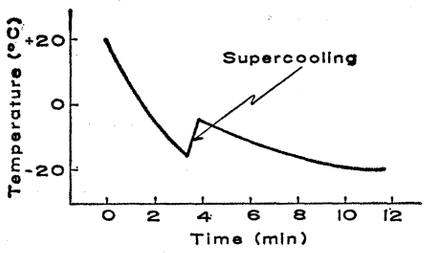

Fig. 1. Cooling rates for *Tetrahymena pyriformis* during the first step of the freezing procedure (+20°C to −20°C).

The frozen material in the vials was thawed by agitating in a water bath at 35°C. Approximately 18 or 36 seconds were needed for a 0.2-ml ice sample to thaw from −20°C or −196°C, respectively, at which time 2 ml of tetrahymena broth were added to the thawed material. After 10 minutes at ambient temperature samples were removed from the vial and examined microscopically for motility. Motility was used as the criterion of survival and was determined quantitatively by counting the number of motile cells in 0.05 ml of the sample distributed in droplets on a slide. The thawed specimens were incubated at 26°C to determine whether the motile cells would reproduce and whether normal subcultures would develop.

As shown in Table 1, the motility of *T. pyriformis* was affected by both steps in the freezing procedure. For strain W the loss in motility was greatest during the first step of freezing and least during the rapid drop from −20°C to −196°C. Regardless of the effect on the motility of the cells during the first freezing step, the number of motile cells in the sample after cooling to −196°C was fairly consistent in all experiments with this strain.

Strain H was more sensitive to the cooling temperatures. It had a greater loss of motility during both stages of the freezing procedure. Also with this strain the total number of motile cells recovered from samples at −20°C and −196°C was less constant from experiment to experiment. Nevertheless, viable cells recovered from samples of both strains developed to apparently normal cultures after 48 hours of incubation at 26°C.

Table 1. Effect of freezing on the motility of *Tetrahymena pyriformis*. Results are based on the number of motile cells per milliliter. Strain W is ATCC No. 10542; strain H is ATCC No. 9357.

Strain	Motile cells × 10^3		
	Before freezing	At −20°C	At −196°C
W	40.0	14.6	9.6
W	40.0	12.0	11.0
W	57.2	30.0	12.2
W	40.0	14.5	12.5
H	51.6	17.7	10.0
H	60.0	21.4	5.0
H	60.0	13.3	7.6

Figure 1 represents a typical curve of cooling rates for the first step of the freezing from +20° to −20°C. At −15°C the cell suspension appeared to be supercooled. The rate of cooling to this point was approximately 9°C per minute. The temperature of the cell suspension then rose rapidly to −5°C and cooled slowly at a rate of approximately 2°C per minute to −20°C, at which point the suspension seemed to be frozen solid. The temperature of the specimen, as recorded, did not go lower than −20°C during the first step of freezing.

Thus, *T. pyriformis* can be frozen to −20°C, then rapidly cooled further to −196°C, and recovered after thawing. Preliminary results of experiments to determine viability of samples stored in the gas phase of liquid nitrogen indicate that after cooling to −196°C, these organisms can be stored between −170°C and −196°C without any further loss of viability. Records are available for 3 months' storage of both strains at these temperatures.

Apparently the critical phase for these protozoa is the initial step from +20°C down to −20°C, and surviving this phase seems to depend on the rate at which the temperature falls. Several different rates of cooling were tried: for example, 1°C or 3°C per minute to −20°C, then directly into liquid nitrogen at −196°C; or rapid cooling from +20° to −76°C by placing the culture directly into dry ice slush; and from +20°C to −196°C by plunging it into liquid nitrogen. However, no viable cells were recovered from samples so treated. Viable cells were recovered only under the conditions reported. Nevertheless, even under the optimum conditions for survival, the proportion of cells that maintained their motility was not more than one-half, and in many cases was as low as one-fifth, of the initial population (Table 1).

The lethal effect upon these living cells seems to take place at the time the liquid phase of the suspending fluid changes into the solid. In other experiments, in which the cooling rate was 1°C per minute, observations made from samples taken out at 1°- to 2°-intervals during the cooling indicated that the loss of motility occurred after the cultures reached the frozen state (between −20°C and −30°C).

If ice formation causes death of the cells, the question arises why these organisms do not respond uniformly at the temperature at which ice formation occurs. Only by changing the cooling rate were conditions created that had less injurious effects on the cells studied.

