1953

The Uptake of Methionine by Tissues of Cancer-Susceptible Mice

Arthur Harvey Goldkamp

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Recommended Citation
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THE UPTAKE OF METHIONINE BY TISSUES 
OF CANCER-SUSCEPTIBLE MICE

by

Arthur Harvey Goldkamp

A Thesis Submitted to the Faculty of the Graduate School 
of Loyola University in Partial Fulfillment of 
the Requirements for the Degree of 
Master of Science

Loyola University Medical Center 
June 1953
LIFE

Arthur Harvey Goldkamp was born in Cincinnati, Ohio, August 8, 1929.

He was graduated from Chaminade High School, Dayton, Ohio, June, 1947, and began his undergraduate studies at the University of Notre Dame, Notre Dame, Indiana, the following September; he was awarded the Bachelor of Science degree, with honors, with a major in chemistry, June, 1951.

He began his graduate studies at Loyola University, Chicago, Illinois, in July, 1951.
ACKNOWLEDGMENT

The author wishes to express his sincere appreciation to Doctor Jacklyn B. Melchior for her suggestion of the problem and for her constant interest and aid throughout the course of the investigation.
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CHAPTER I  

INTRODUCTION

The relation of carbohydrate, protein and fat metabolism to cancer has been of great interest to biochemists for the last three decades. Many of the observations have been concerned with the differences in the levels of the products of metabolism in normal and cancer tissues. The mechanisms by which these differences occur are for the most part obscure and await a clearer picture of the initial biochemical aberration for their elucidation. Though these mechanisms may vary for different tissues, the biochemical characteristics of resulting malignancies are usually very similar; that is, most malignancies, with respect to their biochemistry, appear to converge toward a common tissue type (Greenstein, 1947). It is on this basis that information on the metabolism of various types of tumors is often included under the broader heading of "cancer metabolism." However, extrapolation of data to refer to cancer metabolism in general requires caution and only after having been shown to hold for a number of malignant types may any inclusive generalisations be made; even then, exceptions may arise. In its present status, therefore, the biochemical approach is largely directed toward an understanding of the aspects of general cancer metabolism. The majority of the investigations can be classed
into one of two main groups: (1) those concerned with the metabolisms of tumors, and (2) with the metabolism of the tumor-bearing host.

Among the earliest advances in the biochemistry of cancer tissue were those concerning carbohydrate metabolism. They are summarized in the classical experiments of Warburg (1930). His studies show that cancer tissue has an abnormally high rate of glycolysis when compared to normally functioning adult tissues. Since then there have been a number of observations indicating that the enzymes involved in the terminal oxidation of carbohydrate, the Krebs cycle, may be low (Potter, 1944). Cytochrome oxidase and succinoxidase activities, in particular, have been found to be lower in hepatomas than normal liver homogenates (Schneider and Hogeboom, 1950). However, it has recently been shown (Weinhouse, 1951) that fortification of tumor homogenates with high concentrations of diphosphopyridine nucleotide (DPN) resulted in their ability to oxidize oxaloacetate and pyruvate. These results have been confirmed by Potter and Lyle (1951). From a series of studies Potter and his associates have concluded that loss of oxidative capacity does not always accompany the homogenisation of normal tissue as it does with tumor tissue (for references see: Kameonik, 1952). The effects of homogenization and fortification with DPN might be represented as follows:

\[
\begin{align*}
\text{Substrate} & \quad \text{homogenization} \quad \text{Inactivated enzymes} \quad \text{Fortification} \quad \text{with DPN} \\
\text{O}_2 & \quad \text{DPN-enzyme complexes} \\
\text{CO}_2 & \quad \text{H}_2\text{O} \quad \text{ATP}
\end{align*}
\]
Electron (Dalton, Kahler, Kelly, Lloyd and Striebich, 1949) and ultraviolet (Annau, Manginelli and Roth, 1951) microscopic studies have shown that the mitochondria of many tumor cell types, including hepatoma, are physically quite different from those of normal liver cells. Damage to mitochondria resulting from the technique of homogenizing may vary from normal to cancer tissue and thus account for the differences in oxidative capacity observed. In reviewing some of the later work, Zamecnik (1952) states that there is no conclusive evidence for the qualitative lack in cancer tissue of any enzyme in the Krebs cycle. The problem may reside in either the special location of enzymes and coenzymes in the cell or in the relative concentrations of the various enzymes.

Cytochrome c, a necessary component for most oxidative mechanisms, has often been reported at quite low levels in tumor tissue; however, it is present in even lower concentrations in embryonic tissue, a perfectly good example of a normal, highly glycolysing tissue (Potter, 1944). In this characteristic, as well as in numerous others, it seems, biochemically, as though the hypothetical common tissue toward which most malignant tissues converge is a more primitive and less-differentiated type (Greenstein, 1947).

In studies on protein metabolism, malignant tumors appear to possess a high priority for amino acids, and, in the absence of an adequate diet, can grow at the expense of normal body tissues (Rider, 1951). In tissue slice experiments Zamecnik and co-workers have shown hepatoma to be more active than normal liver in incorporating amino acids into tissue protein, though it is no more active than fetal liver in this respect (Zamecnik, Frants, Loftfield and Stephenson, 1948). This indicates that
amino acid uptake by tumor tissue is a property related to growth rate rather than to the neoplastic process itself. However, some phase of protein metabolism may be involved in the actual transformation.

A study with cell suspensions has been made by Kit and Greenberg (1951b) in which the Gardner lymphosarcoma was compared to lymph nodes and to spleen from tumor-bearing and normal animals. Cell suspensions of the lymphosarcoma were found to be no more active in incorporating radioactive alanine or glycine into protein than were cells from any of the other three tissues. However, in intact animals the rate of glycine uptake by lymph nodes appears to be less than that of the Gardner lymphosarcoma (Norb erg and Greenberg, 1951). The reverse order has been noted in comparing in vivo to in vitro uptake by hepatoma tissue. Administration of labeled glycine by stomach tubes to rats bearing liver tumors resulted in less incorporation into tumor protein than into normal liver protein up to ninety-six hours after administration. However, the two values approached one another as the time from administration to sacrifice was increased (Griffin, Bloom, Cunningham, Teresi and Luck, 1950). On the other hand, hepatoma tissue, as mentioned previously, has been found to be more active than normal liver in tissue slice experiments (Zamecnik, Frauts, Loftfield and Stephenson, 1946). Tracer studies have shown that the vascular portion of the rat hepatoma is largely peripheral, extending only to a depth of about five millimeters, and that the anabolic activity of the tumor tissue is hindered by circulatory restrictions (Zamecnik, Loftfield, Stephenson and Steele, 1951).
In connection with these apparent contradictions of *in vitro* and *in vivo* experiments, it should also be mentioned that the *in vivo* studies are further complicated by the interactions of the tissues of the animal. Moreover, the uptake of label from different amino acids may not give the same comparative results. For example, though the uptake of methylene-labeled glycine *in vivo* has been observed to be slower for hepatome than for normal liver protein (Griffin, Moon, Cunningham, Teresi and Luck, 1950), tyrosine, on the other hand, has been found to be more rapidly incorporated into hepatome than into normal liver (Winnick, Friedberg and Greenberg, 1948).

