rats. The growth reaction or the development of alveoli induced by Tp was enhanced by Ca. Injections of E did not enhance the responses of the mammary glands to $Tp_{0.2}$ or $Tp_{0.1}$. These findings agree with those after local application of testosterone and injections of either cortisone or estrone into castrated adrenalectomized rats(5). The result obtained from Group 4 (Table I) treated with $Tp_{0.2}$ or $_{0.1}$, Ca and E showed an 'androgen' response (alveolar lobules) in 82 or 69% of the mammary glands of 17 and 13 rats studied, respectively. A complete restoration to normal was not achieved. In view of the difficulty of finding properly balanced doses of the 3 hormones, this does not seem surprising. Of importance is that Ca or E given singly enhanced the effects of $Tp_{0.2}$ or $_{0.1}$ slightly (Ca) or not at all (E), but that Ca and E together permitted the stimulation of alveolar development in the mammary glands of a considerable number of gdx adx rats given $Tp_{0.2}$ or $Tp_{0.1}$. The result supports the view(8,9) that the actions of testosterone on the mammary glands of male rats are superimposed on those of estrogens. In agreement with others(5) the present findings show furthermore that adrenal cortex hormones facilitate mammary reactions.

Summary. The reaction of the mammary glands to testosterone propionate (Tp) was studied in gonadectomized (gdx), adrenalectomized (adx) rats given estrone (E) and cortisone acetate (Ca) singly or together. Compared with controls with intact adrenals the mammary responses to Tp were reduced after adrenalectomy. With few exceptions alveolar lobules failed to develop in gdx adx rats injected with 0.1 mg Tp every other day. When daily injections of 0.125 mg Ca were added to the treatment with 0.1 mg Tp the number of glands presenting alveoli increased slightly. E, 0.05 μg daily, failed to modify the mammary response to 0.1 mg Tp. Combined treatment with Ca, 0.125 mg, and E, 0.05 μg, daily and Tp, 0.1 or 0.2 mg, every other day resulted in development of alveolar lobules in a great majority of mammary glands of gdx adx rats. The findings support the view that the mammary response to androgens is dependent on concomitant actions of estrogens and adrenal cortex steroids.

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Growth and Glycolysis in the Human Diploid Cell Strain WI-38.* (30057)

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The serial cultivation of human diploid cell strains provides a unique system for biochemical studies since these cells, in contrast to the commonly used heteroploid cell lines, retain many of the characteristics of normal cells *in situ, e.g.*, a stable diploid karyotype, a limited life span, and non-malignant growth *in vivo*(1,2).

Extensive studies have been carried out

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with these cells and published reports include observations on life span, chromosome number, and virus susceptibility(1,2); however, no published reports on the biochemistry of these cells have appeared.

The carbohydrate metabolism of cultured cells has been reviewed by Levintow and Eagle(3), and although many differences appear among the different cell lines, several properties seem to be common to all cultured cells studied. Among these are: (1) serially cultured cells degrade glucose to either CO_2 or lactate, the latter pathway predominating in most cases; (2) other sugars, *e.g.*, mannose, fructose and, possibly, galactose, can be utilized in place of glucose for cell growth; (3) the metabolism of serially cultured cells will differ qualitatively and quantitatively depending on the method of cultivation, pH and species of serum(3).

Aspects of the carbohydrate metabolism of human diploid cells, and a description of our method for growing them in amounts suitable for biochemical studies and under conditions which are reproducible comprise the basis for this report.

Materials and methods. Starter cultures of strain WI-38 human diploid cells(2) were obtained from Dr. Leonard Hayflick of The Wistar Institute and subsequently subcultured. The composition of the medium used was essentially that described by Hayflick(1). The gas phase was 5% CO_2:95% air and the pH was maintained between 7.2 and 7.4.

A 0.25% solution of trypsin prepared in a buffered balanced salt solution was used for removing the cells from the glass.

Routinely, starter cultures were subcultured into flat 2 liter Povitzky bottles having an available surface area of 250 cm^2 and containing 150 ml of medium. When these cells had reached confluency, they were subcultured further at a ratio of 1:10.

