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THE ASSOCIATION OF HERPES SIMPLEX VIRUS WITH CERVICAL CANCER; A MATHEMATICAL MODEL, AND EXPLORATION OF AN APPROACH TO RETRIEVE VIRAL GENETIC INFORMATION FROM TRANSFORMED CELLS

By

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ABSTRACT

A review of the available evidence relating herpes simplex type 2 (HSV-2) to cervical cancer in humans showed reasonable circumstantial evidence to suspect the participation of HSV-2 in the pathogenesis of the tumors. The best evidence was provided by seroepidemiological data, however, variations were observed among the data obtained from different populations. A mathematical model based on causal considerations and developed as an extension of a model used in chemical carcinogenesis was found to account for a large part of these variations. This model was found to reasonably describe the relation between the incidence rate of cervical cancer and the fraction of women with previous experience with the virus in different human populations. The fitting to the data suggested the existence of two kinds of etiologic factors involved in the pathogenesis of cervical cancer, one related to HSV-2 and another not related to the virus.

The model described for average population values was expanded to age-specific data. This allowed the estimation of the age distribution of primary infection with HSV-2 ($\phi(v)$), the distribution of the age of developing cancer unrelated to HSV-2 ($Y_u(t)$), the distribution of the age of developing cancer associated with HSV-2 ($Y_v(t)$), and the distribution of the time elapsing from the primary infection with HSV-2 up to the development of cervical cancer associated to HSV-2 ($\psi(u)$). The different shapes of $Y_u(t)$ and $Y_v(t)$ supported the existence of the two kinds of etiologic factors in the pathogenesis of the tumors. In addition, $\psi(u)$ was found to correspond to relatively long times suggesting an important role for viral latency or viral reinfections in HSV-2

carcinogenesis.

In view of the fact that some cervical cancers may not be related to HSV-2 the direct detection of HSV-2 genetic information in the cancer cells was considered fundamental for the correct assessment of the viral participation in the pathogenesis of the tumor. Large inconsistencies were found among reports of studies designed to detect HSV-2 genes in cancer cells. As a consequence a new approach was explored. The direct retrieval of viral endogenous genes was attempted from cells biochemically transformed by the virus. Contrary to reports from other systems, rescue was not detected. The frequency of rescue products was estimated as a value below $7x10^{-7}$. These results suggested that rescue experiments which may be suitable for the detection of latent HSV, are not sensitive enough for the exposure of subgenomic viral sequences present in transformed cells.

As a requirement for the rescue experiments, a new technique was developed to quantitate and clone HSV expressing the viral thymidine kinase in stocks predominantly lacking virus able to express this enzymatic activity.

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This thesis is dedicated to:

Q.F. Adela Piccardo de Campione
and
Dr. Adela Campione de Polero

who bear my absence,
and to:

Dr. Martha Ruben de Campione

who bears my presence.

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LIST OF SYMBOLS USED IN MATHEMATICAL EXPRESSIONS

Page First Introduced	Symbol	Meaning
15-17	x,z	fraction of women with antibodies against HSV-2. x: control, z: cancer patients
	y	overall "world" incidence of cervical cancer in a given population
	a, <u>a</u>	intercept of the regression line between y and x (in the text <u>a</u> is underlined to avoid confusion with the indeterminate article)
	b	slope of the regression line between y and x
	y_u	incidence rate of cervical cancer in a population with no HSV-2 (cases unrelated to HSV-2)
	y_v	incidence rate of cervical cancer related to the occurrence of HSV-2 infections
19	C	absolute number of cancer patients
	T	total number of individuals
20	f(y)	a function of the incidence rate of cervical cancer
	y_E	incidence rate of cervical cancer predicted to correspond to extreme (maximum) differences between the fraction of women with previous experience with HSV-2 among cancer patients and in the control population (z-x)
	RR	relative risk
22	P_j	prevalence of previously infected individuals in population j
	j	subindex indicating a given population

LIST OF SYMBOLS USED IN MATHEMATICAL EXPRESSIONS (cont'd)

Page First Introduced	Symbol	Meaning
23	x_i	fraction of women with antibodies for HSV-2 in age-group i among the control population
	i	subindex indicating a given age group
	$T_j, T_j(t)$	total number of women in population j
	$S_j, S_j(t)$	number of women that will eventually become infected with HSV-2 (susceptible)
	$N_j, N_j(t)$	number of women that will never become infected with HSV-2 (non-susceptible)
	$k_j(t), k_j, K_j$	proportion of susceptible women in a given population j (see text for specific definitions)
	$P_j(t)$	distribution of the prevalence of previously infected women as a function of age (t) in a population j
	t	continuous variable indicating age
24	$S_j^+(t)$	number of previously infected women at age t in population j
	$P_s(t)$	distribution of the prevalence of previously infected women as a function of age (t), among the population of women that will eventually become infected (susceptible)
25	$x_{i_{max}}$	maximum value attainable by x_i
26	$\phi(t)$	probability density distribution of the age of primary infection with HSV-2 among susceptible individuals
27	y_i	age-specific incidence rate of invasive cervical cancer in age group i
	$F_j(t)$	cumulative probability distribution of the age (t) of developing a detectable invasive cervical cancer. It also represents the cumulative risk at age t
	$Y_j(t)$	incidence rate of invasive cervical cancer at age t among individuals with no previous cervical cancer

LIST OF SYMBOLS USED IN MATHEMATICAL EXPRESSIONS (cont'd)

Page First Introduced	Symbol	Meaning
28	a_i	intercepts of regression lines between y_i and x_i
	y_{u_i}	incidence rate of cervical cancer in age-group i in a population with no HSV-2
	y_{v_i}	incidence rate of cervical cancer in age-group i related to the occurrence of HSV-2 infections
	$Y_u(t)$	incidence rate of invasive cervical cancer not related to HSV-2 as a function of age (t)
	$Y_v(t)$	incidence rate of invasive cervical cancer related to HSV-2 as a function of age (t)
29	V	time elapsing from birth to primary infection with HSV-2
	U	time elapsing from the primary infection with HSV-2 to the detection of invasive cervical cancer
	T	time elapsing from birth to the clinical detection of invasive cervical cancer related to HSV-2 infection (T=V+U)
	$\phi(v)$	density distribution of V
	$\psi(u)$, $\psi(t-u)$	density distribution of U=T-V
	$\lambda(t)$	density distribution of T=V+U
30	r (in Chapter 1)	correlation coefficient (see below for meaning in Chapter 3)
	P, p	probability
	α	intercept of the regression line between 1/y and 1/z
	β	slope of the regression line between 1/y and 1/z
36	$\bar{x}_u$	mean value of the estimated incidence rates of cancer cases unrelated to HSV-2

LIST OF SYMBOLS USED IN MATHEMATICAL EXPRESSIONS (cont'd)

Page First Introduced	Symbol	Meaning
	s_u	standard deviation of the estimated incidence rates of cancer cases unrelated to HSV-2
	ε	significance coefficient (Usually α is used to design this coefficient. It was changed here to avoid confusion with the meaning already assigned to this symbol)
	$r(t)$	hazard rate associated with the density distribution of the time of primary infection with HSV-2
45	$S^+(t)$	same as $S_i^+(t)$ referred to the population studied by Ng et al. (1970)
	S	same as S_i referred to the population studied by Ng et al. (1970)
	S_i^+	previously infected women in age-group i
	$\bar{x}$	mean of ln t
	s	standard deviation of ln t
49	SD_{b_i}	standard deviation of the regression slopes (b_i)
	RR_i	age-specific relative risks
50	a_i'	relative value of a_i in age group i as defined by equation (47)
	$\bar{x}_a$	mean of normal distribution fitted to a_i'
	s_a	standard deviation of normal distribution fitted to a_i'
54	b_i'	relative value of b in age-group i as defined by equation (48)
	$\lambda'(t)$	relative expression for $\lambda(t)$ as defined by equation (49)
57	ν	degrees of freedom
63	P_b	probability of a woman having cells committed to develop into cervical cancer related to

LIST OF SYMBOLS USED IN MATHEMATICAL EXPRESSIONS (cont'd)

Page First Introduced	Symbol	Meaning
		HSV-2 after infection by HSV-2
	P'_b	fraction of P_b representing events occurring before 80 years of age
	P_a	probability of developing cancer not related to HSV-2
64	RR'	relative risk considered with respect to events occurring before 80 years of age
143	f_s	frequency of rescue products
	f_v	frequency of revertants
	r (in Chapter 3)	number of successes (see above for meaning in Chapter 1)
	n	number of trials
146	F_ε	value of the F distribution for a value ε of the significance coefficient
159	P_{C^+}	probability of developing cervical cancer if not exposed to HSV-2
	P_{C^-}	probability of developing cervical cancer if previously infected with HSV-2
161	F_{ij}	fraction of previously infected women at age-group i in population j
	$F_{ij_{max}}$	maximum value of F_{ij}

LIST OF ABBREVIATIONS

Page First Introduced	Abbreviation	Meaning
2	HSV-2	Herpes simplex type 2
5	*et al.*	and coworkers
7	DNA	deoxyribonucleic acid
8	RNA	ribonucleic acid
9	AG-4	virally induced early antigen
10	HSV	herpes simplex virus
	UV	ultraviolet radiation
	HSV-1	herpes simplex type 1
66	EBV	Epstein-Barr Virus
69	TK	thymidine kinase
	TK^+	thymidine kinase positive
	TK^-	thymidine kinase negative
70	R970-5	TK^+ parental cell line derived from a human osteosarcoma
	143	TK^- cell line derived from R970-5
	BUdR	5-bromodeoxyuridine
	Vero	continuous cell line derived from green monkey kidney
	Hepes	N-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid
	mM	millimolar
	µg	microgram
	ml	millilitre

LIST OF ABBREVIATIONS (cont'd)