Of great advantage in many of the methods employed in cancer research has been the use of highly inbred strains of mice with a high incidence of cancer; through their development and study, investigations on the genesis of cancer have been greatly facilitated.

The C3H strain was originated by Strong in 1920 (1935a and 1935b). The mice used in these studies are of Bittner's C3H subline obtained from Strong in 1931 after twenty-two generations of inbreeding (Bittner, 1935). Bittner has often identified this strain in his publications as strain I. The females of the strain have an incidence of spontaneous mammary tumors of over 90 per cent with an average tumor age of ten months (Bittner, 1943). The following discussion will be limited to this particular type of tumor and to the general characteristics of strains susceptible to its development.

In the numerous inbred mouse strains three factors are generally recognized as essential for the development of spontaneous mammary tumors.
They are: (1) the proper genetic constitution; (2) the milk factor; and
(3) hormonal influence (Shiskin, 1945).

The first of these, the proper genetic constitution, is a rather
evident requirement on the basis of the historical development of the various
strains susceptible to spontaneous mammary tumors. However, it might be
well at this point to make a distinction between susceptibility and in-
cidence. The incidence of mammary tumors is not a reflection of the genetic
susceptibility alone, or, for that matter, of either of the other two factors.
It is merely a measure of the degree to which the three factors approach an
optimum relationship to each other. Thus, any one or two of these factors
may be present without mammary tumor development.

The second requirement, the milk, or extrachromosomal, factor is
normally transmitted through the milk of the mother. It has been shown to
be present in mammary tumors, lactating mammary gland, thymus, spleen and
whole blood, though it does not pass through the placenta (Andervont, 1945).
Foster nursing of high-tumor-strain young by females of low-tumor strains
has markedly lowered the tumor incidence. However, it has also been shown
by suitable breeding experiments that the genetic susceptibility of the
individual is not altered by foster nursing. For example, foster nursing
of high-tumor-strain A young by females of low-tumor strain CBA lowered the
incidence of mammary tumors in breeding A females from 83 per cent to 3.1
per cent. When the foster-nursed males were mated to strain A females
carrying the milk influence, the offspring showed the same high incidence
as did strain A controls. This high incidence was followed for five
generations and showed no effect from the foster-nursed male ancestors
(Bittner, 1941 and 1943).

The resemblance of the milk factor to viruses has frequently been
noted. It differs from tumor viruses mainly in its relatively long latent
period. Susceptible mice ingesting this factor shortly after birth will
develop mammary tumors six to twenty-four months later provided the proper
hormonal influence is present. Usually this agent is transferred in the
absence of any recognized malignancy and this continues for many generations.
It might also be noted that, besides being effective only in susceptible
strains, the milk agent is also specific for a particular tissue (Andervont,
1945). These properties are the primary ones in which a similarity to
latent virus infections has been observed.

Finally, the hormonal influence has a rather easily demonstrable
effect on the occurrence of spontaneous mammary tumors. The specific
hormones to which this requirement refers are the estrogens.

Of historical interest are the findings of Loeb and his co-workers
from 1913 to 1924 that tumors were more frequent in breeding than in non-
breeding females and that the incidence of tumors could be radically reduced
by ovariotomy (Shimkin, 1945). The practical absence of mammary tumors
in male mice led to attempts to produce cancer in castrated males by
transplantation of ovaries. At first these attempts were unsuccessful,
probably because the strains used were not yet sufficiently homozygous for
survival and function of the transplanted tissue. Murray, in 1928, suc-
cceeded in obtaining an incidence of 7 per cent in castrated dba males
with subcutaneous implants of ovarian tissue from dba females.
With the advent of the isolation of the estrogen, Lacassagne, in 1932, reported the appearance of mammary tumors in male mice injected with these compounds. In the following decade it was completely established that the response in males of different strains was in direct relation to the incidence of mammary tumors in the corresponding females. The original interpretation was that the incidence in treated males depended on the genetic susceptibility of the strain. However, with the discovery of the milk influence (Sittner, 1936), it was realized that this incidence was equally dependent on the quantity and potency of the mothers' milk. For example, Andervont, in 1941, showed that introduction of the C3H strain milk influence into strain C females (1 per cent incidence) transformed the latter into a high tumor strain with 60 to 70 per cent incidence.

The general implications derived from inbreeding, foster-nursing and hormone studies are highly significant in establishing the effects of three primary factors for mammary tumorigenesis in mice. However, the difficulties involved in determining the nature and extent of these factors in various strains are numerous. The interaction of these factors add further complications in attempting to interpret the known facts. The genetic constitution is not merely involved in determining the susceptibility at the tumor site, but must also have a profound influence on the various endocrine secretions and, consequently, on the hormonal influence. In addition, the milk factor, when transferred from high-tumor to some low-tumor strains, is capable of propagation; in others, it dies out in the following generations. Finally, environmental factors also exert some influence and add to the difficulties of interpreting experimental data.
Concerning the naturally occurring factors in tumorigenesis, Shinakin, in 1945, made the following observation:

The quantitative aspects of the problem are far from clear, particularly in reference to the interrelationships of the various endocrine secretions, and to the relationship between the hormones, the milk influence, and the genetically determined factors of susceptibility.

An attempt has been made to give a condensed presentation of two important phases of cancer research. The first of these concerned the application of biochemical techniques and was primarily restricted to those investigations which are directly concerned with cancer metabolism.

Secondly, the genetic approach has done much to establish the predisposing factors involved in mammary tumorigenesis in mice. Though a knowledge of these factors is essential to a complete understanding of cancer, no conclusions can be reached from this method alone regarding any precancerous changes closely connected with, or involved in, the actual transformation.

In considering the possible influence of factors other than the three previously mentioned, the known effect of the estrogens in mammary tumor development has led to a certain amount of speculation concerning the role of the other endocrine organs. The pituitary, in particular, because of its general control over the rest of the endocrine system, has been of interest. For example, transplants of anterior lobe tissue have been found to increase mammary tumorigenesis, though this effect was not noted after ovariectomy (Loeb, Burns, Sutinoff and Moskap, 1937). There is no reason at present, however, to suspect that the hormone influence results from any such endocrine imbalance; that is, from any non-physiological state of
the endocrine system such as that produced with transplants of pituitary tissue. However, certain studies indicate that the pituitary may play some part in addition to its normal control of estrogen secretion.