In studies with sugars other than glucose, the sugar was added to the medium in place of glucose and at the same concentration, *i.e.*, 5.5 mM.

Periodically, the cells were tested for mycoplasma contamination by Dr. L. Hayflick and found to be negative.

Cell counts were made daily using a hemocytometer. At least 400 cells were counted each time giving a standard deviation of no more than $\pm$ 5%(4).

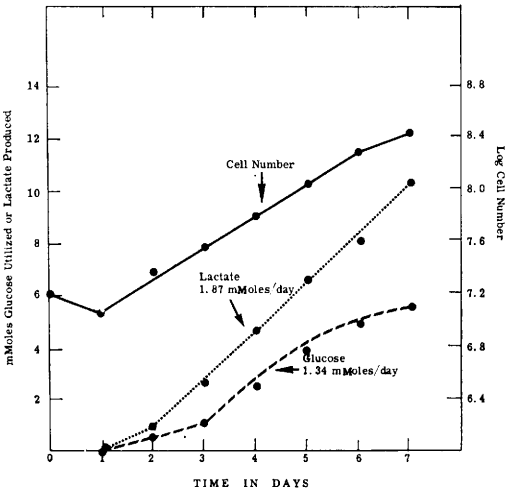

FIG. 1. Glycolysis and cell growth in WI-38.

For determination of sugar and lactic acid concentration, aliquots of the medium were taken each day and, after precipitation of the protein with trichloroacetic acid (final concentration 2%) and centrifugation, portions of the protein-free extract were analyzed. Lactic acid was measured by the method of Barker and Summerson(5). Glucose was determined enzymatically using the glucostat (glucose oxidase) procedure of the Worthington Laboratories, Freehold, N. J. Sugars other than glucose were determined by the anthrone method of Roe(6).

Results. Fig. 1 shows a curve obtained in measuring rate of cell growth and glycolysis. Typically, during the first 24 hours following inoculation, 25-50% of the inoculated cells fail to survive. Cell doubling begins at approximately 24 hours after inoculation and continues at a constant rate for 5 to 6 days yielding 10^8-10^9 cells per 20-2 liter bottles or about 2×10^4 cells per cm^2. This represents a 10-fold increase in cell number and yields about 1 ml of packed cells.

Analyses of replicate growth curves show the cells to have a generation time of 24 hours, confirming the original findings of Hayflick(1,2) and of Defendi and Manson (7) on smaller population densities.

The data in Fig. 1 also show that after an initial lag, glycolysis proceeds at a uniform rate which parallels cell division. From the

TABLE I. Glycolysis in Human Diploid Cell Strain WI-38.

Glucose utilization* μmoles $(10^6$ cells·days$)^{-1}$	Lactate production* μmoles $(10^6$ cells·days$)^{-1}$	$Q_{CO_2}^{O_2}$†
3.34	7.34	11.2

* Rates determined from slope of curve during straightest portion.

† $Q_{CO_2}^{O_2}$ Equivalent microliters CO_2 produced per mg dry weight of cells per hour.

TABLE II. Lactate Production from Different Sugars in WI-38.

Sugar 5.5 mM	Glucose, μmoles/ml 0 time	Harvest	Glucose used, μmoles/ml	Lactate produced, μmoles/ml
Glucose	5.84	2.84	3.00	6.38
Mannose	.61	0	.61	5.90
Fructose	.61	0	.61	4.30
Galactose	.61	0	.61	4.36

slopes of these curves, one can calculate the glycolytic rate on a cellular basis; from these data and the dry weight per cell (mean $= 6.11 \times 10^{-7}$ mg/cell) a Q value (microliters of gas equivalents per mg dry wt per hour) for aerobic glycolysis can be estimated. These results are summarized in Table I. The lactate to glucose ratio is very close to the theoretical 2:1 suggesting that, under these conditions of culture, most of the glucose utilized is converted to lactic acid. The Q value for aerobic glycolysis of 11.2 compares well with values reported by other workers for the glycolytic capacity of cells in culture (8,9,10,11,12).