Page First Introduced	Abbreviation	Meaning
71	MTAGG	medium containing methotrexate (Davis *et al.*, 1974)
	Mtx	methotrexate
	M	molar
	KOS	strain of HSV-1 TK^+
	cl-101	strain of HSV-1 TK^+
	TK^-21	strain of HSV-1 TK^-
	B2006	strain of HSV-1 TK^-
	HSV-1 d_2	strain of HSV-1 TK^- with an engineered deletion within the TK gene
	PFU	plaque forming units
	mm	millimetre
72	cm^2	squared centimetre
	^oC	degree centigrade (Celsius)
	h, hr	hour
	min	minute
	vol	volume
	dThd	thymidine
	$[^3H]$dThd	tritiated thymidine [methyl-^3H]
	Ci	curie
	mmol	millimole
	µCi	microcurie
73	MOI	multiplicity of infection
75	log_{10}	decimal logarithm
79	B_4	a clone of B2006

LIST OF ABBREVIATIONS (cont'd)

Page First Introduced	Abbreviation	Meaning
86	expt	experiment
90	cpm	counts per minute
91	fig.	figure
95	B2006/A-11 B2006/B-11 B2006/C-11	plaque purified clones of B2006
	TK^-21/D-11 TK^-21/E-11 TK^-21/F-11 TK^-21/G-11	plaque purified clones of TK^-21
104	mRNA	messenger RNA
106	AC-1 AC-4 AC-5	clones of 143 cells biochemically transformed with sheared DNA of HSV-2 (219)
	LTK^-	mouse TK^- continuous cell line
	Hela Bu	human TK^- continuous cell line
	148 C-1	clone of LTK^- cells biochemically transformed with the cloned TK gene of HSV-1 KOS
	pTK-1	recombinant plasmid containing the TK gene of HSV-1 KOS
	BamHI	restriction endonuclease
	HSV-2 (219)	strain of HSV-2 TK^+
107	rpm	revolutions per minute
	Tris	Tris hydroxymethyl aminomethane
	Tris-HCl	Tris-hydrochloric acid
	EDTA	disodium ethylenediamine-tetraacetate
	NaCl	sodium chloride

LIST OF ABBREVIATIONS (cont'd)

Page First Introduced	Abbreviation	Meaning
	wt	weight
	pH	$-\log[H^+]$
108	NaI	sodium iodide
	OD_{260}	optical density at wavelength 260 mμ
	OD_{235}	optical density at wavelength 235 mμ
	ρ_{20}	density at 20°C
	DNA_o	DNA in cell lysate
	DNA_1, dna_1	pooled fractions of HSV DNA purified once in a NaI gradient
	DNA_2, dna_2	pooled fractions of HSV DNA purified twice in NaI gradient
	CsCl	Cesium chloride
	gm	gram
	cm^3	cubic centimetre
113	KCl	Potassium chloride
	$CaCl_2$	Calcium chloride
	$Na_2HPO_4 \cdot 2H_{20}$	sodium phosphate (dibasic)
	EcoRI HindIII XbaI	restriction endonucleases
118	ATP	Adenosine triphosphate
	Tris-maleate	Tris neutralized with malic acid
	$MgCl_2$	magnesium chloride
122	ATP^-	without ATP
	ATP^+	with ATP (1 mM)

LIST OF ABBREVIATIONS (cont'd)

Page First Introduced	Abbreviation	Meaning
128	ND	not done
130	kbp	thousand base pairs
131	LKH-1	LTK^- cell line biochemically transformed with HSV-1 KOS DNA digested with HindIII endonuclease
	L2X-1 L2X-2 L2X-3	LTK^- cell line biochemically transformed with HSV-2 219 DNA digested with XbaI endonuclease
133	Rn AC1A1 Rn AC4A2 Rn AC5A3	putative rescue products
135	Rn AC1B4 Rn AC1B5 Rn AC5B7 Rn AC5B8 Rn AC5C9 Rn AC5C10	putative rescue products
138	aTK-1	rabbit serum with antibodies for HSV-1 KOS TK
144	SV-40	Simian virus - 40
148	ts	temperature sensitive
	tsA	early ts mutant of SV-40

CHAPTER 1

THE ASSOCIATION BETWEEN HERPES SIMPLEX VIRUS TYPE 2 AND HUMAN CERVICAL CANCER

1.1 Introduction

In this chapter, the available evidence relating herpes simplex virus type 2 (HSV-2) to cervical cancer will be reviewed. Then a mathematical model based mainly on causal considerations will be presented which satisfactorily describes and unifies the available quantitative data. Probability distributions will be derived for the age of primary infection with HSV-2 and for ages of developing cervical cancer related or unrelated to HSV-2. The probability distribution of the time between infection and detection of the tumor will be derived for the cases associated with HSV-2. The shapes of these distributions allow the evaluation of the carcinogenic potential of the virus and suggest possible mechanisms of transformation by the agent.

1.2 Evidence Relating Herpes Simplex Virus Type 2 with Cervical Cancer

The evidence relating HSV-2 to cervical cancer has been reviewed repeatedly (Rawls *et al.*, 1977; Adam *et al.*, 1974; Aurelian, 1976; Melnick and Adam, 1978; Nahmias and Sawanobori, 1978) and consists of three main streams of data: first, epidemiologic investigations demonstrate a greater relative risk for cancer among women infected with HSV-2 than among women not infected; second, there is some data suggesting the presence of viral genetic information in the cancer cells; and third, the oncogenic potential of the virus can be demonstrated by transforming cells *in vitro*. Although the available data suggest that the virus may cause the cancer, there are sufficient inconsistencies to preclude definitive conclusions. An examination of the data generated from epidemiologic investigations will illustrate some of these inconsistencies.

A number of case-control seroepidemiologic studies have been reported in which antibodies to HSV-2 were found more frequently among cases than among controls. However the percentages of cases and of controls with antibodies to the virus have not been consistent. Fifteen to 100 percent of cases have been considered seropositive in the different populations sampled while seropositivity varied from 7 to 71 percent among control groups (Table 1). The data for incidence of cervical cancer presented in column 3 of table 1 only refers to invasive cervical cancer. The incidence of cancer *in situ* is less reliable, and will not be considered in this thesis. Waterhouse *et al*. (1976) have detailed the reasons for the difficulties in evaluating the incidence rates for *in situ* cervical cancer.

Different techniques were used to detect antibodies to HSV-2 in many of the studies and it is possible that some of the variation observed between populations could be accounted for on this basis. However, differences have been observed when serum samples from different populations were assayed in one laboratory using the same technique (Rawls *et al*., 1977; Rawls *et al*., 1980). Differences could also be due to variation in the methods of selecting the study subjects. This is especially true for control samples since differences in the occurrence of past HSV-2 infections would be expected between segments of the population in accordance with the sexual behavior of the individuals. It is difficult to envision variations in the sampling of cases as an explanation for the differences in the occurrence of antibodies to HSV-2 even though therapy for the malignancy has been reported to alter the antibody status of these women (Christenson and Epsmark, 1976, 1977;

TABLE 1

INCIDENCE OF INVASIVE CERVICAL CANCER AND OCCURRENCE OF ANTIBODIES TO HSV-2 AMONG CERVICAL CANCER CASES AND CONTROLS IN DIFFERENT HUMAN POPULATIONS[a]

Study Area[b]	Fraction with HSV-2 Antibodies		Cancer Incidence (per 100,000) (Standardized for World Population)	Reference[c]
	Controls	Cases		
Chicago, Ill./ Connecticut	.18	.48	9.8	Plummer & Masterson, 1971
Copenhagen, Denmark	.47	.85	13.6	Vestergaard *et al.*, 1972
Johannesburg, S. Africa	.64	.87	50.1	Freedman *et al.*, 1974
Prague, Czech./ Warsaw, Poland	.21	.50	21.5	Janda *et al.*, 1973
Israel	.07	.15	4.5	Menczer *et al.*, 1975
Pecs, Hungary/ Vas, Hungary	.09	.50	16.1	Pasca *et al.*, 1975
Turku, Finland	.18	.47	13.6	Peltonen, 1975
Atlanta, Ga./ Alameda, Ca.	.35	.83	28.0	Nahmias *et al.*, 1970
Aukland, New Zealand	.23	.32	9.9	Rawls *et al.*, 1970
Houston, Tx. (Black)/ Alameda, Ca.	.52	.80	28.0	Adam *et al.*, 1972a

TABLE 1 (cont'd)

Houston, Tx. (White)/ Alameda, Ca.	.23	.54	12.3	Adam *et al.*, 1972
Yugoslavia	.24	.37	18.1	Kessler *et al.*, 1974
Kampala, Uganda	.71	.81	30.0	Adam *et al.*, 1972b
Taiwan/Singapore (Chinese)	.23	.83	18.6	Kao *et al.*, 1974
Montreal, Canada/ Quebec, Canada	.21	.35	14.7	McDonald *et al.*, 1974
Tegucigalpa, Honduras/ Puerto Rico	.46	.68	25.6	Figueroa & Zambrana, 1976
W. Virginia/N.Y. State	.17	.45	10.8	Rawls *et al.*, 1976
Osaka, Japan	.15	.28	16.2	Ozaki *et al.*, 1978
Toronto, Canada/ Manitoba, Canada	.35	.50	18.6	Rawls, *et al.*, 1980
India	.36	.65	23.2	Seth *et al.*, 1978
Manitoba, Canada	.10	.19	18.6	Choi *et al.*, 1977
Ibadan, Nigeria	.11	.71	21.6	Adelusi *et al.*, 1975
Stockholm, Sweden	.27	.81	17.7	Christenson & Epsmark, 1976
W. Indies/Jamaica	.77	.97	40.4	Ory *et al.*, 1974
Norway	.28	.44	18.1	M. Davey, personal communication
Cali, Colombia	.68	.90	62.8	Muñoz *et al.*, 1975

TABLE 1 (Footnotes)

[a] The annual incidence of cervical cancer per 100,000 population (age standardized to world population) for a number of the areas were obtained from data collected by cancer registries and compiled by Waterhouse *et al.*, (1976). Rates for areas not reported in the references were assigned by making several assumptions. The assumptions made for the matching of cancer incidence and antibody prevalence data from different populations were taken from Rawls *et al.*, (1980). Briefly: The black women and white women in Houston, Texas and the black women in Atlanta, Georgia were assigned incidences for the same races living in Alameda County; these are similar for the two racial groups recorded in other U.S. cities. The Chicago sample was from a suburban population and was assigned the incidence of Conneticut. West Virginia was assigned the incidence of New York State and Montreal was assigned the incidence of Quebec: Taiwan women were assigned the incidence for women of Chinese origin living in Singapore. This incidence is quite similar to women of Chinese origin living in the San Francisco area or in Hawaii. Women in Honduras were assumed to have an incidence similar to those in Puerto Rico, the women of the West Indies were assigned the incidence reported for Kingston, Jamaica and Prague was assigned the incidence of Warsaw. The rates of cervical cancer for Johannesburg and Kampala were estimated from data published by Davies *et al.*, (1965).