It is a disappointing fact that organisms do respond differently to cooling rates. Before very low temperatures can be used for the long-term preservation of fastidious microbes a cooling rate that has less injurious effects upon cells must be sought.

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Chromosome Number and Morphology of a Human Preinvasive Neoplasm

Abstract. *The cervical epithelium from five patients with varying degrees of histologically proven intra-epithelial neoplasia can be grown successfully in tissue culture. After four or less successive transfers, the cells are diploid and have a normal karyotype.*

If there is a relationship between chromosome morphology and neoplasia, evidence of the relationship would be obtained most readily by studying cells from early cancers, particularly neoplasms which are designated as preinvasive, rather than cells from advanced cancers (*1*). Preinva-

Table 1. Chromosome numbers of cells grown in tissue culture from patients with varying degrees of cervical neoplasia.

Patient	Histological diagnosis	Chromosome number of 50 cells																	
		37	40	41	42	43	44	45	46	47	48	49	53	81	86	87	89	90	92
1	Mild dysplasia						1	1	44	1	1		1			1			
2	Moderate dysplasia		1				2	1	45					1					
3	Marked dysplasia			1				4	43	1					1				
4	Carcinoma-in-situ	1						2	42			1				1		1	2
5	Carcinoma-in-situ with microinvasion				2	1	1	2	38	3							1	1	1

sive neoplasms have been examined to some extent in experimental animals (*2*), but because of lack of experimental material and effective methods of examination there has been little opportunity for studying such lesions in humans.

We have now devised techniques for identifying accurately cervical intra-epithelial neoplasia and for growing the neoplastic cells in tissue culture (*3*). The cervices of women who are found to have abnormal cells on cytological examination are examined by means of a colpomicroscope and by using toluidine blue dye (*4*). Areas of dysplasia and carcinoma-in-situ on the exposed portion of the cervix can thus be identified and their distribution delineated with confidence. At a subsequent visit, when the cervix is not stained with dye, a biopsy is taken from the previously defined area of neoplasia. The tissue is divided into two parts under a dissecting microscope; one portion is sectioned and stained for routine histological examination, and from the other portion the epithelium is stripped, minced very finely, and placed in a small tissue-culture chamber under perforated cellophane in Eagle's minimal essential media with 15 percent fetal calf serum at 37°C, 5 percent carbon dioxide and air being used as the gas phase. After one or more successive transfers, when the cells are in the most active phase of growth, they are processed by accumulating metaphases with colchicine, expanding the cells in hypotonic solution, and fixing them in acetic alcohol. The cells are then air-dried on slides and stained with acetic orcein. The slides are scanned and metaphase plates which appear to be complete and show little or no overlap are photographed, counted, and karyotyped.

Data from five such patients are shown in Table 1. These patients had cervical neoplasia of varying degrees of severity as determined histologically. Fifty consecutive unruptured cells with well-distributed chromosomes were selected from each tissue culture. In each instance the cells were found to have a normal diploid chromosome distribution and in no instance was there evidence of significant abnormal variation in chromosome morphology (*5*). A photomicrograph of the tissue from which the culture of patient 4 was derived is shown in Fig. 1, together with a representative normal metaphase plate and karyotype.

The relationship between cancer and chromosomes has been of interest to investigators since early in this century (*6*). It is generally agreed that, whereas 97 to 99 percent of normal somatic cells are diploid with a consistent morphology, well-established malignant neoplasms are frequently heteroploid, often hypotetraploid (*7*). Diploid neoplasms do occur, particularly when the lesion is virally inducted (*8*), but established cervical malignancies, in particular, have consistently been found to be heteroploid (*9*). Previous investigation of the chromosomes from cervical intra-epithelial neoplasia by means of direct squash, without the use of tissue culture, has shown in a limited number of cells a high proportion of tetraploidy and some chromosomal morphological changes (*10*).

