block audiogenic seizures has also been used to assess tranquilizing activity of drugs(3), it lends further support to the assumption that Br^- does not possess tranquilizing properties in non-toxic doses.

Summary. The effect of 5 doses of bromide was tested for effect on conditioned avoidance response in rats. Blockade of response occurred at dose of 500 mg/kg of Br^-, but this effect was non-specific, since it was accompanied by ataxia and loss of righting reflex.

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Inheritance of Capability of Human Esophageal Epithelial Cells to Grow with Diverse Carbohydrates.* (25464)

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Several carbohydrates have been reported sufficient for growth of human cells in continuous culture in a medium composed of inorganic salts and dialyzed serum(1,2). Additionally, Chang(3) derived variants of HeLa and conjunctival cells able to grow in medium containing d+xylose, a usually insufficient sugar, as sole carbon-energy source. Previous work has not determined whether such capacity of human cells to grow with particular carbohydrates is constitutive or adaptive, or how such capacity is inherited. This paper is concerned with capacity of cells in culture to initiate and continue multiplication, given particular carbohydrates. Capacity of cultures to grow in particular carbohydrate media was employed as a measure of cell capacity. This measure was influenced by the proportion of capable or potentially capable cells in culture inocula, degree of capability of such cells, and ability of these cells to become established in surface culture. When capability to grow in a medium containing a specific carbohydrate was independent of continued addition of that carbohydrate during subculture, the capability was considered hereditary. If capability to grow in a particular medium was attenuated reversibly by substitution of another carbohydrate during subculture, the capability was considered adaptive, as opposed to constitutive. By definition, the presence of a constitutive enzyme and the power to make an adaptive enzyme are hereditary. The expression of an adaptive enzyme is dependent upon the substrate and therefore nonhereditary. Irreversible alteration of culture capability by presentation or withdrawal of the carbohydrate was viewed as evidence of selective enrichment of a culture with capable or incapable cells. This report is concerned with propagation of human esophageal epithelial cells in medium with various carbohydrates, derivation of substrains by serial culture in media containing fructose, d+xylose or d+galactose, and determination of heritability of capacity to grow in these carbohydrate media.

Materials and methods. Cells of human esophageal epithelium (Minn. EE 55-12-1) have been carried as a stable line for 4 years after establishment from tissue of a male infant with tracheo-esophageal fistula(4). *Medium* for growth of the parental (uncloned) EE strain contained 105 mM sodium chloride, 5 mM potassium chloride, 0.8 mM disodium

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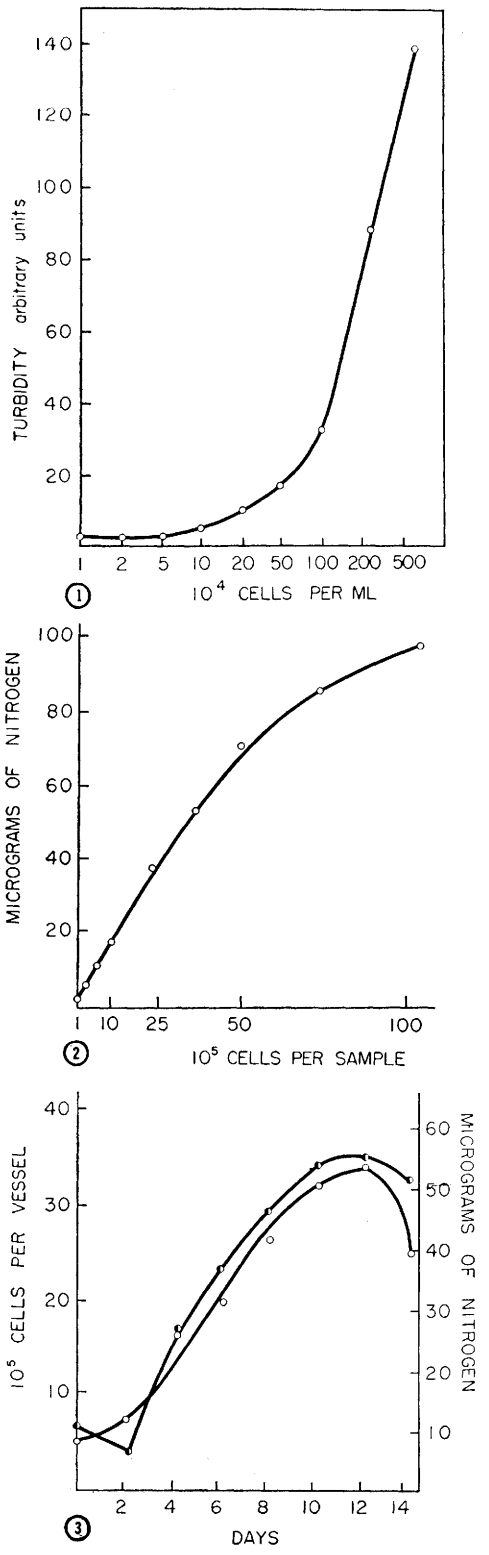