[b] When two geographical locations are given the first one corresponds to the area of study for the prevalence of antibodies, and the second one represents a matched population with available data for cancer incidence.

[c] These references correspond to the study providing data for the fraction of the population with antibodies against HSV-2.

Thiry et al., 1974; Catalano and Johnson, 1971).

The inconsistencies in prevalence of antibodies to HSV-2 found in different study groups could also reflect true differences in past infections by HSV-2. If it is assumed that the estimates of the occurrence of antibodies to HSV-2 are a reasonable approximation of past HSV-2 infections in the different populations, then it is apparent that HSV-2 is not a necessary cause for all cases of cervical cancer. The virus, could, however, be a necessary cause for some cases of cancer and the proportion of cases attributable to the virus may vary between populations.

It is also appropriate to point out that cervical cancer occurs more commonly among women with attributes that increase their risk of acquiring a venereally transmitted disease (Rotkin, 1973; Kessler, 1976). Since HSV-2 is venereally transmitted, the possibility of the cancer and HSV-2 infections being covariables of a sexual behavior pattern is difficult to exclude. A number of studies have been reported which suggest that this is not the case (Royston and Aurelian, 1970a; Adam et al., 1974; Schneweis et al., 1975). The particular attributes contributing to the behavior associated with an increased risk of acquiring these diseases are not completely understood, thus, it is difficult to exclude a covariable relationship with certainty.

A currently accepted concept in viral oncogenesis is that the transformation events are triggered and maintained by the continuous expression of a viral gene (Tooze, 1980). This requires the continuous presence in the transformed cell of viral genomic sequences. This idea has proven very fruitful in the study of transformation by DNA viruses

which are less complex than the herpesviruses. The transforming capacity of papovaviruses and adenovirus seems to be mediated by the expression in the transformed cells of specific genes. In some cases specific enzymatic activities have been associated with polypeptide products of the viral transforming genes (Langan, 1980). Since there is no a priori reason to suspect that herpesviruses would mediate transformation in a substantially different way, the detection of viral genetic information in human cancers has been considered as a fundamental evidence for the oncogenic capacity of the virus in humans.

Molecular hybridization techniques (Frenkel et al., 1972; Zur Hausen et al., 1974; Pagano, 1975; Copple and McDougall, 1976; Jones et al., 1979; McDougall et al., 1980) and the detection of virus induced antigens (Royston and Aurelian, 1970b; Melnick et al., 1979; Dressman et al., 1980; Suh et al., 1980; Rawls et al., 1979) have been proposed as possible approaches to this problem. These techniques have been successfully used to detect HSV-DNA sequences or the products of their expression in biochemical and growth transformants obtained "in vitro" (Duff and Rapp, 1971b; McNab, 1974; Kraiselburd et al., 1975; Reed et al., 1975; Flanery et al., 1977; Gupta and Rapp, 1977; Pellicer et al., 1978). However, the suitability of these techniques for the detection of HSV-DNA sequences in naturally occurring human tumors remains uncertain (Spear and Roizman, 1980; Zur Hausen, 1980). To date, viral DNA has been detected in only one case (Frenkel et al., 1972; Pagano, 1975; Zur Hausen, 1974). Using a different approach, HSV-2 messenger RNA was detected in 5 of 8 (63%) specimens examined by in situ hybridization (Jones et al., 1979). In another study, 25 of 41 (60%)

carcinoma *in situ* specimens were positive for viral RNA while no viral RNA was found in 5 specimens from cases of invasive cancer (McDougall *et al.*, 1980). Variable numbers of cells from neoplastic lesions of the cervix have been found to contain antigens detected by antisera to various HSV-2 antigens. The percent of cancer cases with detectable viral antigens were reported to be 100 (Royston and Aurelian, 1970a), 62.5 (Nahamias *et al.*, 1975), 50 (Notter *et al.*, 1978) and 40 (Dreesman *et al.*, 1980). Thus, the percent of cases with evidence of viral genetic information in cancer cells has been found to vary between studies and in a substantial proportion of the cases no such evidence could be found.

Indirect evidence suggesting that HSV-2 genetic information is present in cancer cells also comes from reports of antibodies to AG-4, a virus induced antigen. Antibodies to this antigen were found to correlate with the presence of cervical cancer which implies that the antibodies reflect the expression of viral antigen in the cancer cells (Aurelian *et al.*, 1977). In Baltimore, Md., where essentially all cases have antibodies to HSV-2 detected by neutralization test, 85 percent of cervical cancer cases had antibodies to AG-4. However, in Japan only 47 percent of cancer cases had antibodies to AG-4 (Kawana *et al.*, 1976). The incidence of cervical cancer and the percent of cancer cases with antibodies to HSV-2 detected by neutralization are lower in Japan than in Baltimore. Taken together these observations suggest that the virus is not etiologically related to all cases of cervical cancer.

Morphological transformation induced in vitro by specific fragments of HSV DNA has also led to some inconsistencies. Originally, Duff and Rapp (1971a) showed that morphologically transformed cells could be obtained at low frequencies in primary cultures of rodent cells previously infected with UV-inactivated virus. More recently Camacho and Spear (1978) indicated that similar results could be obtained with a specific subgenomic fragment. Reyes et al. (1980) expanded these results and found striking differences in the transforming capacity of equivalent regions in both serotypes of human HSV. The results of these studies suggest that the transforming capacity of HSV may be less than that of papova and adenoviruses. Also a major problem is the retention of viral sequences by the transformed cells after the transforming event. Several reports in the literature have suggested the loss, after numerous passages, of the viral DNA sequences integrated in cells originally transformed by Duff and Rapp (1971a) (Frenkel et al., 1972; Copple & McDougall, 1976; Collard et al., 1973; Minson et al., 1976; Davis and Kingsbury, 1976). Reyes et al. (1980) found a similar result with one cell line transformed by one of the putative transforming regions of HSV-1 DNA. These findings considered together with the finding that HSV-DNA can transiently activate endogenous oncornaviral sequences in mouse cells (Boyd et al., 1978) has led to the idea that HSV may transform cells by a hit-and-run mechanism similar to chemical carcinogens (Reyes et al., 1980).

If HSV-2 caused cervical cancer in humans by a hit-and-run mechanism, the detection of molecular markers of viral genes in tumor cells will cease to be an appealing approach for the evaluation

of HSV-2 as a cause of the tumor. This evaluation would rely in the future on conceptual patterns more similar to those followed in the study of chemical carcinogens, and on the existence of overwhelming circumstantial evidence rather than in clearcut answers obtained from experimental approaches. It is in this context that the development of the model presented in the following sections may be considered appropriate.

1.3 Models and Causality

With the exception of radical empiricists, most scientists will accept that the primary aim of science is the explanation of phenomena (Bunge, 1963a,b). This difficult task is attempted by the establishment of law-statements or conceptual reconstructions of the actual laws ruling the evolutive changes of phenomena. Although it had been claimed in the past that all scientific law-statements were causal, it now seems clear that both causal and non-causal scientific laws can be stated (Bunge, 1963a). Even if this is the case, the establishment of causal connections is a very important approach to explanatory knowledge in modern science.

The actual existence of causal connections in the real world is still a matter of controversy. From a certain point of view causality may be considered as a simplification in the conceptualization of the evolution of natural systems. But from a practical aspect causality can be considered as a conceptual model of the actual connection existing in reality, and as such it can be fruitfully used.

In the absence of a direct experimental approach, the evaluation of the causal nature of a relationship can be difficult and subjective.

There is not a mathematical expression or a statistical test specific for causal connections. It has been indicated that scientific laws could only be expressed as differential equations (Russell, 1927). This point may be argued in Physics, but it is certainly not true in Biology (Williams, 1977), and in any case differential equations describe continuous associations without any causal implication or polarization of the connection. On the other hand most causal connections have a formal or logic component which can be satisfactorily represented by a differential equation. This component corresponds to the mathematical description of the continuous association. Although it is clear that such an equation is devoid of any causal implication, its empirical consistence very often suggests the existence of a causal link (MacMahon and Pugh, 1970; Susser, 1973).

The main interest in the detection of putative causal connections lies in their use to design causal models from which a testable hypothesis can be deduced. The overpowering logics of Popper's approach to Epistemology (Popper, 1972, Popper, 1975) and his proposed solution to the problem of induction have shown that true knowledge is made of all the hypotheses which have been shown to be false, and that those that have been accepted represent only provisional knowledge. This means that if a model is based on causal assumptions, its empirical validity will not imply the actual existence of the causal link. In fact even in the event of an empirical failure it would be difficult to "disprove" the existence of a causal connection, an experimental approach being the only way to solve such a problem. In the case where an experimental approach is not possible, the use of models in the study of causal connections is, nevertheless, justified. Depending on the

degree of certainty of preexisting knowledge about the system, the empirical failure of the model may strongly argue against the existence of the causal connection or may suggest the existence of more complex relationships within the system. If the model is empirically successful, the causal connection will not be proven, but the degree of confidence on the causal connection will be increased, or, to put it in Bayesian terms, the likelihood of the hypothesis will have increased (Lindley, 1965).