The earliest investigations of the pituitary were made on cancerous rather than precancerous animals. X-ray treatment of the pituitary region was found to retard the growth of spontaneous, transplantable and 3,4-dibenzpyrene-induced tumors (Bischoff, Maxwell and Ullmann, 1934). With rat carcinomas 256 the growth rate was restored by anterior lobe extracts. Also, growth of the Jensen carcinoma was found to be inhibited by hypophysectomy (Salye, 1944). The ways by which such treatments affect tumor growth are not known.

However, more recent evidence may be cited which points toward a direct pituitary influence in carcinogenesis. Numerous neoplasms have been obtained by treating rats with the growth hormone, but, despite continuous body growth, these neoplasms were not obtained with hypophysectomized rats (Moon, Simpson, Li and Evans, 1951). The daily dosage of growth hormone was so large (0.4 to 2.5 milligrams) that the absence of normal growth hormone secretion in the hypophysectomized rats is negligible. Therefore, some other pituitary influence must also be involved. Inhibition of methylcholanthrene carcinogenesis by hypophysectomy has also been reported (Simpson, Evans and Moon, 1952). Even in cancerous animals the growth hormone has been found to exert an effect; increase in tumor weight was considerably greater in treated than in control animals bearing transplantable mammary adenocarcinomata (Smith, Slattery, Shiskin, Li, Lee, Clarke and Lyons, 1952).
An earlier study by Wolfe and Wright (1943) may indicate a "precancerous" pituitary condition. They made histological comparisons of the anterior hypophyses of two strains of rats, one of which (the Albany strain) is characterized by a high incidence of mammary fibroadenomas. They found that the eosinophile level was lower and the chromophobe higher in the Albany rats than in the normal Vanderbilt strain. Other characteristics, such as low fertility, failure of ovulation and an abnormal estrous cycle, were also noted.

In view of these cancer-pituitary relationships, it seems quite possible that the pituitary bears some fundamental relationship to cancer development other than its control of estrogen production. However, no definite proof of any such relationship exists.

The effect of the estrogens on mammary tumor development is attributed primarily to their stimulation of mammary gland development. The rarity with which cancer develops in a previously unaltered tissue has been mentioned by Dunn (1945). Studies on the mammary duct system in male mice treated with crude estrone preparations have shown the necessity of rather extensive duct development prior to the appearance of mammary cancer (Gardner, 1935). This *in vivo* action of the estrogens is believed to be primarily an indirect one, since they appear to elicit from the pituitary hormones which are referred to as mamrogen I and mamrogen II (Best and Taylor, 1950). In hypophysectomized animals estrogens cause little, if any, mammary proliferation unless prolactin is also administered (Gardner and White, 1941). The mamrogenic hormone is thought to be a component of prolactin, or crude lactogenic hormone preparations. However, if pro-
gesterone or desoxycorticosterone is given along with estrogen to hypophysectomized mice some mammary growth is noted (Gardner, 1960). That estrogens do not act exclusively through some intermediary process in the pituitary was confirmed by painting experiments with mice. When estrone was applied to a single nipple, localised proliferation was found to occur (Gardner and Chamberlin, 1941).

The measurement of amino acid uptake by tumor tissue has already been discussed. To the author's knowledge, however, this type of study has not been applied to the precancerous metabolism of tissues from cancer-susceptible mice. The method has been successfully used in determining the relative concentrations of protein-synthesizing enzymes in pituitary tissue from male and female rats of varying age and physiological condition (Melchior and Halikis, 1952). Considerable evidence has been accumulated for the enzymic nature of in vitro amino acid incorporation into protein. For example, aside, fluoride and cyanide, typical respiration inhibitors, have been found to inhibit the uptake of labeled methionine by Escherichia coli (Melchior, Rellody and Klots, 1948), while glucose and succinate increase the uptake of certain amino acids by cells of the Gardner lymphosarcoma (Kit and Greenberg, 1951a). Other observations on stimulation of amino acid uptake by ATP and citrate and inhibition by dinitrophenol have been summarized by Tarver (1952). The uptake of amino acids by tissues, therefore, appears to depend upon a source of ATP, either directly, or indirectly through operation of respiratory systems. In addition, net productions of albumin in chicken slice experiments and amylase by slices of pigeon pancreatic gland have been observed by Peters (1953) and Nokin (1951), respectively.
CHAPTER II

STATEMENT OF THE PROBLEM

Female mice of the C3H strain have been shown to exhibit a high incidence of mammary tumors, while the males of the strain will not develop these tumors unless estrogens are administered. Also, a factor normally transmitted to the young through the milk of the mother has been shown to be essential for mammary tumor development. This investigation is concerned with an attempt to relate these phenomena to the rate of incorporation of an amino acid into protein in vitro by two tissues closely related to the development of tumors.

The protein-labeling capacities of pituitary tissue and tissue from the site of expected tumor development have been chosen as useful indices for any precancerous changes in protein metabolism which might occur in these tissues. These studies deal in part with a comparison of the protein-labeling capacities of these tissues from two groups of C3H females, one of which was not subjected to the milk influence.

The effect of sex and sex hormones on amino acid uptake by protein of the tissues mentioned above constitutes another phase of the problem. Finally, the protein-labeling capacities of other tissues more frequently studied have been included for the purpose of orientation.
CHAPTER III

MATERIALS AND METHODS

Animals

Mice of Bittner's C3H subline (Bittner, 1935) were used throughout the study. The majority were received from the Jackson Memorial Laboratory at four to five weeks of age, though some of the mice used in the later experiments were first-generation offspring of our colony. The TC3H females (transplanted, foster nursed C3H females) were also obtained from the Jackson Memorial Laboratory; these mice, lacking the milk factor, do not develop tumors, and were used for comparison with the cancer-susceptible C3H females. Some of the tumor-bearing C3H females were received at ten to eleven months of age; others had developed tumors while in our colony. Adult mice were used in all experiments. Their diet consisted of Purina Foxchow standard pellets and water fed ad libitum up to the time of sacrifice.

Hormone Injections

Male mice of three to four months of age were injected subcutaneously in the back with twenty-two micrograms of estrone suspended in a total of one-tenth milliliter Nasola oil; control animals were injected with Nasola oil alone. Injections were given once a day, five days a week. The amount of estrone injected was based on a study by Burns and Schenken (1940).
in which one hundred rat units of estrogen per week administered for six-
teen to twenty weeks gave a maximal incidence (approximately 50 per cent) of
mammary cancer in C3H males; this corresponds to approximately sixty
micrograms of estrone per week. Since the purpose of the experiments re-
ported here was to determine any precancerous effects which might arise, the
total weekly injection was increased and the total period of injections
decreased. The number of injections up to the time of sacrifice is given
in the results.

Removal of Tissue

The animals were sacrificed either by decapitation or by breaking
the neck with a pair of blunt scissors. Both of these methods killed the
animals within ten seconds, thus minimizing any stress reaction.

For each pituitary measurement, the glands were removed from four
to six animals and weighed on a Roller-Smith torsion balance. The weights
of the glands varied from 0.75 to 2.85 milligrams.