hydrogen phosphate, 0.3 mM sodium dihydrogen phosphate, 1 mM magnesium sulfate, 0.6 mM calcium chloride, 12 mM glucose, 0.002% (w/v) phenol red, 0.1% (w/v) Difco yeast extract, 10% (v/v) human serum, and sufficient sodium bicarbonate to adjust the pH to 7.6. Ten-times concentrated stock solutions of a) magnesium sulfate, calcium chloride and yeast extract, and b) remaining salts and phenol red, were sterilized by autoclaving; stock carbohydrate solutions were sterilized by Selas filtration. For use as culture medium, sterile stock solutions were diluted with sterile deionized water and mixed with serum. *Subculture of cells* was accomplished by a) washing cultures with 2-3 changes of NaCl-KCl solution; b) detaching cells from glass by incubation with 0.5% Difco "certified" 1:125 trypsin in NaCl-KCl solution for 5 minutes at 37°C; c) harvesting cells by centrifugation and resuspending them in NaCl-KCl solution for counting with a hemocytometer, and diluting to desired density; and d) inoculating cells into growth medium warmed to 37°C and equilibrated to pH 7.2-7.4. Cultures were fed every third day by complete medium change; acid production was countered by appropriate addition of bicarbonate to maintain pH. *Measurement of culture* growth was compared for a) hemocytometer counts of cells dispersed with 0.5% trypsin, collected by centrifugation, and diluted to required population densities; b) dry weight estimation (4 x 10^7 EE cells weighed 0.7 $\pm$ 0.2 mg); c) turbidity of dispersed cell suspensions (Fig. 1); and d) assay of cell nitrogen by Koch-McMeekin Nessler method and use of Klett-Summerson photoelectric colorimeter with No. 54 filter. Dry weight was an insensitive and inaccurate measure; turbidity was measurable accurately but re-

FIG. 1. Turbidity of human EE (Minn. 55-12-1) cell suspensions, as read in a Klett-Summerson photoelectric colorimeter, filter 42. Cell suspensions were prepared from 10-day cultures.

FIG. 2. Total nitrogen content of human EE (Minn. 55-12-1) cells, determined by the Koch-McMeekin Nessler method.

FIG. 3. Growth of human EE (Minn. 55-12-1) cells at 37°C in medium containing 10% whole human serum, 0.1% yeast extract and 12 mM glucose, measured by total cell count (half-solid circle) and nitrogen determination (open circle).

quired too many cells. Cellular nitrogen was directly proportional to cell count over a wide range (Fig. 2). Growth curves based on cell count and nitrogen measurement were compared for EE cells grown in standard medium with glucose (Fig. 3). During days 1 and 2 of culture, a drop in cell number was not accompanied by a reduction in cellular nitrogen. Toward the end of the growth cycle on days 10-12 nitrogen continued to rise although cell count leveled, and on days 12-14 cellular nitrogen dropped more rapidly than cell count. These discrepancies were compatible with assumption of differences in associated rates of protoplasm synthesis and cellular division(5). Since the present study was concerned with qualitative capacity of individual carbohydrate media to support production of new cells rather than with quantitative capacity to apply derived energy or metabolites to protein synthesis, cell count was adopted as a sufficient measure of culture growth. *Viable inoculum* for dispersed plane cultures was measured as efficiency-of-cell establishment (EOE), *i.e.,* the fraction of the cells in the inoculum that attached to the glass. EOE was measured by hemocytometer counts of a) cells and cell ghosts floating in medium 18 hours after culture inoculation, or b) cells of washed monolayers completely dispersed by trypsin. Agreement of the two measures is illustrated by a typical experiment where 3.6 x 10^5 cells and ghosts were counted in medium taken from cultures inoculated with 6 x 10^5 cells: 1.8 x 10^5 cells were recovered after removal of medium and detachment of cells from glass. *Glucose content* of media was determined as a) reducing sugar measured by Folin-Wu method and colorimetric assay with Klett-Summerson photoelectric colorimeter (filter No. 42); and b) fermentable substrate measured manometrically by use of *Candida stellatoidea*(6). Eighteen-hour yeast cultures were harvested by centrifugation, washed 4X with cold deionized water, depleted of fermentable reserves by incubation for 4 hours at 20°C in 0.06 M phosphate buffer at pH 4.5, and added to Warburg vessels in 2 ml of phosphate buffer to a density of 1.5 x 10^8 yeasts/ml. Side arms were loaded with test medium diluted appropriately in phosphate buffer. Manometric measurement of carbon dioxide was continued until evolution was less than 1 μl/15 minutes by 2 consecutive readings.