A mathematical model is a set of mathematical expressions attempting to simulate the behaviour of a natural system under study. There are basically two ways to create a mathematical model: one is to find a set of functions which reasonably describe the behaviour of the system. A function selected in this way gives an empirical description of the biological process. Another way to proceed is to derive from acceptable assumptions based on preexisting knowledge a group of equations describing quantitative relations between the variables. Usually these equations are stated as differential equations and their solutions represent a theoretical description of the biological process. Models which ignore the existence of statistical variation are called deterministic as opposed to more complex stochastic models, which are based on probabilistic considerations. The model that will be presented in this chapter is deterministic ~~and its~~ derivation is a mixture of theoretical and empirical approaches.

The three main uses for a mathematical model are the smoothing of the data, making predictions, and the use of the function itself to gain further insight into the nature of the process under study

(Causton, 1977). Logical considerations restrict this last use almost exclusively to theoretically derived models. In the model presented in the next pages most of the parameters and functions which are assigned a biological meaning and which are utilized in further theoretical considerations belong to expressions which have been theoretically derived.

1.4 The Model. Theoretical Aspects

1.4.1 Basic Development

Intuitively, it seems reasonable to expect that if an agent or factor causes a tumor then there should be some kind of relation between the incidence rate of the tumor and the occurrence of that factor within a given population. Crump and coworkers (Crump et al., 1976) have analyzed the relation between the incidence rate of developing cancer and the dose of a carcinogen which is adding its effect to an already ongoing process of spontaneous transformation. They derived an expression linking these two variables which is independent of the model chosen to describe the particular carcinogenic mechanism. They concluded that at low dose rates the response should be linear. This is specially true within the range where the extra risk introduced by the carcinogen is less than the preexisting spontaneous risk. I have expanded these concepts to develop a model describing the relation between the occurrence of HSV-2 and the incidence of invasive cervical cancer in different human populations.

HSV-2 does not elicit effective immunity and the virus may remain latent. Recurrent infections and reinfections are also possible (Rawls, 1973). Therefore, it seems reasonable to assume that the average dose rate received by each individual will be proportional to the occurrence of the virus within the population. Also antibodies are

induced following the primary infection and in most cases may last throughout life (Wentworth and Alexander, 1971). The occurrence of the virus should then correlate with the number of individuals with past experience with the virus. If it is assumed that the type specific antibodies against HSV-2 last at detectable levels throughout the life of the individual, then the fraction of individuals with antibodies in a given population can be taken as an estimate of the fraction of individuals with past experience with the virus. The fraction of individuals with type specific antibodies against HSV-2 can be defined as the occurrence of HSV-2 type specific antibodies (x) and if the virus acts as a carcinogenic agent, the overall incidence of invasive cervical cancer (y) in different populations would be expected to correlate with x. The development followed by Crump et al. (1976) for chemical carcinogens, applied to viral systems allows the expectation of linear relations between the dose (viral occurrence) and the response (incidence rate of cancer induced by the virus).

$$y = a + bx \tag{1}$$

where a and b represent parametric values of the linear expression. Within such a model a biological meaning can be assigned to these parameters; a represents the incidence rate of cancers in a population with no virus and bx is the incidence rate related to the occurrence of virus infections. The symbols y_u and y_v will be used to indicate that those biological meanings have been assigned to a and bx. Thus,

$$y = y_u + y_v \tag{2}$$

$$y_u = a \tag{3}$$

$$y_v = bx \tag{4}$$

$$b = y_v/x \tag{5}$$

y_v represents the incidence rate of cancer cases associated with the occurrence of the virus and y_u represents the incidence rate of cases independent from the virus occurrence. If it is assumed that y_v represents cases causally related to the virus infection, then y_u must represent cases caused by a different factor independent from the virus. The validity of this kind of model would then strongly suggest the existence of more than one causal factor in the pathogenesis of the tumor.

Also, from (4), $b = y_v$ when $x = 1$. The biological meaning implicitly assigned to b is then the following: b represents the cancer incidence rate associated with HSV-2 in a theoretical population where all individuals have been exposed to the virus (ie.: $x = 1$).

It should be noted that the empirical success of expression (1) will not imply the existence of a causal relation between the cancer and the virus. Any carcinogenic factor correlating in its occurrence within the population with HSV-2 but otherwise independent from it would yield the same result. If this was the case, the cancer cases correlating with HSV-2 may not necessarily appear in HSV-2 infected individuals. The consistent reports of greater numbers of HSV-2 positive individuals among cancer patients than in matched normal controls suggest otherwise (Rawls _et al_., 1980).

1.4.2 Implications of the Causal Assumption

As indicated above, causality cannot be assessed directly. However, it is possible to derive hypotheses from the model and the test of these hypotheses may provide empirical answers which support or reject the existence of a causal connection.

Two extreme situations can be considered: If all the cancer cases correlating with the occurrence of the virus are caused by the viral infection, then all the individuals with this kind of cancer must have been exposed to the virus. On the other hand if none of these cases is caused by the virus and the correlation predicted by equation (1) is circumstantial, then there is no reason to expect a higher exposure to the virus among cancer cases than among controls. If z represents the fraction of cancer patients with previous exposure to the virus, then the relations between z, y and x predicted by the two situations can be postulated: The first situation predicts that all cases represented by y_v will be positive, then:

$$z = \frac{y_u \cdot x + y_v}{y} \tag{6}$$

or using the parametric symbols:

$$z = \frac{ax + bx}{y} . \tag{7}$$

As detailed in appendix 1, this expression can be rearranged to yield:

$$y = \frac{a(a + b)}{a + b - bz} \tag{8}$$

which by inversion can be transformed into a linear expression:

$$\frac{1}{y} = \frac{1}{a} - \frac{b}{a(a+b)} \cdot z \ . \tag{9}$$

The second situation predicts that the cases represented by y_v will be positive in the same proportion detected among the normal control population (x):

$$z = \frac{y_u \cdot x - y_v \cdot x}{y} \tag{10}$$

$$z = \frac{(y_u + y_v)x}{y} \tag{11}$$

$$z = x \ . \tag{12}$$

A common assumption implied by both expressions (6 and 10) is that the fraction of y_u cases with previous exposure to the virus is always x (the fraction in the normal control population). This assumption seems justified because, by definition, those cases do not correlate in their occurrence with the prevalence of HSV-2 primary infections. Because of this, they cannot be caused by the virus. A proportion of positives higher than the normal population would have indicated that they are being caused by some factor correlating in its occurrence with the virus, which, as stated above, is not possible by definition. Since there is no formal or biological reason indicating that the proportion of positives in y_u are lower than the proportion in the normal population, the assumption that the cases reflected y_u have the same proportions of positives as the normal controls is then justified. The validation of the prediction of equation (12) by empirical data would seriously question the causal connection between HSV-2 and

cervical cancer. The data in table 1 clearly indicates that equation (12) does not have any empirical support. This means that a rejection of the possibility that HSV-2 causes cervical cancer is not justified, and that, independently from any causal consideration, a risk of developing cervical cancer can be assigned to the HSV-2 infection.

1.4.3 Other Formal Implications of the Model

Expression (7) has some interesting implications. From it an expression can be derived which relates y_u with variables y, x and z without the intervention of any estimated parameter (see appendix 1).

$$y_u = a = y(1-z)/(1-x). \tag{13}$$

The biological meaning of this equation is better appreciated if it is realized that y = C/T where C is the absolute number of cancer patients and T the total number of individuals in the population. Then

$$y_u = a = C(1-z)/T(1-x) \tag{14}$$

which is another way for stating that y_u represents the fraction of cancer patients among individuals with no past experience with HSV-2. Using expression (14), the values of y_u can be independently determined for each population considered. Their independence from x can be assessed and the probabilistic limits for their mean value can be determined independent from the accuracy of the estimation of parameter b.

The second implication is that the value z-x should not be used in studies of single populations as an indirect way to evaluate in those populations the involvement of HSV-2 in the pathogenesis of cervical

cancer. The function $z-x = f(y)$ predicted by the model is a complex expression (see appendix 1) with a maximum at:

$$y_E = (a^2 + ab)^{\frac{1}{2}} . \qquad (15)$$

The fact that this function has a maximum in its middle range indicates its poor suitability for the task outlined above. It also predicts that z-x may be very low in populations with high and low incidence of cervical cancer. The observation of values of z similar to x in these populations is not a valid reason to discredit the association of HSV-2 with cervical cancer.

Another implication is the prediction of the relation between z and x:

from (1) and (7):

$$z = (a+b)x/(a+bx) \qquad (16)$$

which by inversion can be transformed into a straight line:

$$1/z = b/(a+b) + (a/(a+b)).1/x \qquad (17)$$

An expression can be derived from the model relating the relative risk (RR) with parameters a and b. The relative risk is a parameter widely used in Epidemiology which relates the probability of developing a disease among individuals with some special characteristic, repect to the same probability in individuals lacking the characteristic. As detailed in appendix 1:

$$RR = \frac{a + b}{a} \qquad (18)$$

1.5 A New Variable: Age

1.5.1 General Considerations

One of the weaknesses of the analysis described above is that it is based in the use of single values for cancer incidence and antibody prevalence for each population considered. Both of these values are known to be functions of age and the introduction of this variable would considerably increase the scope and accuracy of the predictions.

This analysis has two interesting aspects: one of them is that it would allow an assessment of the age distribution of the cancer cases attributed to either of the two etiological factors assumed by the model. The detection of different age distributions for these cancers would constitute strong evidence for the existence of the two etiologic factors. Also age-specific rates attributed to either factor could be predicted. The second interesting aspect is the prediction of the density distribution of the time elapsing from the first contact with HSV-2 to the detection of an invasive cervical cancer. An estimate of this distribution of the age of primary infection may enable the prediction of the fraction of infected women developing cells committed to become cancerous. The following paragraphs present the theoretical basis of such analysis.