The data presented here, which indicates that cells grown in tissue culture from preinvasive cervical neoplasms are normally diploid, suggests that the cells which constitute the growing portion of such early lesions are primarily diploid, whereas the cells with visibly abnormal chromosomes are principally nonreproductive. It would be of interest to discover at which stage in the transition progression from nor-

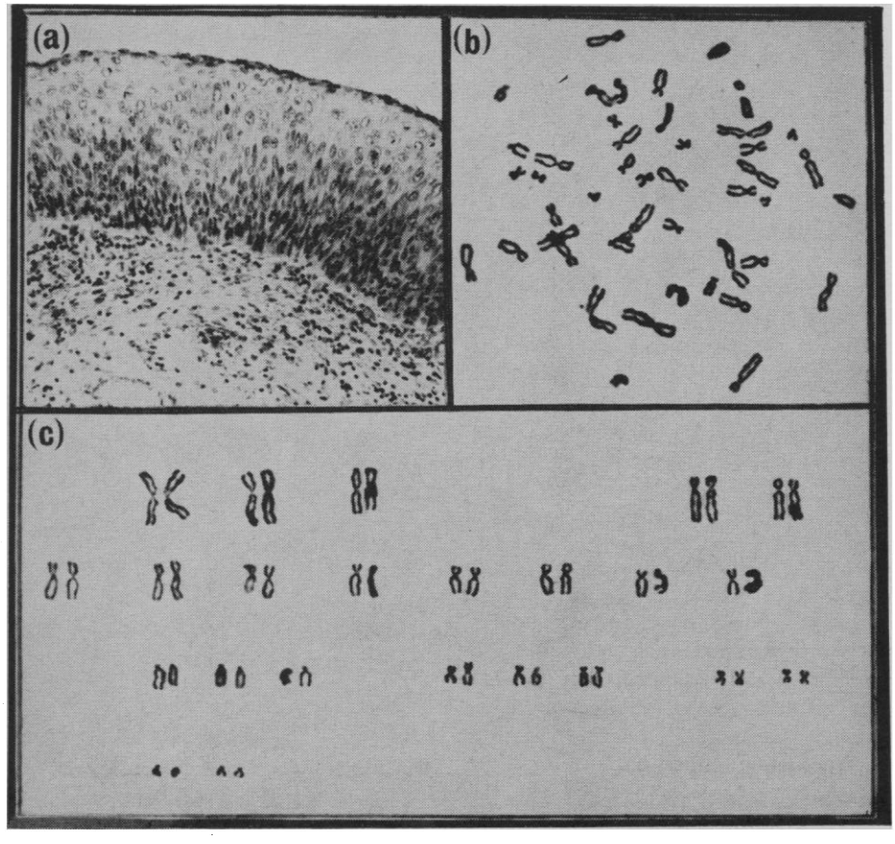

Fig. 1. (*a*) Section of tissue derived from patient No. 4 and identified histologically as carcinoma-in-situ. (*b*) Normal metaphase plate. (*c*) Normal karyotype.

mal epithelium to carcinoma changes in chromosome number and morphology in the dividing cells first appear.

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29 January 1964

Alpha-Ketoglutaric Semialdehyde: A Metabolic Intermediate

Abstract. *α-Ketoglutaric semialdehyde has been obtained as a product of hydroxyproline through reactions catalyzed by purified enzymes from* Pseudomonas. *It has been characterized both by chemical and enzymatic derivatives and by comparison with the chemically synthesized compound. This reactive compound, not previously known as a product of biological reactions, may participate in other metabolic pathways.*

α-Ketoglutaric semialdehyde (2,5-dioxovalerate) was earlier proposed (*1*) as an intermediate in the inducible degradation of hydroxyproline to α-ketoglutarate by extracts of *Pseudomonas striata.* This compound has now been isolated as an enzymatic product of hydroxyproline.

Hydroxyproline is degraded by extracts of *Pseudomonas* through the reactions shown in Fig. 1. The immediate precursor of α-ketoglutaric semialdehyde, Δ^1-pyrroline-4-hydroxy-2-carboxylate, has been obtained as an enzymatic product of reaction 2; the enzymes catalyzing reactions 3 and 4 have been purified from soluble extracts of *P. striata* grown on hydroxyproline (*2*). α-Ketoglutaric semialdehyde (KGSA) was made enzymatically on a millimolar scale and purified by chromatography through Dowex-1-Cl. Eluates in 0.1*N* HCl were concentrated and assayed by the use of enzyme 4 (Fig. 1). The following observations support the identification of the semialdehyde.

1) The reaction catalyzed by partly purified enzyme 4 leads to the formation of TPNH (reduced triphosphopyridine nucleotide) and α-ketoglutarate in quantities equivalent to the KGSA utilized. α-Ketoglutarate was measured by oxidation of DPNH (reduced diphosphopyridine nucleotide) with crystalline glutamic dehydrogenase and was identified as the 2,4-dinitrophenylhydrazone by paper chromatography.