Results. Glucose depletion of serum-yeast-extract growth medium was accomplished by microbiological treatment. Dialysis was avoided since it might deplete growth factors present in the complex basal medium. *Candida stellatoidea* from 18-hour broth cultures was washed twice with cold deionized water (7) and added to growth medium to a concentration of 5 x 10^7 yeasts/ml. Seeded medium was incubated stationary at 30°C for 4-6 hours before the yeasts were removed by centrifugation. Growth of EE cells in treated medium with and without added glucose indicated that yeast treatment had depleted glucose satisfactorily, without apparent production of toxic material (Fig. 4). By colorimetric reducing-sugar assay, untreated medium contained 186 $\pm$ 38 mg glucose/liter, and treated medium 22 $\pm$ mg/liter (88% depletion). These media had been prepared without added glucose: standard medium with 12 mM added glucose contained 2,328 $\pm$ 294 mg glucose/liter, in close agreement with the expected value. By manometric assay, untreated no-sugar medium contained 154 mg glucose/liter (42.1 $\pm$ 4.3 μl CO_2 evolved from 1 ml), treated no-sugar medium contained 31 mg glucose/liter (8.4 $\pm$ 1.3 μl CO_2 from 1 ml), and standard 12 mM glucose medium contained 2,206 mg/liter. Results obtained by chemical and microbiological assays thus were similar. *C. stellatoidea* grown in glucose broth fermented fructose and glucose, but not lactose, sucrose, d+maltose, d+galactose, mannitol, cellobiose, d+xylose, l+arabinose, d-melibiose, d+melezitose, d+trehalose, d+turanose, d+raffinose, sodium lactate, sodium pyruvate, ethanol or sodium acetate. Representative values of carbon dioxide evolution are shown in Table I. By these results, *C. stellatoidea* fermentation was sufficiently selective to be a reliable method for glucose depletion of cell culture media.

Toxicity of sugars for EE cells was tested by adding sucrose, maltose, galactose, lactose, arabinose, xylose, fructose, melibiose, melezitose, trehalose, raffinose, or mannose at con-

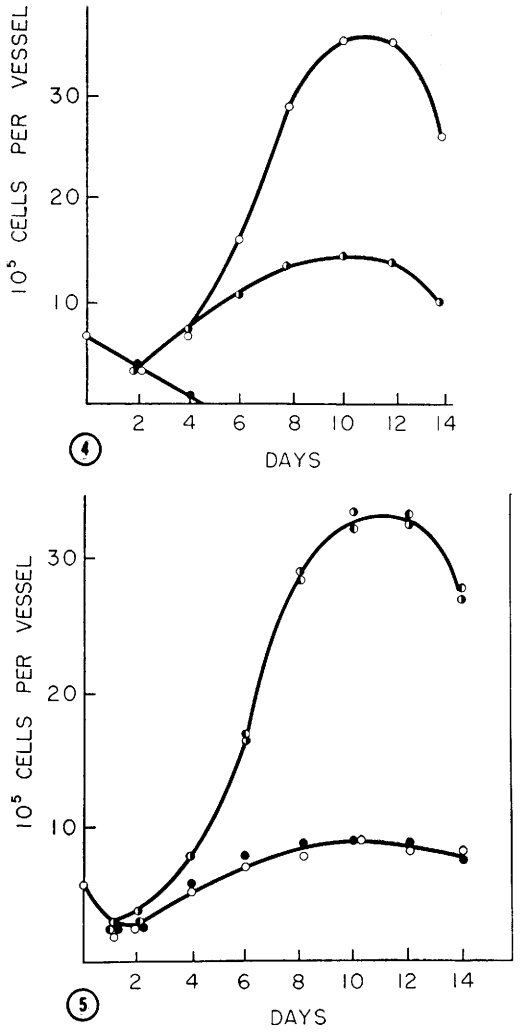


FIG. 4. Growth of human EE cells at 37°C in medium containing 10% whole human serum and 0.1% yeast extract, and depleted of glucose by treatment with *Candida stellatoidea*. The 3 curves depict growth obtained with no added glucose (solid circle), with 1 mM added glucose (half-solid circle) and with 12 mM added glucose (open circle).