1.5.2 Age-Specific Prevalences of HSV-2 Antibodies.* A Model

The introduction of the age variable in the model requires the availability of age-specific incidences for invasive cervical cancer as well as estimates of age-specific prevalences for HSV-2 antibodies. The

*The term prevalence of antibody is applied to the prevalence of women with antibodies.

data on cancer incidence are available (Waterhouse et al., 1976). The estimation of the age-specific prevalences of HSV-2 antibodies in different populations cannot be obtained directly from published data, these being very scant and restricted to a small number of populations. When the published data are examined, two problems are immediately apparent. One is the difference in the techniques used by different investigators to detect HSV-2 antibodies. The other problem is the fact that in most publications age-specific prevalences are presented in a minimum of capriciously chosen age groups making it very difficult to detect trends in the change of antibody prevalence with age. The latter problem can be overcome by the standardization of the age intervals in all the populations included in the study. This can be done assuming a uniform occurrence of primary infections within each reported age group. The choice of five year age groups was influenced by the availability of cervical cancer age-specific incidence rates for such age intervals.

In the previous section, the variable x represented the fraction of individuals with antibodies against HSV-2 in the control population. Since the cases are a small fraction of the population, x can satisfactorily represent the fraction of positives in the overall population. This reasoning considered together with the implicit assumptions that HSV-2 antibodies are efficiently detected and that they last during the whole life of the individual, allows x to be an estimate of the prevalence (P_j) of infected individuals in a given population j:

$$x \approx P_j \ .$$

These variables may themselves be functions of age. If i indicates each age group, the discrete distributions of the x_i values should approach $P_j(t)$. It is evident that in each human population there is a certain number of women susceptible (the term susceptible is here taken to identify the females which will eventually become infected by the virus) to HSV-2 infection and a certain number which will never by exposed to the virus. For a given population j, one can write:

$$T_j = S_j + N_j \tag{19}$$

where T_j represents the total number of women in population j, S_j the number of susceptible and N_j the number of non-susceptible females. The susceptible women will be present in a proportion given by K_j:

$$K_j = S_j/T_j \tag{20}$$

and

$$1-K_j = N_j/T_j \ . \tag{21}$$

Considering age-specific data (19) can be transformed into:

$$T_j(t) = S_j(t) + N_j(t) \tag{22}$$

$$k_j(t) = S_j(t)/T_j(t) \tag{23}$$

but unless susceptible and non-susceptible females have different death rates, it can be assumed that $k_j(t)$ is independent from age: $k_j(t)=k_j$.

If $S_j^+(t)$ is defined as the number of female individuals of age t in population j with antibodies against HSV-2, then:

$$\frac{S_j^+(t)}{T_j(t)} \approx P_j(t) \tag{24}$$

which can be estimated by the age distribution of the x_i values. Similarly, an age-specific prevalence can be defined for the susceptible individuals only:

$$P_s(t) = \frac{S_j^+(t)}{S_j(t)} . \tag{25}$$

The assumption that $P_s(t)$ is independent from the population being considered is a basic requirement for the development detailed below. This assumption means that $P_s(t)$ is common for every population and that it is being assumed as a constant for the species. In other words, the probability of becoming infected for an individual at risk (susceptible) is independent from the population considered. The present status of the knowledge about HSV-2 epidemiology does not permit a careful assessment of the validity of this assumption. On the other hand, it is to a certain extent justified since the acquisition of a venereal infection will primarily depend on the sexual development of the individuals in the population. Considered as a biological variable, there does not seem to be any reason to believe that major differences will be encountered among different populations in the human society. On the other hand, cultural and socioeconomic factors may highly

influence the distribution. Unfortunately, there is no reliable way to measure these kinds of parameters. It is also possible that most individuals at high risk of acquiring a venereal infection differ less in their sexual behaviour from one population to another than the average individuals in the same populations.

Now, $P_s(t)$ is of little operative value unless it is linked with $P_j(t)$ which can be estimated from the data available for x_i:

$$P_j(t) = \frac{S_j^+(t)}{T_j(t)} \tag{26}$$

$$P_j(t) = \frac{S_j^+(t)}{S_j(t)} \cdot \frac{S_j(t)}{T_j(t)} \tag{27}$$

$$P_j(t) = k_j \cdot P_s(t) \ . \tag{28}$$

To use this last expression an estimate of k_j is required. If it is assumed that all the individuals which are at risk of acquiring an HSV-2 infection will eventually become infected during their lifetime, then for old age groups, x_i should approach k_j.

$$x_i \approx \frac{S_j^+(t)}{T_j(t)} \approx \frac{S_j(t)}{T_j(t)} = k_j \qquad \text{(for large i and t)} \tag{29}$$

In fact, k_j represents the limit value for x_i:

$$x_{i_{max}} = k_j \ . \tag{30}$$

As detailed below, the analysis of the available data for age-specific prevalence of HSV-2 antibodies suggest that t values greater than 40 years of age can be assumed to provide a satisfactory approxi-

mation of $x_{i_{max}}$. The maximum value attained by x_i in each of the populations for which age-specific prevalence of HSV-2 antibodies data were available, always occurred beyond 40 years of age. Then:

$$\frac{x_i}{x_{i_{max}}} \approx \frac{P_j(t)}{k_j} = P_s(t) \ . \tag{31}$$

The $x_i/x_{i_{max}}$ values were calculated for each population and age group where data were available.* These values represent independent estimates of $P_s(t)$. Since $P_s(t)$ is assumed to be constant for all populations, the values obtained could be averaged for every age-group i. When this was done it was apparent that the mean estimates of $P_s(t)$ were not random, and a cumulative lognormal distribution empirically fitted to them was found to satisfactorily predict their trend. If $\phi(t)$ represents the probability density distribution of the age of primary infection by HSV-2 among the population of individuals at risk ($S_j(t)$), then:

$$\frac{dP_s(t)}{dt} = \phi(t). \tag{32}$$

Since the prevalences of antibodies in the normal populations (x) used in the first part of this section are largely based on determinations in age-matched controls, and the average age for invasive cervical cancer is above 40 years of age, it may be considered that the x values reasonably represent estimates of $x_{i_{max}}$. This assumption allows the use of $\int_0^t \phi(t)dt$ to estimate the age-specific prevalences for each of the populations included in the study.

*The final procedure used is described in appendix 2.

1.5.3 Age Specific Incidence Rates

The age specific incidence rates (y_i) used in this part of the study (Waterhouse et al., 1976) are the ratios of the average number of annual new cases of invasive cervical cancer occurring within each given five-year age group over the average total number of individuals in the same age group.

According to this definition and for a given population j, the y_i values in age group i with an interval centre at age t provides a estimation of the incidence rate at age t. If $F_j(t)$ represents the cases of cervical cancer within a given group which appeared at or before a certain age t:

$$y_i \approx \frac{\Delta F_j(t)/\Delta t}{1-F_j(t)} . \tag{33}$$

The limit of y_i when Δt becomes very small is:

$$\lim_{\Delta(t)\to 0} y_i = Y_j(t) = \frac{dF_j(t)/dt}{1-F_j(t)} . \tag{34}$$

Integrating this expression yields:

$$F_j(t) = 1 - e^{-\int_0^t Y_j(t)dt} . \tag{35}$$

$F_j(t)$ represents the cumulative probability distribution of the age (t) of developing a detectable invasive cervical cancer. It can also be defined as the cumulative risk, the risk of an individual

developing cancer within a certain age period if no other causes of death were in operation (Waterhouse et al., 1976).

Since for cervical cancer $F_j(t)$ values are small (Waterhouse et al., 1976), equation (35) can be approximated by:

$$F_j(t) \approx \int_0^t Y_j(t)dt \tag{36}$$

or

$$Y_j(t) \approx dF_j(t)/dt \tag{37}$$

which means that the density probability function of the age of developing cervical cancer can be directly estimated by $Y_j(t)$. This last conclusion allows the use of the age-distribution of age-specific incidence rates y_i to estimate probability density distributions of age random variables. This condition is a prerequisite for the subsequent developments described in this chapter.

1.5.4 Estimates for Distributions of Transforming Times*

The age-specific values for incidence rate of cervical cancer and prevalence of HSV-2 antibodies can be considered within the model developed for overall population values. In this way, age-specific values can be obtained for parameters a and b (a_i and b_i). Then, biological meanings similar to those assigned to a and b can be attributed to the age-specific parameters ($a_i = y_{u_i}$, $b_i \cdot x_i = y_{v_i}$).

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*Here the term transforming time is taken in a broad sense to include the time of the transforming events plus the time of growth of the tumor to a detectable size. For $Y_u(t)$ it spans from birth to the development of a detectable tumor, while for $Y_v(t)$ it goes from the primary infection with HSV-2 to the clinical detection of cervical cancer.

The age-distribution of the y_{u_i} values can be interpreted as an estimate of the age distribution of the incidence rate of cervical cancer in a theoretical population with no cases associated with HSV-2, $Y_u(t)$. If HSV-2 is assumed to be the etiological factor, then this would be the case of a population with no HSV-2 infections.

In a similar way the age distribution of the values for y_{v_i} represents the distribution of the incidence rates of invasive cervical cancer associated with HSV-2 in a population with all of the individuals infected by the virus, $Y_v(t)$. A difference between $Y_u(t)$ and $Y_v(t)$ would support the empirically derived conclusion about the existence of at least two etiological factors in the pathogenesis of cervical cancer.