A comparison of the utilization of mannose, fructose, galactose and glucose is shown in Table II. No attempt was made to eliminate the small amount of glucose contributed by the serum in the medium. This glucose was used rapidly and completely in all cases, suggesting that glucose is utilized preferentially by these cells. However, the finding that the cultures become confluent and that the amount of lactic acid produced is greater than can be accounted for by the amount of glucose utilized indicates that these other hexoses can substitute for glucose in WI-38 cells.

Lactate production from mannose is almost as great as from glucose, while fructose and galactose seem to be converted to lactate to a lesser extent.

In another series of experiments, total sugar utilized was correlated with lactate production and these data are summarized in Table III. Mannose disappears at 87% the rate of glucose and both of these sugare are, almost stoichiometrically, converted to lactic acid. Galactose is metabolized at a somewhat slower rate, and the conversion of galactose to lactic acid is considerably less efficient. Fructose, on the other hand, while exhibiting the slowest rate of utilization, seems to be more efficiently converted to lactic acid than is galactose.

Discussion. Other investigators have reported studies correlating glycolysis with growth in cultured cells of various origins(8, 9,10,11,12). Our work extends these observations to a strain of human diploid cells and, for the first time, provides data on growth and glycolysis for cells which, in culture, retain many of the characteristics of normal cells *in situ*(1). In a few instances, our results differ from those obtained by workers using heteroploid cell lines.

Munyon and Merchant(8) have reported that there is no constant relationship between cell division and glycolysis and that glycolysis occurs most rapidly during the early log phase of growth; as growth proceeds, lactate disappears from the medium and then a production of lactate occurs again

TABLE III. Sugar Utilization and Lactate Production in WI-38.

Sugar, 5.5 mM	Sugar utilized, μmoles/ml	%	% of control*	Lactate produced, μmoles/ml	Lactate: hexose
Glucose	3.61	66	100	8.33	2.3
Mannose	3.14	57	87	6.37	2.03
Fructose	1.58	29	44	2.36	1.49
Galactose	2.78	50	77	2.50	1.11

* Control value, glucose $= 100\%$.

at a later stage in growth. Our data (Fig. 1) indicate that, although glucose utilization is somewhat more rapid during early log phase, it shows a direct correlation with cell division. Lactic acid is produced at a constant rate through the period studied. This constant relationship between glycolysis and growth in WI-38 has recently been confirmed by Kruse.† A partial explanation of the difference between the results of Munyon and Merchant(8) and the data presented here may lie in the relative constancy of pH at which our experiments were carried out. Zwartouw and Westwood(9) have shown that the glycolytic rate is profoundly affected by the pH and suggest that this effect may constitute a homeostatic mechanism by which the cell controls the environmental pH.

A Q value of 11.2 for aerobic glycolysis compares favorably with data published for other cultured cells. Graff and McCarty(10) report that growing strain L fibroblasts produce 10.4 μmoles of lactate per 10^6 cells per day (Q approximately 9.5) while Leslie(11) has reported a mean Q value of 9.9 for some embryonic human cells of unspecified karyotype.

A Q lactate of 11.2 is relatively high as compared with the glycolysis of many normal tissues *in situ,* ranging from 0.3 for rat kidney to 35 for rat retina(13). The Q lactate for serially cultured cells varies within a much smaller range and has no apparent relationship to the origin of the cell line. Thus, the Q lactate values for normal intestine and conjunctiva are 9.2 and 12.5, respectively and for HeLa and KB cells are 6.2 and 12.5 (14). In the course of other experiments involving the SV_{40} virus transformed human diploid cells‡ we have observed that the Q lactate of these cells is in the same range as that in Table I. The data presented here, obtained with cells which fulfill all the generally accepted criteria for normal cells, support the suggestion resulting from earlier work with heteroploid cell lines(14) namely, that the glycolytic rate cannot be used as a criterion for alterations in cells since, metabolically, cells grown in culture resemble each other more than they resemble their respective tissues of origin.