In other experiments a precancerous tissue was chosen which
corresponded to the normally expected site of tumor development. In the
females this tissue consisted of a piece of skin approximately five milli-
meters square containing and surrounding the nipple. Since any precancerous
changes in the activity of this tissue should be due mainly to the mammary
tissue which it contains, it will be referred to hereafter as mammary tissue
alone. This choice of tissue, for use in in vitro experiments, was based on
the practical absence of distinctive mammary tissue in non-pregnant mice.
This practical difficulty has also been reported for the rat (Essential and
Drabkin, 1943). In the case of recent mothers, the young had been weaned
within the previous week; for purposes of comparison the same choice of
tissue, also to be termed "mammary" tissue, was maintained. In the males,
tissue was chosen from the same area. This tissue, though probably con-
taining a rudimentary mammary duct, will be referred to as skin tissue; this
term will also be used for the same tissue from hormone treated males. The
tissues described above were cut away after first stripping the skin from
the underlying subcutaneous layer. Four or five pieces of mammary tissue,
or two to four pieces of skin, all from a single animal, were used in each
measurement. In the experiments on the in vitro effects of hormones on amino
acid uptake, corresponding tissue from the same animal was used for a control
value.

For measurements on liver and tumor slices a standard tissue
slicing technique was employed. Either one or two slices was used in each
determination, depending upon the size. For measurements on subcutaneous
tissue, the slices were taken from the tissue immediately beneath the skin.
In the case of diaphragm, a naturally occurring tissue slice, one half of
the whole muscle was used for each measurement.

Incubation Period

The tissue for the determination was placed in the center com-
part ment of a Warburg flask which contained one-half milliliter Singer's
bicarbonate buffer (Welchier, 1946) supplemented with one milligram sodium
succinate hexahydrate. In determining the in vitro effect of hormones on
amino acid uptake, one to three micrograms of hormone in a solution of
approximately 10 per cent ethyl alcohol in water was evaporated to dryness
in the center compartment at eighty to ninety degrees centigrade prior to
the experiment. Pituitary glands were either partially severed at their
midpoint or completely cut in half with the point of a dissecting needle as
they were placed in the flask.

One-half milliliter of Singer's bicarbonate buffer containing one
micromole $^{35}$S-labeled L-methionine, was placed in the side arm of the
Warburg flask. This compound was obtained from Doctor E. L. Tabern of the
Abbott Laboratories and had a specific activity of 12.6 microcuries per
gram as of July 20, 1952.

The flask was then placed in a water bath maintained at 37.5
degrees centigrade and shaken at one hundred twenty revolutions per minute.
Since the buffer used was originally equilibrated with a mixture of 5 per
cent carbon dioxide and 95 per cent oxygen, the system was evacuated and
filled with this gas mixture three times in order to maintain the buffer
composition. The contents of the side arm were then tipped into the main
chamber to initiate the reaction. The final pH was 7.40 to 7.45.

The final concentration of $^{35}$S-L-methionine was one micromole per
milliliter. Using the data of Simpson and Tarver (1950) with liver slices,
this corresponds to excess substrate concentration and should give a linear
rate of synthesis up to four hours. Linearity was confirmed with one and
two hour determinations for skin tissue. Also, preliminary two hour
pituitary measurements with a final concentration of one-half micromole
$^{35}$-dl-methionine per milliliter gave results practically identical with
those employing one micromole L-methionine, thus indicating maximal sub-
strate concentration.
In the experiments on the effect of previous hormone injections on the uptake by skin tissue the reaction was initiated within ten minutes after sacrifice of the animal. In the other experiments the tissue remained in the center compartment of the flask in the Ringer's bicarbonate buffer at room temperature from thirty to ninety minutes before initiating the reaction, depending on the procedure used. This "pre-incubation" time had no observable effect on the uptake during the reaction period.

Homogenisation, Washing and Hydrolysis

Following the incubation period (one or two hours as noted) the flask was removed from the bath and one milliliter of 0.67 M (10 per cent) trichloracetic acid (TCA) added to stop the reaction. The contents of the flask were then transferred with three washings of 0.33 M TCA to a fifty milliliter centrifuge tube. The tube was then centrifuged and the supernatant liquid decanted from the whole tissue. The tissue was then transferred in the manner described above to an all glass homogenizer as described by MoShan and co-workers (MoShan, Davis, Soukup and Meyers, 1950). After homogenizing, it was returned, with liberal washing, to the original centrifuge tube, centrifuged, and the supernatant liquid decanted. The homogenized tissue was washed again with 0.33 M TCA in the same manner; the tissue in this and the following washings was treated directly in the centrifuge tube.

After decanting, ten milliliters of tenth normal sodium hydroxide was then added to dissolve the protein material and free any absorbed methionine not accessible to TCA alone. This resulted in a slightly turbid solution with pituitary, liver and diaphragm and somewhat greater turbidity
with skin and mammary tissues. Two drops of phenolphthalein indicator was then added, followed by dropwise addition of two normal hydrochloric acid until the color change was observed. Ten milliliters of 0.67 N TCA was then added and the mixture allowed to stand for one hour to ensure complete precipitation of protein material. The tube was then centrifuged, the supernatant liquid decanted, and the residue redissolved, reprecipitated, centrifuged and the supernatant liquid decanted as before. In all of the steps in the washing procedure the volume in the tube before centrifuging was twenty milliliters. Following this final washing, two milliliters of a solution containing 12 per cent formic acid in concentrated hydrochloric acid (Miller and Du Vigneaud, 1937) was added to the residue. Hydrolysis was effected by heating under an air condenser at 100-105 degrees centigrade for thirteen to fifteen hours.

At the end of this period the hydrolysate was evaporated to dryness on the steam bath with the aid of sublimation and a correspondingly reduced pressure. Ten milliliters of distilled water was then added and the solid material stirred to dissolve the products of hydrolysis. Any labeled cystine or cysteine produced from the methionine during the incubation was then removed by the method of Zittle and O'Dell (1941) which involves precipitation as the cuprous salt. Two and four-tenths milligrams of carrier cystine in ten milliliters of water was added prior to the addition of cuprous oxide, which was used in slight excess. The tube was then centrifuged and the supernatant liquid (twenty milliliters), containing methionine as the only labeled material, decanted into a fifty milliliter beaker. The residue was washed once with ten milliliters of 0.035 N acetate buffer of pH 4.3 to 4.5,
corresponding closely to the point of least solubility of the cuprous salts. After centrifuging, this supernate was also decanted into the beaker. The beaker content was then evaporated to dryness on a steam bath and the residue quantitatively transferred with the aid of tenth normal hydrochloric acid to a paraffin planchet one and one-quarter inch square, the inside bottom surface of which was completely covered with a square of Whatman No. 1 filter paper. Final evaporation in the planchet occurred at room temperature. The sample was counted with a proportional counter and an internal counting tube. In each experiment eight determinations were run in parallel through the experimental procedure described above.