If, as assumed in the model, all cases which correlate with the prevalence of HSV-2 antibodies are caused by the virus, then they must all have had a previous experience with HSV-2 before developing a tumor. The time from birth to the development of a detectable tumor has to be the result of the summation of the time elapsing from birth up to primary infection (V) plus the time from primary infection up to the development of the tumor (U). If these two times are considered as independent random variables with density distributions $\phi(v)$ and $\psi(u)$, the age of developing a tumor (T) will have a distribution $\lambda(t)$ which will be given by the convolution of the distributions of V and U (Lindley, 1965; Defares et al., 1973)

$$\lambda(t) = \int_0^t \phi(v) \cdot \psi(t-v)\, dv \,. \tag{38}$$

It is immediately apparent that $\phi(v)$ represents the distribution $\phi(t)$ described above to which a lognormal distribution has been successfully fitted. Also $\lambda(t)$ can be estimated through $Y_v(t)$. This should allow the derivation of an estimate for $\psi(t-v) = \psi(u)$. After solving the problem posed by the fact that $Y_v(t)$ represents a truncated distribution, different functions with different parametric values can be assigned to $\psi(t-v)$ to find out which one allows the best fit of equation (38) to the data for y_{v_i} estimating $Y_v(t)$.

1.6 The Model. Its Fitting to Empirical Data

1.6.1 Fit of a Linear Function to y and x

The fitting of equation (1) to the data for incidence of invasive cervical cancer (y) and prevalence of HSV-2 antibodies in the normal population (x) shown in table 1, is presented in figure 1. The estimated values for each of the parameters and for the correlation coefficient (r) are the following:

$$a = 5.72 \times 10^{-5}$$

$$b = 50.08 \times 10^{-5}$$

$$r = 0.793 \quad (P<0.001).$$

1.6.2 Fit of a Hyperbola to y and z

The data for y and z in table 1 may be used to fit equation (8) through the use of equation (9), (figure 2). The intercept α and the slope β predicted by equation (9) are:

$$\alpha = 1/a = 1.246 \times 10^4$$

$$\beta = b/(a^2+ab) = 1.071 \times 10^4$$

$$r = -0.635 \quad P < 0.001$$

Figure 1

Correlation between the incidence of cervical cancer (y) and the fraction of women in the control population with antibodies against HSV-2 (x). The incidence values are the annual incidence of invasive cervical cancer per 100,000 individuals, standardized to the World population, reported by Waterhouse et al. (1976). The estimated fraction of control women age-matched to cervical cancer cases with antibodies against HSV-2 are those listed in table 1. The line represents the linear function providing the best fit to the data.

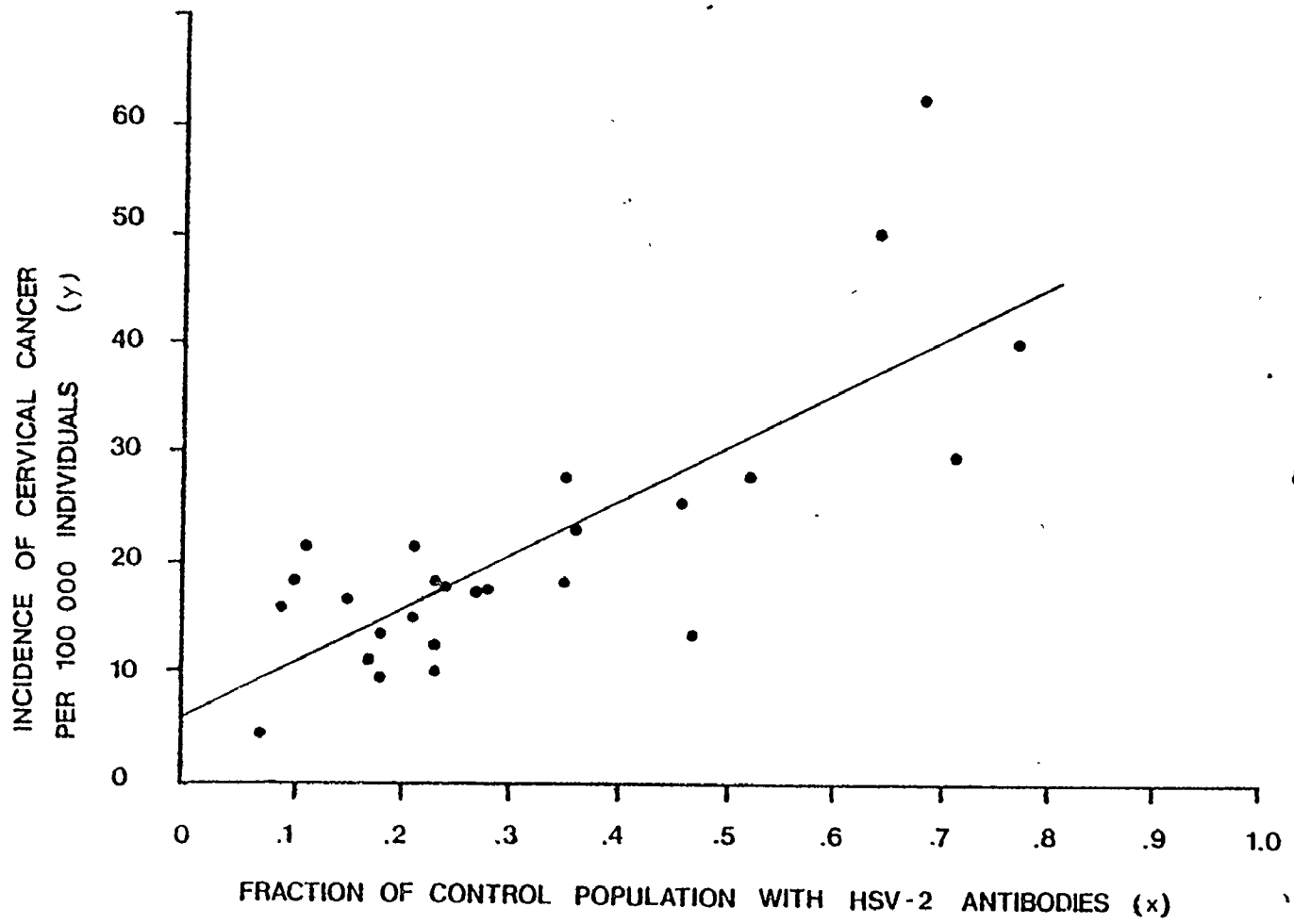
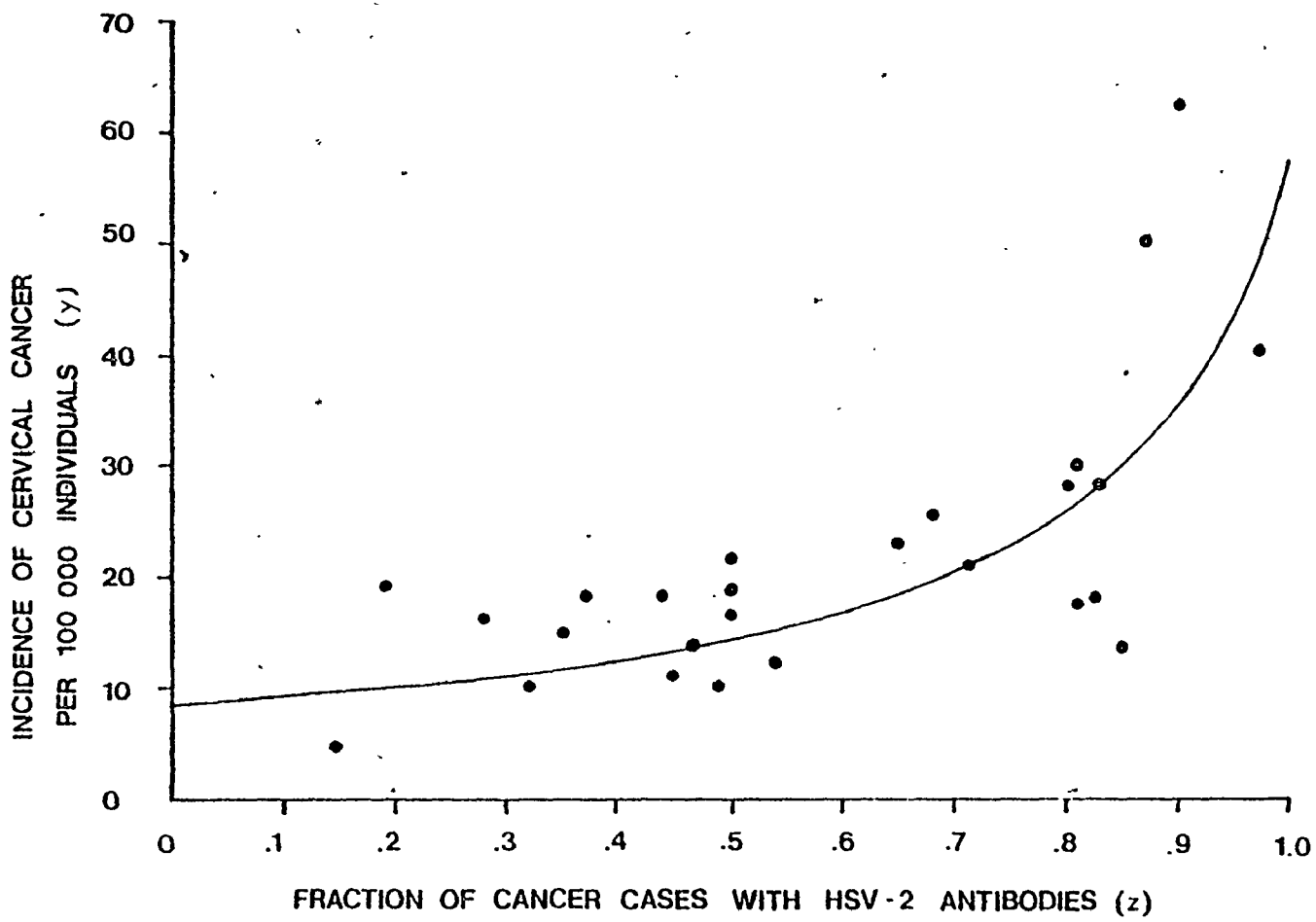

INCIDENCE OF CERVICAL CANCER
PER 100 000 INDIVIDUALS (y)
0
10
20
30
40
50
60
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
FRACTION OF CONTROL POPULATION WITH HSV-2 ANTIBODIES (x)

Figure 2

Correlation between the incidence of cervical cancer (y) and the fraction of cancer cases with antibodies against HSV-2 (z). The y and z data are the same presented in table 1. The line represents the best fit hyperbolic equation predicted from the assumption that the correlation shown in figure 1 only occurs at the level of the individual.