Eagle(12) has reported that a number of carbohydrates when substituted for glucose can support the growth of cell lines derived from normal as well as neoplastic human tissue. The data given here extend these findings to human diploid cells. In agreement with Eagle's findings WI-38 cells will convert mannose to lactate at almost the same rate as glucose. However, fructose and galactose, although able to support growth of WI-38 are utilized at a somewhat slower rate.

Summary. Aspects of the carbohydrate metabolism of a strain of serially cultured human diploid cells which fulfill the criteria for normal cells have been studied. These cells show an initial 24-hour lag in cell division and glycolysis upon subcultivation. Following this, cell division proceeds rapidly for 5 to 6 days. Rates of glucose utilization and lactic acid production generally follow the same time course. The glycolytic rate of these cells resembles that of other serially cultured cells.

In the absence of added glucose: mannose, galactose and fructose were able to support the growth of human diploid cells. Mannose was converted to lactic acid at almost the same rate as glucose while galactose and fructose were converted to lactate at a somewhat slower rate.

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Antigenicity of Polypeptides (Poly Alpha Amino Acids). XIV. Studies on Immunological Tolerance with Structurally Related Synthetic Polymers.* (30058)

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For the past 10 years, synthetic polymers of amino acids have been used to study some of the molecular parameters of immunogenicity and antigenic specificity (Reviews in 1,2, 3). By altering the nature and amounts of the amino acids in the polymers, it is possible to study the effect of such variables as charge, conformation, optical configuration of the amino acids, helix content, etc. on immunogenicity. From studies of cross reactions with antisera and related polymers, it is becoming possible to learn the nature of the immunogenic determinants in these polymers(4). Although the structures of synthetic random copolymers of amino acids are not known, they are not as complicated as those present in natural proteins made up of many amino acids which contribute to secondary and tertiary structures. Therefore, in addition to studies on the immunogenicity of polymers, investigations in our laboratory, as well as in others(5,6) were undertaken on the production of acquired specific immunological tolerance towards a variety of synthetic antigens. A preliminary account of some of this work appeared several years ago (7). The present report deals with the effect of neonatal injections of synthetic polymers on the subsequent response of the adult rabbits against related cross reacting polymers.

Materials and methods. Table I lists the polymers employed in this study. The random copolymers and the homopolymer of glutamic acid were prepared by the polymerization of the appropriate N-carboxy-α-L-amino acid anhydride by methods referred to previously(8,9). The following abbreviations are used for the amino acids: G—glutamic acid, L—lysine, A—alanine, T—tyrosine. Subscripts refer to the mol percent composition of the amino acid. All rabbits employed were New Zealand white and were injected according to the following schemes:

TABLE I. Synthetic Polymers Studied for Immunological Unresponsiveness.

Polymer*	Nomenclature	Approx avg molecular wt
$Glu_{60}ala_{40}$	$G_{60}A_{40}$	33,000
$Glu_{42}lys_{28}ala_{30}$	$G_{42}L_{28}A_{30}$	65,000
$Glu_{60}lys_{40}$	$G_{60}L_{40}$	64,000
Glu_{100}	G_{100}	60,000

* Subscript refers to mol % of amino acid in polymer.

Experiment 1. ($G_{60}A_{40}$). The rabbits were injected intraperitoneally within 24 hours after birth with 100 mg of the antigen. At day 75, the rabbits were divided into 2 groups. Animals in Group A (1A) were injected in the toe pad with 20 mg of the polymer in complete Freund's adjuvant, and then bled weekly for 3 weeks (Course I). The following week (day 103), the rabbits were boosted with another injection of the polymer in adjuvant and bled again after 2 and 3 weeks (Course II). The rabbits in the second group (1B) received 3 intravenous injections of 20 mg of the polymer per injection on days 75, 82 and 96. One week later, the animals were injected with 20 mg of the $G_{60}A_{40}$ in adjuvant and bled and injected

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