Zero time controls were run by adding TCA immediately before or after tipping in the methionine; the order had no effect on the zero time values. In some cases the contents of the flask were incubated for two hours and in others started directly through the washing procedure. This variation also had no effect on the zero time value. The zero times measure the physically adhering and non-biologically incorporated methionine. This value was always less than 10 per cent of the total incorporation and for the more highly active tissues it was less than 5 per cent.

**Counting Procedure**

The final sample for counting, consisting of tissue hydrolysate and small amounts of the copper and sodium salts of the reagents previously mentioned, presented a rather heterogeneous sample for assay. At this point it seemed highly desirable to circumvent the lengthy procedure usually adopted which involves digestion followed by precipitation and counting of the label as the barium or benzidine sulfate (Henriques, Kistiakowsky,
Margonetti and Schneider, 1946). Therefore, a method for direct plating of the heterogeneous mixture was chosen. However, to obtain quantitative, as well as comparative results, it was first necessary to investigate the absorption characteristics of a number of substances in order to correct for the total weight of sample.

The method used to investigate the counting procedure involved the addition of given amounts of various absorbing materials to parafilm planchets lined with filter paper, containing a constant amount of radioactive methionine. This same procedure was also used without filter paper in ordinary aluminum planchets 3.6 centimeters in diameter; in this way a comparison was made to a more common method of sample preparation (Heuvel, Roth, Leifer and Langham, 1946). In all cases the level of liquid in either type of planchet was adjusted to the same depth. The samples were allowed to evaporate at room temperature and then counted. The per cent total (control) activity, or its log, was then plotted as a function of total added absorber. The control samples consisted of radioactive methionine alone in amounts giving a total activity of the same order of magnitude as those observed in the experiments. All samples were counted for a sufficient length of time to give a total of one thousand counts or more, and this was repeated at least once for each sample; this corresponds to a counting error of 3 per cent or less.

It is important here to realize that the absorption is not necessarily a simple function of the total weight of absorbing material, but also depends on the substances present. This is brought out in Figure 1 which shows the extreme dependence of absorption on the absorbing material
% TOTAL ACTIVITY

FIGURE 1

EFFECT OF INERT ADDITIONS ON THE COUNTING RATE OF $^35$S-LABELED METHIONINE SAMPLES PREPARED BY EVAPORATION IN ALUMINUM PLANCHETS

Each point represents an average of ten separate samples.
when the sample is plated out in aluminum planchets without filter paper.
The rapid drop in activity on addition of very small amounts of absorber
should be borne in mind for comparisons with the use of the parafilm planchets
with paper.

In Figure 2, self-absorption curves, obtained by adding inactiv
dl-methionine, are given for both the aluminum and paper-lined parafilm
planchets. The slight initial rise in activity shown here (Figure 2) for the
parafilm-filter paper planchets is characteristic of a phenomenon referred to
as self-focusing. For window counters, it has been suggested that this is
due to the deflection of angular radiations into the counting tube, and that
this effect is greater than any additional self-absorption or scattering of
properly directed radiations (Kamien, 1951). For internal counters and the
parafilm-paper combination, this effect is probably associated with the
distribution of sample material in the filter paper.

The results seen in Figure 2 are seen to be quite different for
the two types of planchets. Thus, the filter paper exerts a considerable
leveling effect on the reduction in activity due to added materials, and, as
shown in Figure 3, eliminates in large part the variance in absorption
between different materials (compare to Figure 1).

The curve drawn in Figure 3 expresses the absorption due to added
amounts of sodium dihydrogen phosphate. This substance was found to give a
representative absorption curve for most of the materials tested in the
paper-lined parafilm planchets. Therefore, its effect is shown separately
in Figure 4, the log of the activity being used in this case. Since the final
stages of evaporation occur primarily from the top of the filter paper, an
FIGURE 2

SELF-ABSORPTION CURVES FOR METHIONINE

Curve 1: In parafilm-filter paper combination planchets.

Curve 2: In aluminum planchets.

Each point represents an average of ten separate samples.
FIGURE 3

The effect of various additions on the counting rate of samples prepared in the parafilm-filter paper combination planchets.

Each point is an average of ten samples.

All points indicated by the metals are for the common chloride salt.
FIGURE 4

THE EFFECT OF SODIUM DIHYDROGEN PHOSPHATE ON THE COUNTING RATE OF $^{35}$-LETHINOLINE SAMPLES PREPARED IN PARAFILM-FILTER PAPER COMBINATION PLACHERS.
uneven distribution of sample material should occur; also, the mixing of absorber and labeled material would be expected to result in rather complicated absorption characteristics. In view of these considerations, it is somewhat surprising that the linear relationship in Figure 1 corresponds to what would normally be the case for solid absorbers such as lead or aluminum when the sample and absorber are not intermixed.

In Figure 3, the divergence for some of the heavier metals suggests the possibility of complex formation which might alter the distribution of methionine in the paper. Since a small amount of copper, known to be a good complex-former, was present in all the final samples for the determination of methionine uptake, the effect of comparable amounts of copper chloride on the sample activity in the paraffin-filter paper combination planchets was investigated. The data are presented in Figure 5 and clearly indicate anomalous behavior in the presence of copper. It seems likely that complex formation, here, as well as in other cases with the heavier metals, alters the distribution of methionine in the paper. This is further emphasized by the data in Table 1, where the effect of cupric ion is reversed by adding an excess of glycine to compete for the copper ion, or by making the solution acid, which reduces complex formation. In each of these latter cases it is seen that the values of the per cent total activity as a function of the total weight of absorber now fall on the normal absorption curve (Figure 3). Thus the hydrochloric acid, used in transferring the final experimental samples to the planchets, should minimize any variations in methionine distribution due to complex formation.
FIGURE 5

THE EFFECT OF COPPER ON THE COUNTING RATE OF $^{35}$S-METHIONINE
SAMPLES PREPARED IN PARAFILM-FILTER PAPER
COMBINATION PLANCHETS

Each point is an average of ten separate samples.
<table>
<thead>
<tr>
<th>Additions</th>
<th>% Total Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 mgm. CuCl₂·2H₂O</td>
<td>82.0</td>
</tr>
<tr>
<td>6 mgm. glycine</td>
<td>67.3</td>
</tr>
<tr>
<td>9 mgm. CuCl₂·2H₂O + 6 mgm. glycine calculated</td>
<td>69.3</td>
</tr>
<tr>
<td>observed</td>
<td>63.0</td>
</tr>
<tr>
<td>9 mgm. CuCl₂·2H₂O + 0.1 Meq. HCl</td>
<td>90.0</td>
</tr>
</tbody>
</table>

1. Each value represents an average of ten determinations.
2. Calculated on the assumption that the effects of cupric chloride and glycine are additive.