FIG. 5. Growth of human EE (Minn. 55-12-1) cells in yeast-treated medium containing 10% whole serum, 0.1% yeast extract and glucose or fructose, as measured by hemocytometer counts. The 4 curves show growth with 12 mM glucose (circle, solid right), 1 mM glucose (solid circle), 12 mM fructose (circle, solid left) and 1 mM fructose (open circle).

centrations of 1.08, 2.16 or 4.32 g/liter to glucose medium. None of the carbohydrates prevented growth of EE cells or affected morphology at these concentrations.

Growth in carbohydrate media was measured for an inoculum of 6 x 10^5 EE cells in culture bottles containing 5 ml of carbohydrate medium pretreated by yeast fermentation. In *turanose, melezitose, melibiose* or *arabinose* medium, only about 10% of inoculated EE cells were attached to glass after one day of incubation, and these were detached by the fourth day. In *lactose* medium, 15% of cells attached themselves to the glass and flattened as if to initiate growth, but then detached rapidly as if some toxic product had been formed. In *raffinose* and *trehalose* media the cells adhered to the glass and were maintained for several days but without significant growth. In other experiments, EE cultures established on glass in glucose medium were washed repeatedly with NaCl-KCl solution after 3 days of incubation, and overlaid with yeast-treated sugar medium. After 8 days no cells survived in medium containing lactose, turanose, melezitose, melibiose or arabinose. Raffinose and trehalose maintained the cells but did not support multiplication. Cultures started in fructose or glucose medium exhibited identical EOE of 30%, and similar growth curves (Fig. 5). Although EE cells grew well in yeast-treated maltose medium, this glucose disaccharide was not studied further because manometric assay of glucose in yeast-treated maltose medium showed an increase from an initial concentration of 50 mg glucose/liter to about 750 mg/liter after 12 hours incubation at 37°C. Under these conditions growth permitted by maltose could not be differentiated from that permitted by glucose possibly released by serum maltase. In initial experiments, sucrose medium maintained EE cells for several days

TABLE I. Ability of *Candida stellatoidea* to Ferment Carbohydrates.

Substrate	CO_2 evolved, μl/hr	Substrate	CO_2 evolved, μl/hr
glucose	550	melibiose	19
fructose	465	cellobiose	18
galactose	262 (1st hr)	melezitose	9
	110 (2nd ")	sucrose	18
	43 (3rd ")	trehalose	9
	0 (4th ")	mannitol	12
maltose	35	raffinose	19
arabinose	10	turanose	4
lactose	29	buffer	8
xylose	23		

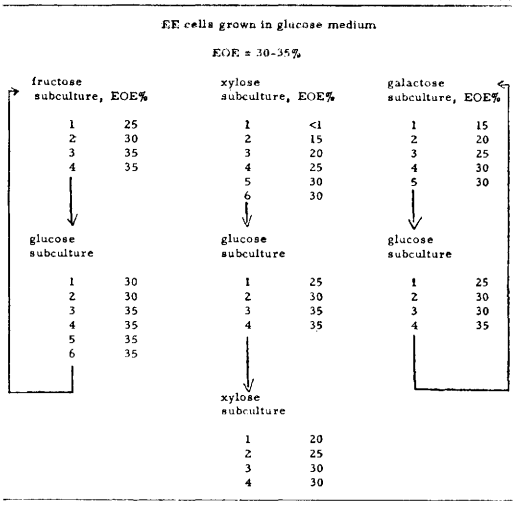
with little or no growth. Occasionally 20% of the inoculum survived and almost immediately multiplied. This irregular behavior suggested a) adaptive enzyme formation or b) uncontrolled variation of culture medium, rather than selection of pre-existing capable cells. Ability of sucrose to permit multiplication of EE cells was dependent on the serum pool employed. To detect any serum invertase, yeast-treated sucrose medium with and without added EE cells was assayed for reducing sugar by the Folin-Wu method. Although yeast-treated sucrose medium alone showed no increased content of reducing sugar after 4 days of incubation, medium with added EE cells rapidly accumulated reducing sugar. In one experiment, reducing sugar concentration increased from zero-time level of 28 mg/liter to 474 mg/liter after 12 hours, and to 840 mg/liter after 24 hours; during the following 48 hours reducing sugar concentration varied from 600 to 800 mg/liter. Results by manometric determination of fermentable substrate were similar. Since neither EE cells nor serum alone was able to convert sucrose to reducing sugar, it appeared that EE cells interacted with certain serum pools to manifest invertase activity. The cells may have activated a serum invertase precursor or deactivated an inhibitor. With the above described carbohydrates then, EE cells were a) unable to use the carbohydrate during growth, b) able to use the unaltered carbohydrate during growth or c) able to grow in medium containing products produced from the presented carbohydrate by the serum or the serum and cells together.