INCIDENCE OF CERVICAL CANCER
PER 100 000 INDIVIDUALS (y)
70
60
50
40
30
20
10
0
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
FRACTION OF CANCER CASES WITH HSV-2 ANTIBODIES (z)

New estimates for a and b can be derived from these values:

$$a = 1/\alpha = 8.02 \times 10^{-5}$$
$$b = \beta a^2/(1-\beta a) = 49.09 \times 10^{-5} .$$

The fitting of equations (1) and (9) to the data was done using a linear regression analysis estimating the linear function which provided the least mean square deviations (Dixon and Massey, 1957). The validity of the linear regression analysis used to fit equations (1) and (9) is based on the assumption that the variance of different estimates of the independent variable for given values of x is independent of the value of x, or, in other words, that the independent variable is homoscedastic. When a linear form of a hyperbolic function is used to fit a hyperbola to the data, one common problem is that it requires the use of a transformed variable, usually the inverse of the original. Equation (9) is the linear transform of the hyperbolic function indicated in equation (8). In this case the new independent variable used in the regression analysis is 1/y, which is the inverse of the original one (y). The assumption of homoscedasticity may not hold true for the 1/y transform and a weighted regression analysis should be preferred (Bliss, 1970). The investigated analysis is, nevertheless, justified because the heterogeneity of sources among the data is a much larger source of error than the one introduced by the heteroscedasticity of 1/y, especially since the range of the y values only spans a ten-fold change in magnitude. The unweighted linear regression analysis should provide a reasonable approximation.

1.6.3 Independence of y_u from the Occurrence of HSV-2

Expression (13) allows the estimate of the value of y_u for each of the populations included in the study. Figure 3 shows the relation between the estimated y_u values and the prevalence of HSV-2 antibodies (x). The correlation coefficient (r) of the relation is not statistically significant ($r = 0.371$, $P>0.05$).

Also, the mean and standard deviation can be calculated for the values of y_u. As indicated in the previous section, this allows the assessment of the probabilistic limits of y_u=a independently from the variation for the estimates of the slope b.

$$\bar{x}_u = 10.764 \times 10^{-5}$$

$$S_u = 4.973 \times 10^{-5}$$

$$[0.867 \leq a \leq 20.660] \qquad 1-\varepsilon = 95\% .$$

This means that y_u is significantly larger than zero.

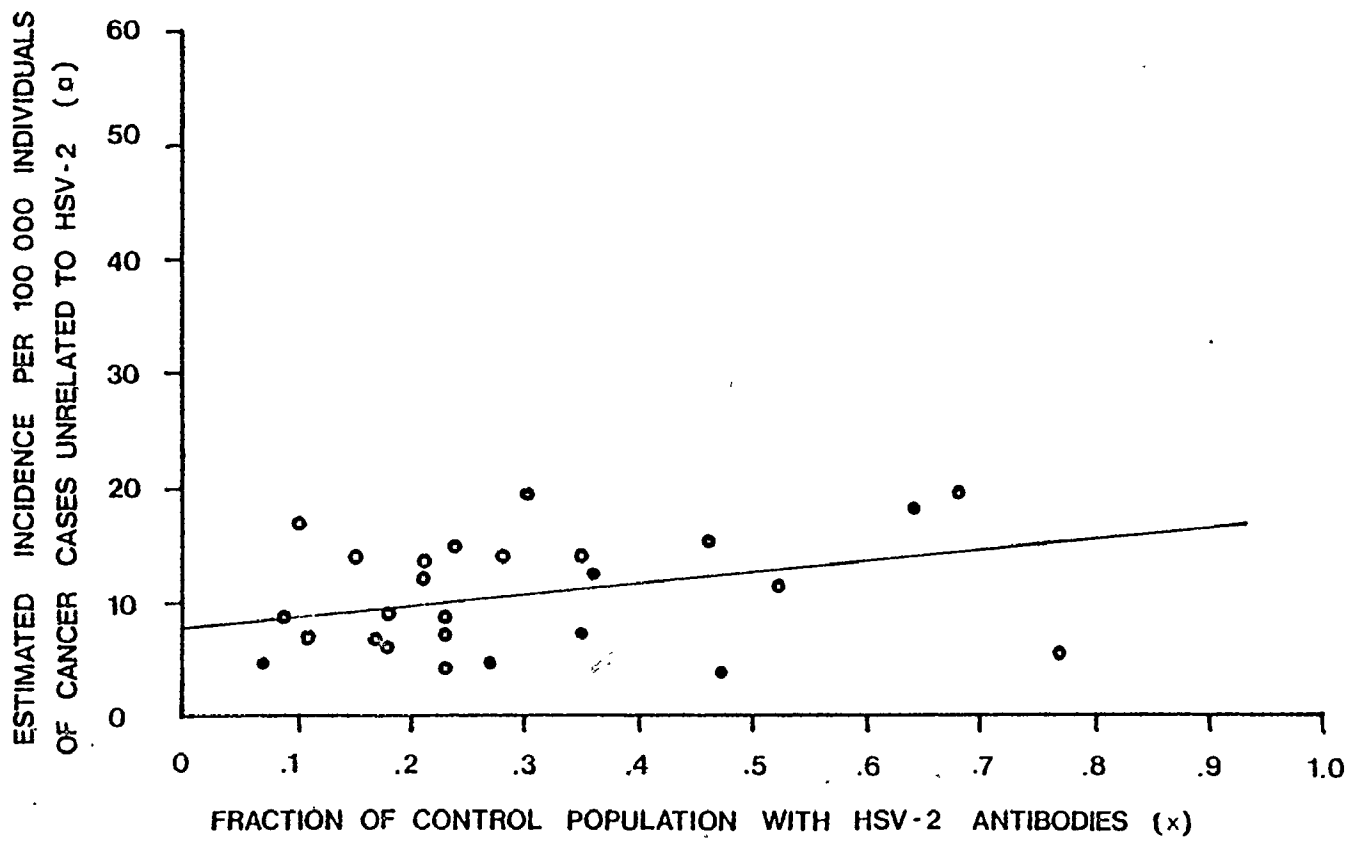
1.6.4 Age-specific Prevalences of HSV-2 Antibodies

A lognormal distribution was fitted to the available data estimating age-specific prevalences of HSV-2 antibodies in different human populations. The choice of this distribution was suggested by the fact that it had been successfully used in the past to describe the onset of different variables associated with sexual maturation in women (Nydell, 1924; Wicksell, 1917; Aitchison and Brown, 1957).

The data estimating age-specific prevalences of HSV-2 antibodies is presented in the literature in heterogeneous age-groupings (Adam *et al.*, 1972a; Adam *et al.*, 1973; Rawls and Adam, 1977; Nahamias *et al.*, 1970; Menczer *et al.*, 1975; Ory *et al.*, 1975; Ozaki *et al.*,

Figure 3

Correlation of the estimated incidence of cervical cancer cases unrelated to HSV-2 (y_u) and the fraction of women in the control population with antibodies against HSV-2 (x). The data for x are those presented in table 1. The values of y_u were estimated independently for each population as described in the text, utilizing the values of x, y and z in table 1.


ESTIMATED INCIDENCE PER 100 000 INDIVIDUALS
OF CANCER CASES UNRELATED TO HSV-2 (a)
60
50
40
30
20
10
0
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
FRACTION OF CONTROL POPULATION WITH HSV-2 ANTIBODIES (x)

1978; Ishiguro and Ozaki, 1978; Rawls et al., 1969; Royston and Aurelian, 1970a). For this reason, the number of susceptible individuals (appendix 3) and the number of positives reported in each study for each age group were reassorted in 7 standardized age groups each spanning 10 years of age (table 2). A weighted probit analysis was then performed with the pooled data (Colquhoun, 1971; Finney, 1964). The pooling together of the data from different populations is allowed by the assumption that $P_s(t)$ is constant. The 95% confidence limits of the estimated $P_s(t)$ are shown in figure 4 together with the empirical pooled data (broken line). The lognormal distribution providing the least square fit has the mode at 19.2 years and the median at 24.7 years of age.

Figure 5 shows the density distribution:

$$\phi(t) = \frac{dP_s(t)}{dt} \tag{39}$$

and the hazard rate:

$$r(t) = \frac{dP_s(t)/dt}{1-P_s(t)} \tag{40}$$

which represents the incidence rate of HSV-2 primary infections among individuals not previously infected.

The empirical validity of the lognormal distribution fitted to $P_s(t)$ can be assessed by comparing it with the age-specific occurrence of herpes genitalis. Data for age-specific incidence of herpes genitalis is provided in a report by Ng and coworkers (Ng et al., 1970). These authors gathered 256 cases of first occurrence of herpes genitalis in a population of predominantly Black women.

TABLE 2

POOLED DATA IN STANDARDIZED AGE-GROUPS FOR NUMBER OF INDIVIDUALS WITH ANTIBODIES AGAINST HSV-2 (POSITIVES) AND TOTAL NUMBER OF SUSCEPTIBLE WOMEN.[a]

Age group	Positives	Estimated susceptible women[b]	% Positives
0-10	1	68	1.5
10-20	32	108	29.6
20-30	78	160	48.7
30-40	116	196	59.2
40-50	167	191	87.4
50-60	116	127	91.3
60-70	47	62	75.8

[a]The data in this table represents the pooled data in standardized age groups for 10 samples of control individuals studies for antibodies against HSV-2. The samples were obtained from different human populations. (See text for references).

