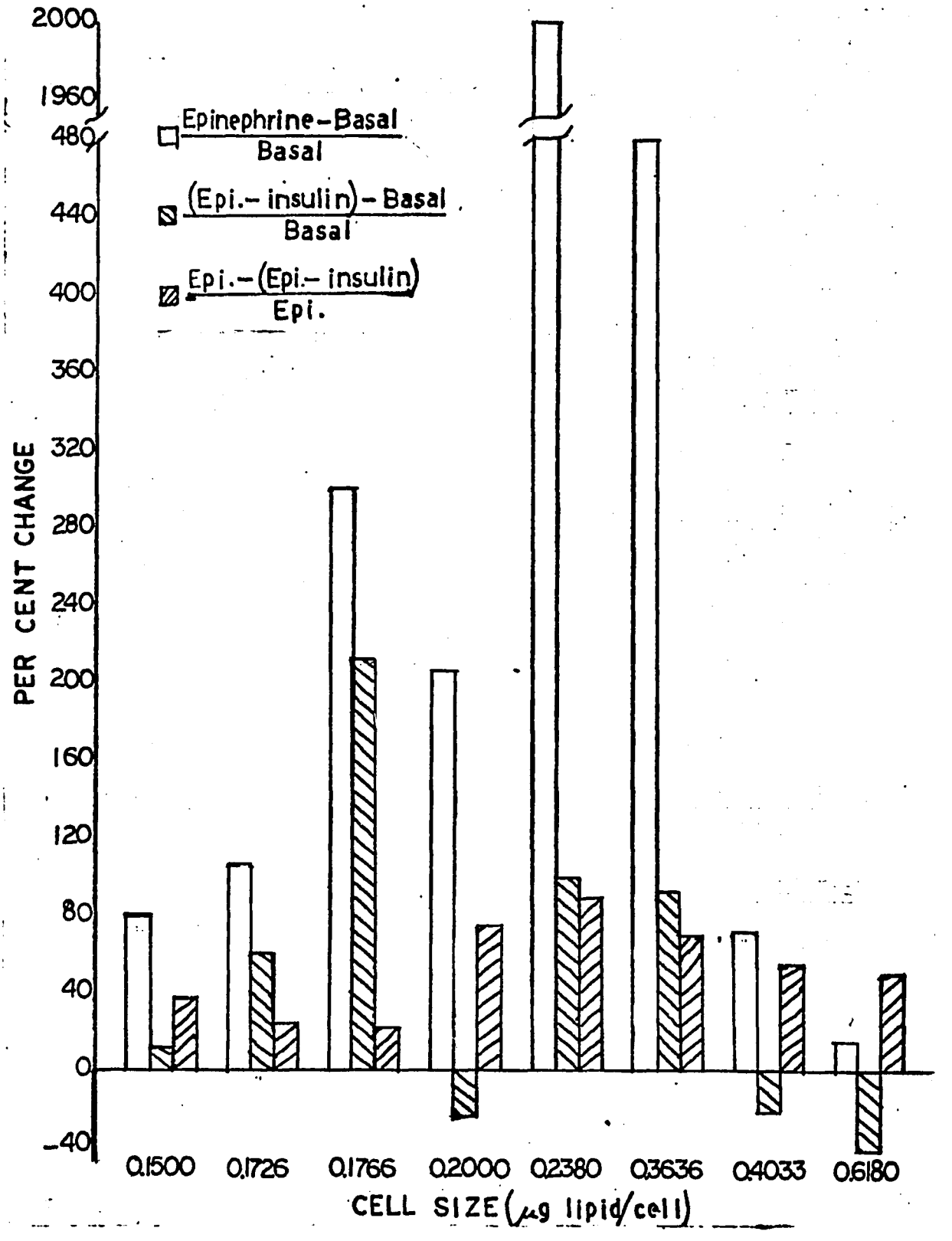
Graph 8: Per cent change in ^{14}C incorporation into triglycerides in human fat cells