Selective growth in carbohydrate media was observed with 2 sugars. Commercially available *galactose* was freed of up to 2% contaminating glucose by yeast-treatment. EE cells transferred from glucose medium to galactose medium established themselves with 15-20% efficiency, compared to 30-35% EOE for glucose-to-glucose subculture. Cells transferred to galactose medium initially were smaller and grew slower than cells in glucose medium. Equivalent cell populations in the same time period frequently lowered the pH of glucose medium to 6.5, but rarely lowered the pH of galactose medium below 7.0. On second transfer in galactose medium, cells attained more nearly the size of cells in glucose medium; again the pH of galactose medium was reduced less than that of glucose medium by cell multiplication. By the fourth subculture in galactose medium, cells were as large as glucose-grown cells and their EOE had increased to 30-35%. *Xylose* medium was yeast-treated for uniformity, although commercial xylose contained very little contaminating glucose. Only 7 of 46 EE *cultures* survived transfer from glucose to xylose medium, and these grew out from a small number of cells (EOE $<1\%$). Efficiency of establishment increased to 10% with the second xylose subculture and progressively to 35% by fourth subculture. After several transfers in xylose medium, cells multiplied as rapidly as in glucose medium; again, minimal lowering of xylose-medium pH was seen. With galactose and xylose, then, ability to grow in the carbohydrate medium could be acquired by EE cultures.

Heritability of power to multiply in particular carbohydrate media was tested by serial subculture. Cells transferred from glucose medium to fructose medium exhibited an EOE of 25-30% and grew immediately. Continued fructose-to-fructose subculture raised the EOE to 30-35%. About 30% of fructose-grown cells survived transfer to glucose medium and multiplied. EOE and growth rate were not affected greatly by retransfer of these glucose-grown cells to fructose medium (Table II). Growth curves were not significantly different from those shown in Fig. 5. Therefore ability to multiply in fructose medium was hereditary and constitutive; that is, the ability was transmitted through many cellular generations in the absence of substrate.

Only small numbers of parental-culture EE cells grew on transfer from glucose to xylose medium: these achieved a 30% EOE after 4-6 subcultures representing 20 to 40 cell generations in xylose medium. The maximum efficiency corresponded to that of glucose-to-glucose subculture of control cells. Xylose-grown cells established themselves with efficiency of 25-30% on transfer to glucose medium, and with 20% efficiency on retransfer to xylose medium (Table II). Thus the altered capacity of EE populations to grow in

TABLE II. Hereditability of Capacity of Human Esophageal Epithelial Cells (Minn. 55-12-1) to Grow in Particular Carbohydrate Media.



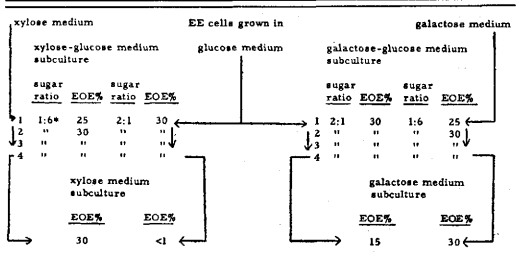
xylose medium, once obtained by selection in xylose, was hereditary and constitutive.

Results for galactose were less decisive but clearly different from those for xylose. As with xylose medium, EE populations propagated by galactose-to-galactose transfer exhibited an EOE of 30%, like that of control populations propagated by glucose-to-glucose transfer (Table II). Galactose-grown cells were transferred readily to glucose medium (EOE = 25%), but were retransferred less easily to galactose medium after limited growth in glucose medium (EOE = 15%). It appeared that the increased ability of galactose-grown cells, contrasted to glucose-grown cells, to initiate growth in galactose was adaptive; that is, the capacity to grow in galactose medium was transmitted from culture to subculture in the absence of galactose but was quantitatively dependent on presence of galactose. Since full efficiency of culture capacity to grow with galactose was not achieved with one passage of glucose-grown cells in galactose medium (Table II), it is not clear whether adaptive enzyme production was the sole mechanism operating.