It was also of interest to determine the effect of planchet size on the counting rate for a given amount of labeled material. The data are given in Table II. It is apparent that the observed activity increases as the planchet size is diminished. However, an increase of over tenfold in the planchet size is seen to be necessary to decrease the counting rate by something less than a factor of two. For all practical purposes, therefore, it is evident that the limits for paper area are quite wide.
TABLE II

EFFECT OF AREA OF PAPER ON COUNTING RATE
IN THE PAPER-LINED PARAFILM PLANCHETS

<table>
<thead>
<tr>
<th>Area, Square Inches</th>
<th>Count Per Minute</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.56</td>
<td>1051</td>
</tr>
<tr>
<td>0.56</td>
<td>1356</td>
</tr>
<tr>
<td>0.14</td>
<td>1825</td>
</tr>
</tbody>
</table>

* Each value represents an average of ten separate samples.

For all of the salts tested in this investigation, the weight is expressed as that of the hydrate formed on drying at room temperature. It should also be mentioned that the total added $^{35}$S-methionine was in the order of one-thousandth of a micromole and contributed no significant amount to the total weight of absorbing material.

The only practical difficulty in using the paper-lined parafilm planchets would be that observed with weak samples, since, due to absorption by the filter paper and the relative backscattering properties of parafilm and aluminum, the activity for a given amount of sulfur-$^{35}$ by this method is approximately one-fifth of that for the aluminum planchets. However, the final samples in measuring methionine uptake were sufficiently active to warrant use of the parafilm-filter paper combination in view of its leveling effects.
Calculations

All sample activities were corrected to August 25, 1952, on the basis of the 57.1 day half-life for sulfur-35 (Kamen, 1951). The specific activity of the 35S-L-methionine on the same date, counted in the parafilm-filter paper planchets, was 2.1 x 10^5 counts per minute per micromole. The planchets were weighed before and after transfer of the final sample material and the incorporation values corrected for the total weight of material from the curve in Figure 3 (page 25). From these data and the weight of tissue used, the results were calculated as micromoles 35S-L-methionine incorporated into protein per gram of tissue (wet weight). The results are expressed in these units in the following tables so that direct comparisons can be made.

Statistical analysis was carried out using the Fisher "T" method (Mills, 1937).
CHAPTER IV

RESULTS AND DISCUSSION

Preliminary Experiments

Table III presents preliminary determinations on the uptake of labeled methionine by pituitary, mammary and diaphragm tissues, using $^3$S$^5$-dl-methionine at a concentration of one-half micromole per milliliter. Each value represents an average of two to four determinations. The tissues were not washed with trichloroacetic acid prior to homogenizing and the zero times were high and variable. This was believed to be due to non-biological incorporation during the homogenizing of the tissue. This is particularly reflected in the two higher values for diaphragm, which, as will be evident from Table IV, do not represent true biological incorporation.

The pituitary and mammary measurements, though not of the reliability desired, were fairly constant. These measurements show essentially the same degree of incorporation as in the following tables, which refer to the procedure described using $^3$S$^5$-l-methionine at a concentration of one micromole per milliliter. Thus, maximal substrate concentration is indicated, since a fourfold increase in l-methionine concentration has no apparent effect on the rate of incorporation.
comparison of tissue activities

In Table IV are given the values for methionine uptake by a number of tissues, listed in the order of increasing activity.

The insignificant value for the subcutaneous tissue provides a striking comparison with that of the skin. Determinations on the nitrogen content of these tissues have shown that they both contain approximately thirty-five milligrams of nitrogen per gram of fresh tissue. Assuming this to be primarily protein nitrogen, the physiological distinction between the functions of these two tissues is seen to be sharply reflected in amino acid uptake.

The uptake by diaphragm of C\textsuperscript{14}H\textsubscript{2}COOH from carboxyl-labeled alanine has previously been determined by Sinex, MacKerron and Hastings (1952); the values found, when expressed in units corresponding to those in Table IV, were 0.03 to 0.05. The value listed in Table IV for diaphragm is seen to be in the same order of magnitude, though in view of the different substrates
used, no quantitative comparison can be made. Also, the measurement of total \( ^{14} \text{C} \) in the former investigation does not account for incorporation of other metabolites produced from the alanine.

### Table IV

**Comparison of Tissue Activities**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Number of Determinations</th>
<th>Methionine Uptake ( ^{\text{1}} ) (micromoles/g/Hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcutaneous</td>
<td>4</td>
<td>0.003 ± 0.002</td>
</tr>
<tr>
<td>Liver</td>
<td>6</td>
<td>0.027 ± 0.003</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>13</td>
<td>0.032 ± 0.004</td>
</tr>
<tr>
<td>Skin and Mammary(^{2} )</td>
<td>14</td>
<td>0.032 ± 0.002</td>
</tr>
<tr>
<td>Pituitary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>6</td>
<td>0.132 ± 0.007</td>
</tr>
<tr>
<td>Female</td>
<td>12</td>
<td>0.164 ± 0.015</td>
</tr>
<tr>
<td>Tumor</td>
<td>8</td>
<td>0.215 ± 0.024</td>
</tr>
</tbody>
</table>

1. Expressed as the average ± the standard error; pituitary values were calculated from values for two hour incubations.
2. Males and females combined.

A previous study on the uptake of methionine by rat pituitary has indicated that this tissue is the most active normal tissue so far reported in this regard (Melahier and Malikis, 1952). No other investigations of this aspect of metabolism in pituitary tissue have been made.

Hepatoma tissue has been shown to be six to seven times more active than normal liver in the uptake of certain labeled amino acids (Zassenhik and Frants, 1949). A similar comparison of tumor and liver tissue in
Table IV shows a slightly higher ratio, though essentially the same qualitative relationship. Thus, these two malignant types appear to resemble one another in this biochemical aspect; this seems to further emphasize the similarity of different malignancies in regard to their relatively intrinsic capacity for growth.

In comparing mammary tumor tissue to the related, normal mammary and skin tissues (Table IV), it is apparent that mammary carcinogenesis is accompanied by an almost threefold increase in protein-labeling rate. The activity found for the tumor tissue indicates that it is the most active tissue so far investigated in protein-labeling studies in vitro.

**Effect of Foster-nursing on Mammary Activity**

In Table V are presented the activities for female mammary tissue and male skin from the mammary region. As is evident from the values obtained, no significant difference for any of the three tissues was observed. Moreover, the resting mammary gland appears to have no effect on the activity of the female tissue. This may be due to masking of mammary tissue activity by dilution with epidermal cells not a part of the gland structure.