2) On treatment with H_2O_2, KGSA releases a molar equivalent of CO_2. The residual product appeared to be succinic semialdehyde by paper-chromatographic comparison of the 2,4-dinitrophenylhydrazone with that derived from chemically synthesized succinic semialdehyde (*3*). The succinic semialdehyde-2,4-dinitrophenylhydrazone was also reduced (PtO_2, H_2, at 1.4 atm), yielding γ-aminobutyrate as the only ninhydrin-positive product. This reduction product migrated identically with authentic γ-aminobutyrate on paper chromatography.

3) Enzymatically formed KGSA could not be distinguished from chemically synthesized KGSA (*4*) by paper chromatography of the free KGSA (Table 1) or of the 2, 4-dinitrophenylhydrazones, or by elementary analyses and melting points. The 2,4-dinitrophenylhydrazine derivative of chemically or enzymatically synthesized KGSA is relatively insoluble in ethyl acetate and other common organic solvents and was recrystallized from nitrobenzene after Murakami *et al.* (*6*). Enzymatically derived KGSA-2,4-dinitrophenylhydrazone was reduced to ornithine (the only ninhydrin-positive product) with PtO_2 and H_2 at 1.4 atm. Analysis (percentage by weight) of the recrystallized phenylhydrazones gave the following composition: Chemically made: C, 41.5; H, 3.24; N, 22.3. Enzymatically made: C, 41.8; H, 2.76; N, 22.5. Calculated (for bis-2,4-dinitrophenylhydrazone, $C_{17}H_{14}N_8O_{10}$): C, 41.6; H, 2.83; N, 22.9. The melting point for the chemically made derivative of KGSA was 231° to 233°C; for the enzymatically made derivative it was 227° to 229°C. The melting point of the mixture was 229° to 233°C.

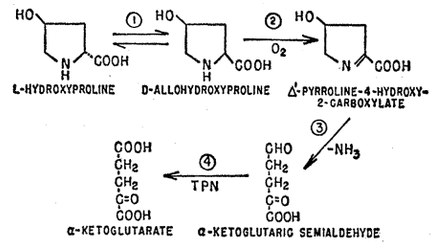

Fig. 1. Individual enzymatic steps in the inducible degradation of hydroxyproline by extracts of *P. striata.*

Little attention appears to have been paid to the chemical characterization or reactivity of free KGSA. The 2,4-dinitrophenylhydrazone of KGSA methyl ester has been synthesized by Clauson-Kaas and Limborg (*5*) from methyl-2-furoate, by way of 2,5-methoxylation and reduction. The 2,4-dinitrophenylhydrazone of KGSA itself has been obtained by Murakami *et al.* (*6*). Reductive amination of KGSA to proline has also been noted (*7*).

The semialdehyde appears to be formed from Δ^1-pyrroline-4-hydroxy-2-carboxylate (reaction 3, Fig. 1) in an irreversible reaction catalyzed by a single enzyme. No evidence has been obtained for the participation of a cobamide coenzyme or of pyridoxal phosphate. 2-Keto-3-deoxy-L-arabonate (*8*) has been ruled out as a free intermediate in the reaction.

In addition to its position as an intermediate in the bacterial degradation of hydroxyproline, KGSA has been provisionally identified by Dr. R. H. Abeles (*9*) as an intermediate in the oxidation

Tabel 1. Chromatographic behavior of KGSA made by chemical or enzymatic synthesis. Ascending chromatograms were run on Whatman No. 1 paper at room temperature. Spots were made visible by spraying with alkaline $AgNO_3$ (*11*), 2,3,5-triphenyltetrazolium (*11*), or *o*-phenylenediamine (*12*).

R_F of KGSA	
Enzymatic	Chemical
n-*Butanol:pyridine:*H_2O *(6:4:3)*	
0.38	0.42
Ether:benzene:formic acid: H_2O *(70:30:14:10)*	
0.14	0.15
n-*Propanol:0.2*N NH_4OH *(3:1)*	
0.33	0.34
t-*Butanol:formic acid:*H_2O *(70:15:15)*	
0.52	0.52
n-*Butanol:ethylmethyl ketone:*H_2O*: triethylamine (40:40:20:4)*	
0.34	0.34