Similar experiments were performed with growth media containing mixed sugars. Grams-per-liter ratios of glucose:xylose or glucose:galactose of 1:2 and 6:1 were used. Ability of glucose-grown EE cells to initiate growth in xylose or galactose medium was not increased by prior subculture in 12 mM glucose medium, supplemented with xylose or galactose respectively. Conversely, once the cells had achieved increased EOE during passage in xylose or galactose medium, this capacity was maintained during passage in medium containing 6 times as much glucose as xylose or galactose (Table III). Supplementation of glucose medium with xylose conferred no selective advantage on xylose-capable cells in glucose-grown populations, and glucose reinforcement of xylose medium conferred no selective advantage on any xylose-incapable cells in xylose-grown populations. Subculture in glucose-fortified galactose medium did not reduce efficiency of galactose-grown cells for establishment in galactose medium, nor did subculture in glucose-fortified galactose medium increase efficiency of glucose-grown cells for establishment in galactose medium.

Discussion. Capacity of human cells in continuous culture (Minn. EE 55-12-1 strain) to multiply in various carbohydrate media was studied with parental rather than clonal populations, to observe the widest range of behavior. The parental EE stock was able to grow in media containing glucose and fructose whether cultivated previously in glucose or in fructose medium. Lack of similar capability for use of sucrose and lactose contrasts with ability of the human animal. *In vivo,* sucrose and lactose capability may result from cellular interaction or from adaptive production of invertase and lactase under peculiar inductive conditions. Xylose capability of xylose-

TABLE III. Effect of Subculture in Carbohydrate Mixtures on Capacity of Esophageal Epithelial Cells (Minn. 55-12-1) to Grow in Carbohydrate Media.



* g/liter ratio of sugars as indicated by subheading.

grown EE cells was not dependent on presence of xylose but was limited to a few cells in glucose-grown parental populations. Experiments presented here did not establish whether the selected xylose-capable cells were mutants or progeny of cells existing in the tissue source of the EE strain. Galactose capability by contrast appeared adaptive. Results of experiments with mixed sugars indicated that xylose and galactose capabilities were alternative rather than substitutive to glucose capability. Given interpretations of data are subject to the reservation that analyses were based on establishment efficiencies of less than 50%. It is not yet known what additional selective forces thus were imposed by conditions of experiment. Before undertaking determinative studies of representative numbers of clones separated from the parental population, it appears desirable to investigate possible effects of very small amounts of one sugar on capacity of cells to make primary use of another sugar for growth. The necessarily complex media used herein for propagation, as noted, were highly depleted but not totally freed of glucose.

Summary. Human esophageal epithelial cells (Minn. 55-12-1 strain) in serum medium microbiologically depleted of glucose were unable to grow with lactose, raffinose, trehalose, turanose, melezitose, melibiose or arabinose as sole carbon sources. Cultures in depleted medium grew well with maltose and occasionally with sucrose by production of reducing sugar through serum or cell-serum activity. Ability to grow in glucose-depleted medium containing fructose, xylose, and galactose was inherited. Acquired xylose capability of cultures appeared to result from selective enrichment of populations with constitutively xylose-capable cells. Expression of capability of cultures to grow in galactose medium was modified reversibly by prior exposure to galactose or glucose.

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Dissociation of Some Injected Antigen-Antibody Complexes *in vivo.* (25465)

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As a natural outgrowth of many studies (1-9) exploring the fate of antigens in normal, x-rayed or sensitized animals, recent investigations(10-12) have probed certain aspects of biological activity or metabolism of antigen-antibody complexes directly. Antigen-antibody (Ag-Ab) complexes prepared *in vitro* using radio-iodinated antigen or antibody(10, 11) were injected intravenously into rabbits and elimination from serum was traced as was their initial deposition and subsequent catabolism in various tissues(11). We previously found(11) that clearance from blood of intravenously injected, insoluble, suspended Ag-Ab complexes as indicated by trace labeled antigen proceeds more rapidly in sensitized than in control animals. It seemed to us that the circulating antibody in sensitized rabbits reduced the dissociation of injected complexes thus expediting their removal from the circulation. We also showed that in normal animals antigen-antibody complexes do, at least to some extent, dissociate. We present here further evidence for (a) dissociation of antigen-antibody complexes in sera of rabbits (normal and sensitized) *in vivo* and (b) rapid