In a thorough study of certain aspects of mammary gland structure, areas of acinar hyperplasia ordinarily observed in high-cancer-strain mice were not found when these mice were deprived of the milk influence (for reference see: Dacewski, 1953). However, in comparing the values for the two female groups (Table V), no significant difference was observed. Thus, the milk factor appears to have no effect on the protein-labeling rate in the absence of tumors.
TABLE V

EFFECT OF FOSTER-NURSING ON MAMMARY ACTIVITY

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Number of Determinations</th>
<th>Methionine Uptake* (micromoles/g/2 Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H Male</td>
<td>4</td>
<td>0.162 ± 0.012</td>
</tr>
<tr>
<td>(skin tissue)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3H Female</td>
<td>4</td>
<td>0.183 ± 0.017</td>
</tr>
<tr>
<td>TC3H Female</td>
<td>6</td>
<td>0.152 ± 0.007</td>
</tr>
<tr>
<td>All Groups</td>
<td>14</td>
<td>0.164 ± 0.007</td>
</tr>
</tbody>
</table>

* Expressed as the average ± the standard error.

**In Vitro Effect of Hormones on Tissue Activities**

It has been found with mice that localized proliferation of mammary epithelium will occur when estrone is painted on a single nipple (Gardner and Chamberlin, 1941). Growth of benign mammary tumors of the rat has been found to be stimulated by estradiol (Haiman, 1963). Also, estrogen in elderly women, and testosterone in some young patients, have given therapeutic response in cases of breast cancer (for reference see: Zamecnik, 1952). In view of these and similar reports, it was of interest to look for an in vitro effect of these substances on similar tissues.

Observations on the effects of steroids on tissue slice respiration, as well as enzymes, have been reviewed by Dorfman (1952). The great majority of the reports have indicated a rather strong inhibition, and where there have been instances of stimulation, this effect was very small. It is worth noting in regard to this review that, of a number of steroid hormones studied,
inhibition was relatively non-specific; that is, it occurred with either all or most of the enzyme systems studied. Also, in view of the concentrations of steroids employed in these in vitro experiments, the indications are that variations in toxicity have been observed, rather than any physiological influence on the system being studied. For example, testosterone, at a concentration of approximately five hundred micrograms per millilitre, has been found to inhibit the respiration of brain cell suspensions whereas the normal blood level for testosterone is less than twenty micrograms per millilitre (Borth and de Matteville, 1950). To the author's knowledge, no investigations on the effect of steroid hormones in vitro on protein synthesizing enzymes have been made.

Borth and de Matteville (1950) have given the highest normal blood level for estradiol in the human subject as approximately two micrograms per millilitre. As shown in Table VI, hormone concentrations in approximately the same order of magnitude (the final volume was one millilitre) had no effect on the protein-labeling rate of the tissues listed.

The possibility that either localised stimulation or the short interval employed in these in vitro hormone studies was not sufficient for any noticeable effects led to the experiments given in the following section.
### Table VI

**In Vitro Effect of Hormones on Tissue Activities**

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Tissue</th>
<th>Number of Determinations</th>
<th>Hormone(^1)</th>
<th>Methionine Uptake(^2) (micromoles/g/Hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H Male</td>
<td>Skin</td>
<td>12</td>
<td>None</td>
<td>0.079 ± 0.004</td>
</tr>
<tr>
<td>C3H Male</td>
<td>Skin</td>
<td>12</td>
<td>Estradiol or estrone</td>
<td>0.051 ± 0.005</td>
</tr>
<tr>
<td>C3H M others</td>
<td>Mammary</td>
<td>6</td>
<td>None</td>
<td>0.085 ± 0.009</td>
</tr>
<tr>
<td>C3H M others</td>
<td>Mammary</td>
<td>7</td>
<td>Estrone</td>
<td>0.094 ± 0.006</td>
</tr>
<tr>
<td>Tumor-bearing</td>
<td>Tumor</td>
<td>8</td>
<td>None</td>
<td>0.215 ± 0.024</td>
</tr>
<tr>
<td>Tumor-bearing</td>
<td>Tumor</td>
<td>5</td>
<td>Estrone</td>
<td>0.207 ± 0.019</td>
</tr>
<tr>
<td>Tumor-bearing</td>
<td>Tumor</td>
<td>3</td>
<td>Testosterone</td>
<td>0.246 ± 0.022</td>
</tr>
</tbody>
</table>

1. One to two micrograms per flask.
2. Expressed as the average ± the standard error.

**In Vivo Effect of Estrone on C3H Male Skin Activity**

In a study on hormone induced tumors in C3H male mice, it has been shown that doses of twenty-five micrograms of estradiol benzoate weekly resulted in approximately an 80 per cent incidence of mammary tumors. With continuous treatment, the average survival was nine months. Larger doses of fifty micrograms weekly resulted in fewer tumors, though the survival was practically the same as with the smaller dose (Gardner, 1941). It has also been shown by histological studies that moderate to extensive mammary gland development will occur in male mice injected with as little as two micrograms of estrone daily for a period of sixty to three hundred days (Gardner, 1935).
This corresponds to about one-fourth of the dose necessary for maximal tumor production in C3H males (Burns and Schanken, 1940). However, comparisons between different estrogens assume that their relative effects in mammary tumor production are in the same ratio as their pharmacologic potency. The validity of this assumption is somewhat doubtful, however, since, as mentioned above, the doses needed for maximal carcinogenesis seem well in excess of those which produce extensive gland development. It has also been reported by Gardner (1941) that large amounts of estrogen will inhibit mammary growth.

Table VII shows the results of measurements on related tissue from the normally expected site of cancer development. Males were chosen for this study since they would not be subject to the physiological variation which might occur in the females due to endogenous estrogen effects. It is apparent (Table VII) that no significant difference was found for any of the values.
TABLE VII

IN VIVO EFFECT OF ESTRONE ON C3H MALE SKIN ACTIVITY

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Paired Determinations</th>
<th>Number of Injections</th>
<th>Methionine Uptake (micromoles/g/Hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4</td>
<td>None</td>
<td>0.079 ± 0.004</td>
</tr>
<tr>
<td>Masola</td>
<td>4</td>
<td>10 to 50</td>
<td>0.092 ± 0.005</td>
</tr>
<tr>
<td>Estrone</td>
<td>3</td>
<td>10</td>
<td>0.100 ± 0.006</td>
</tr>
<tr>
<td>Estrone</td>
<td>3</td>
<td>23</td>
<td>0.079 ± 0.007</td>
</tr>
<tr>
<td>Estrone</td>
<td>2</td>
<td>50</td>
<td>0.076 ± 0.006</td>
</tr>
</tbody>
</table>

1. Two determinations were made on each animal.
2. Expressed as the average ± the standard error.
3. From Table VI.

Effect of Sex and Foster-nursing on Body and Pituitary Weights

The pituitary size for males has been noted to be less than that for females in numerous species (Reece and Turner, 1937). However, for the females of a given mouse strain, the milk influence has been regarded as having no effect on the precancerous health and well-being of cancer-susceptible mice (Amberg, 1945).