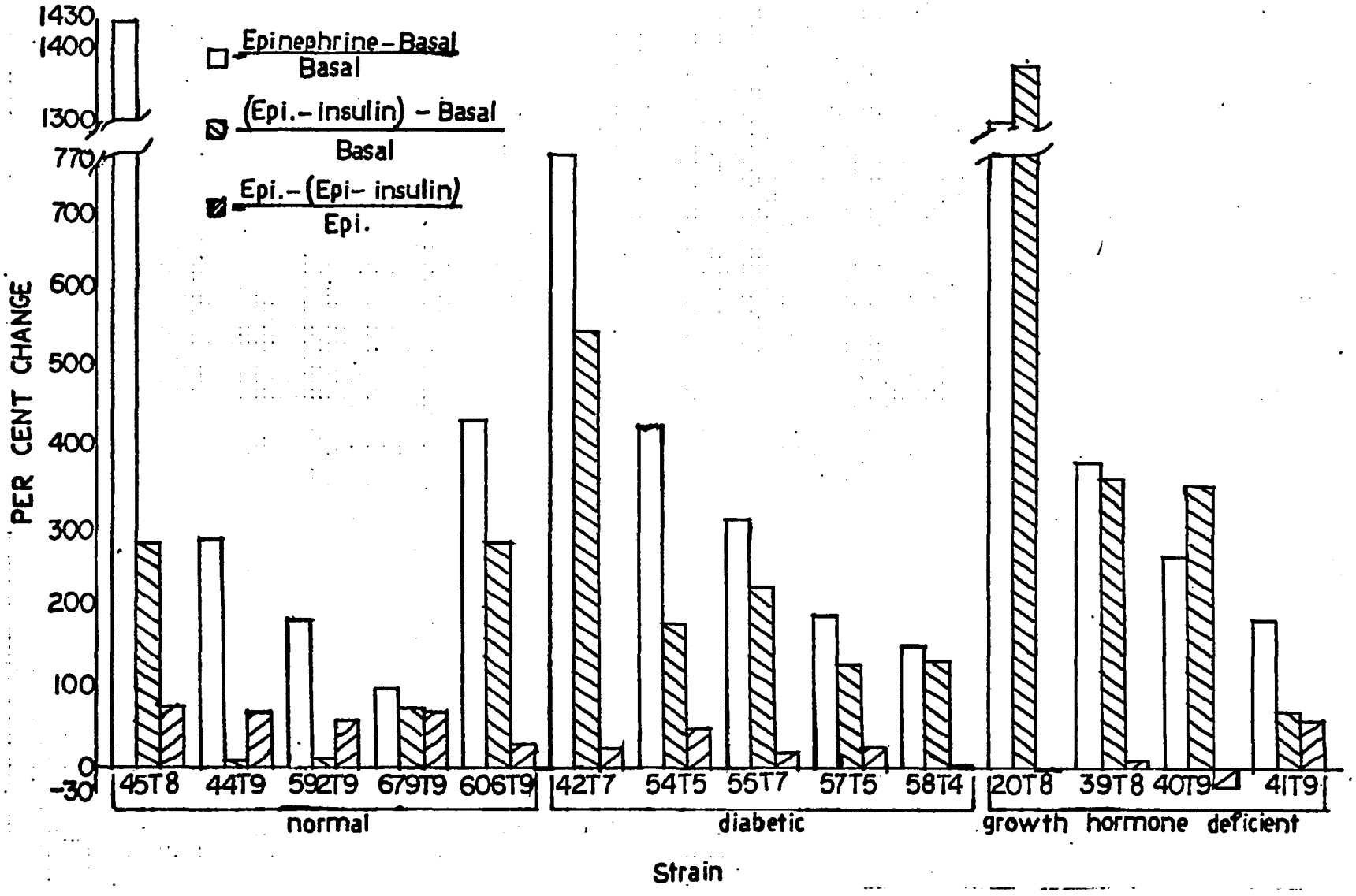
Graph 9: cAMP levels in rat adipocytes



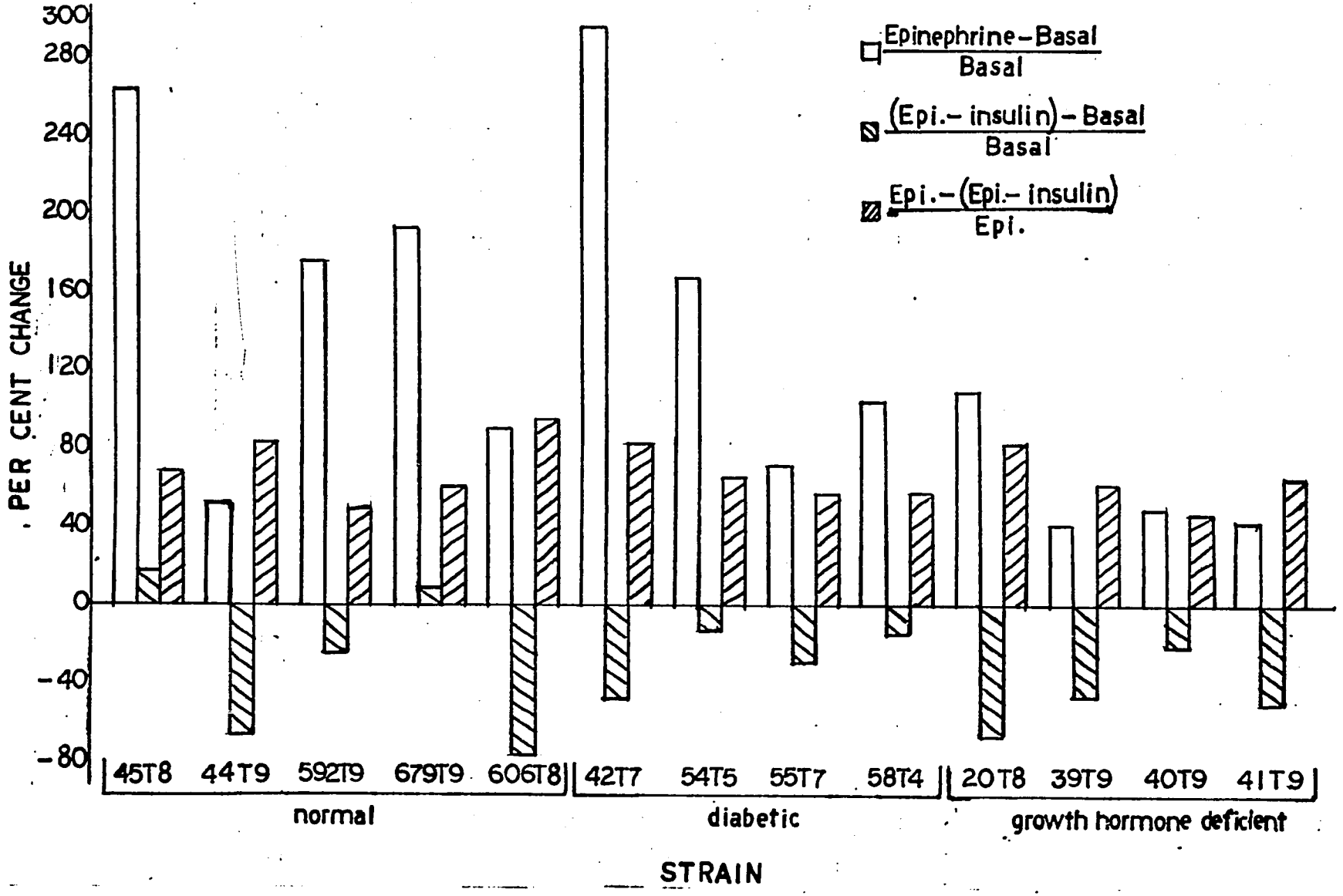
Graph 10: Per cent change in cAMP content by stimulation in rat adipocytes



Graph II: Per cent change in cAMP levels with stimulation in human fibroblasts



Graph 12: Per cent change with stimulation in adenyl cyclase activity/ hour in human fibroblasts



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