In Table VIII are presented average values for all of the non-cancerous, adult mice used for the pituitary measurements on methionine uptake. Though their ages ranged from four to eleven months, the comparison of body weights is assumed valid since the distribution according to ages was approximately the same for each of the three groups and the mice showed very little weight increase after adulthood (approximately three months)
was reached. Typical growth curves for white mice also show no apparent
increase in weight for the females after four to five months of age, though
the males gained approximately two grams from the fourth to the sixth month
(Retslaflf, 1939).

TABLE VIII

EFFECT OF SEX AND FOSTER-NURSING ON BODY AND PITUITARY WEIGHTS

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Number of Values</th>
<th>Body Weight* (g)</th>
<th>Pituitary Weight* (mg)</th>
<th>Micrograms Pituitary per Gram Animal*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H Male</td>
<td>44</td>
<td>28.1 ± 0.4</td>
<td>1.53 ± 0.04</td>
<td>54.3 ± 1.4</td>
</tr>
<tr>
<td>C3H Female</td>
<td>55</td>
<td>23.0 ± 0.4</td>
<td>1.68 ± 0.04</td>
<td>72.2 ± 1.4</td>
</tr>
<tr>
<td>T C3H Female</td>
<td>39</td>
<td>27.4 ± 0.3</td>
<td>2.26 ± 0.04</td>
<td>62.5 ± 1.3</td>
</tr>
</tbody>
</table>

* Expressed as the average ± the standard error.

Table IX shows the same qualitative body weight relationship for
the three groups when given at differing ages. The only adult value which
seems to vary considerably is the one for eleven month old C3H females, though
this represents only four mice. The four to six month values, however, show
the same relationship as in Table VIII. One month values have also been in-
cluded in Table IX and, in comparison to the four to six month values,
indicate an earlier leveling in weight for females possessing the milk factor
than for the foster-nursed females.

An even greater difference is observed (Table VIII) in the average
pituitary weights for the two groups of females. Assuming a proportionately
higher pituitary weight for the larger females, it is apparent from the last
column that even this consideration does not explain the difference observed. Though conditions were not specifically controlled for growth studies, these observations, if verified, would establish some effects of the milk factor in addition to its observed influence on tumor production.

**TABLE IX**

**EFFECT OF AGE ON BODY WEIGHTS**

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Body Weighta (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C3H Males</td>
</tr>
<tr>
<td>1</td>
<td>19 (47)</td>
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<tr>
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<td>5-6</td>
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<td>11</td>
<td>26 (5)</td>
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</table>

* The number of animals averaged is given in parentheses.

**Effect of Sex and Foster-nursing on Pituitary Activity**

In regard to "precanerous" pituitary metabolism, no differences in the pituitaries of females with and without the milk factor was reflected in the protein labeling rate (Table I), although, as suggested in the comparison of pituitary weights (Table VIII), the milk factor does seem to alter some aspect of pituitary metabolism. Thus, the suggestion (Zamenhik, 1952) based on the growth hormone studies previously mentioned, that an altered physiology of the pituitary may contribute to the carcinogenic transformation,
though reflected in pituitary weights, shows no correlation with pituitary activity in Table I.

**TABLE I**

**EFFECT OF SEX AND FOSTER-NURSING ON PITUITARY ACTIVITY**

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Number of Determinations</th>
<th>Methionine Uptake* (micromoles/g/2 Hours)</th>
<th>Micromoles per Kg Animal* x 10² (2 Hours)</th>
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<td>6</td>
<td>0.265 ± 0.025</td>
<td>1.46 ± 0.19</td>
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<td>6</td>
<td>0.325 ± 0.032</td>
<td>2.36 ± 0.22</td>
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<td>TC3H Female</td>
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<td>0.326 ± 0.015</td>
<td>2.62 ± 0.09</td>
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<tr>
<td>Tumor-bearing</td>
<td>2</td>
<td>0.316</td>
<td>0.213</td>
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* Expressed as the average ± the standard error.

The males, on the other hand, appear to have somewhat less active pituitaries than either the combined or foster-nursed female groups, though this difference is not highly significant (0.01 < p < 0.05). If this could be shown to be related to the synthesis of protein hormones, it would seem to be of greater significance to express the total uptake as a function of body weight. Calculated in terms of activity per kilogram animal, the female gland is seen to be approximately 70 per cent more active than that of the male. For albino rats the same qualitative sex differences has been noted, the female gland showing 30 per cent more activity than that of the male on the basis of body weight; no sex difference, however, was noted on a basis of tissue weight (Waldorph and Malikis, 1952).
Though the availability of tumor-bearing animals was restrictive, two measurements on pituitaries of these mice were obtained (Table I). No explanation for their variance can be given.
CHAPTER V

SUMMARY

1. A method for counting sulfur-35 in heterogeneous, water soluble mixtures has been described.

2. The abilities of various whole tissues of C3H mice to incorporate methionine into protein in vitro have been compared. By including two commonly employed tissues, liver and diaphragm, as a reference level, the positions of a few other tissues in the series have been established. The order found was as follows: subcutaneous < diaphragm; liver < skin; mammary < pituitary < mammary tumor. The subcutaneous tissue showed practically no activity. Skin and mammary tissues were more than twice as active as diaphragm or liver and about one-half as active as pituitary tissue. Mammary tumor tissue was the most active of all tissues studied, being half again as active as pituitary.

3. Cancer susceptibility was found to exert no effect on the in vitro protein-labeling rate of normal, "precanecous" tissue related to mammary tumor tissue.

4. Estrogens, either in vitro or in vivo, produced no change in the "precanecous" protein-labeling rate of tissue from the site of expected tumor development. Neither estrogens nor testosterone produced any effect in vitro on mammary tumor tissue activity.
5. The average body weights for the C3H males and C3H females were approximately the same, though both were significantly higher than that of the C3H females. The foster-nursed (TC3H) adult females had significantly larger pituitaries than either the males or C3H females. Differences in pituitary weight per unit of body weight were noted for all three groups. These differences were significant and followed an increasing order for C3H males, C3H females and TC3H females, respectively.

6. The in vitro activity of pituitary tissues of C3H males appeared to be significantly less ($0.01 < p < 0.05$) than that of the combined females. Expressed on a basis of body weight, the female gland is approximately 70 per cent more active than that of the male. Comparison of the values for the two groups of females (C3H and TC3H) showed no effect of the milk factor on pituitary activity.
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Library
Stritch School of Medicine
Loyola University

APPENDIX

ORIGINAL DATA

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<th>ANIMAL GROUP</th>
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IN VITRO EFFECT OF HORMONES ON TISSUE ACTIVITIES (1 Hour)

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**PITUITARY DATA AND BODY WEIGHTS**

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APPROVAL SHEET

The thesis submitted by Arthur H. Goldkamp has been read and approved by three members of the faculty of the Stritch School of Medicine, Loyola University.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

5-27-53
Date

[Signature]
Signature of Adviser