[b]The number of susceptible women in each age-group were computed as described in appendix 2.

Figure 4

Relative age-specific prevalences of HSV-2 infections ($P_s(t)$) estimated by probit analysis from the data for age-specific prevalence of HSV-2 antibodies in different populations. The lines represent the cumulative lognormal distribution providing 95% confidence limits of the best fit line. The broken line shows the estimates from the pooled empirical data.

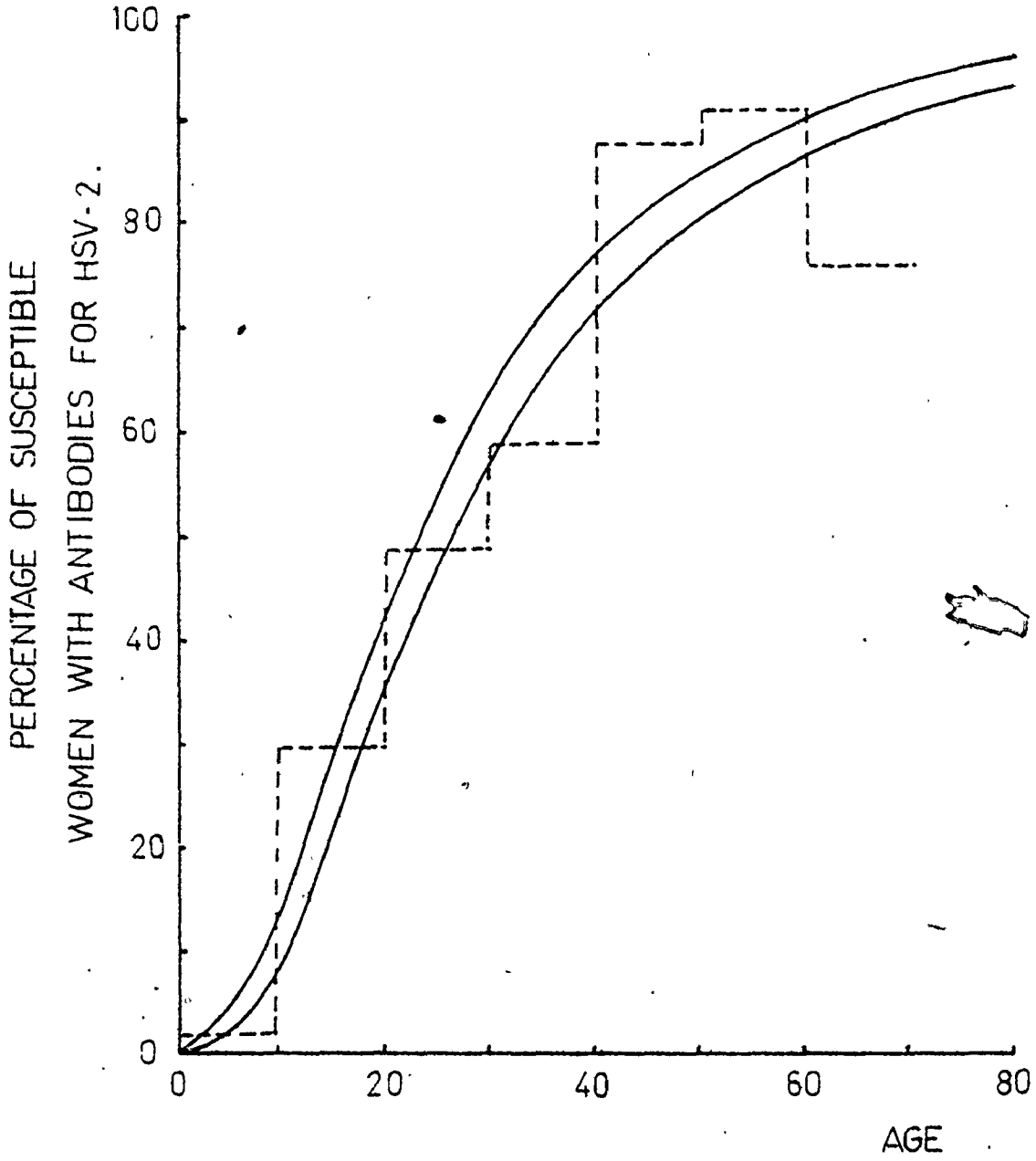
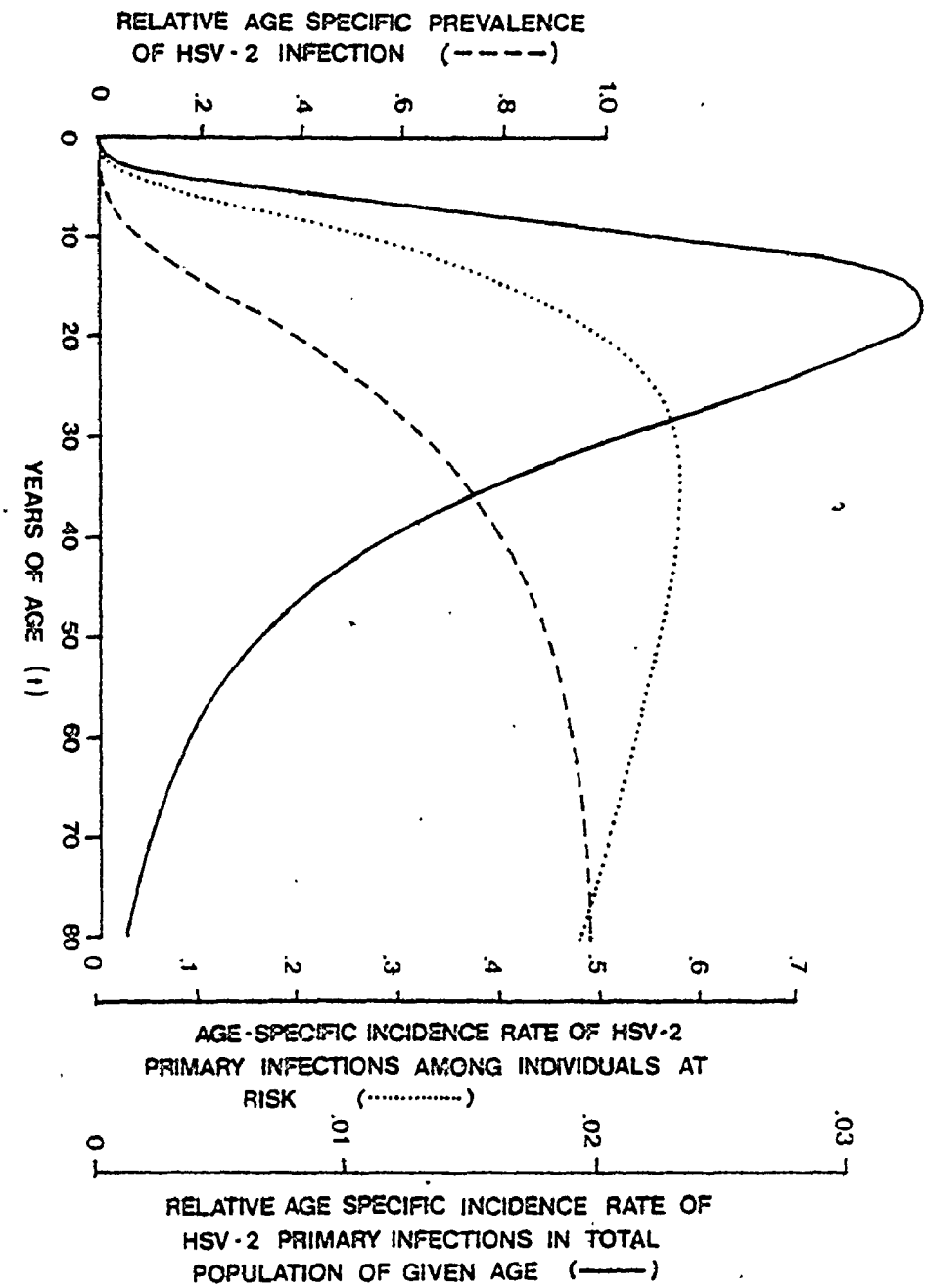

PERCENTAGE OF SUSCEPTIBLE
WOMEN WITH ANTIBODIES FOR HSV-2.
100
80
60
40
20
0
0
20
40
60
80
AGE

Figure 5

Cumulative lognormal distribution fitted to the relative age-specific prevalences of HSV-2 antibodies ($P_s(t)$), its density distribution ($\phi(t)$) and the hazard rate distribution ($r(t)$).


RELATIVE AGE SPECIFIC PREVALENCE OF HSV-2 INFECTION (- - - -)
0
.2
.4
.6
.8
1.0
YEARS OF AGE (t)
0
10
20
30
40
50
60
70
80
0
.1
.2
.3
.4
.5
.6
.7
AGE-SPECIFIC INCIDENCE RATE OF HSV-2 PRIMARY INFECTIONS AMONG INDIVIDUALS AT RISK (.....)
0
.01
.02
.03
RELATIVE AGE SPECIFIC INCIDENCE RATE OF HSV-2 PRIMARY INFECTIONS IN TOTAL POPULATION OF GIVEN AGE (——)

From expressions (25) and (39) one can write:

$$\phi(t)dt = [d(S^{+}(t)/S(t))/dt]dt \ . \tag{41}$$

If other competing risks are disregarded, S(t) can be treated as a constant:

$$\phi(t)dt = \frac{(dS^{+}(t)/dt)dt}{S(t)} \ . \tag{42}$$

The data provided by Ng et al. (1970) represent the percentages of new cases occurring among individuals of given ages in a certain period of time, with respect to the total amount of cases diagnosed during the same period. Considered as a fraction and for intervals of age Δt during an interval of observation $\Delta\tau$:

$$\frac{\text{New cases in age group i per year of age, during interval } \Delta\tau}{\text{Total number of new cases per year of age, during interval } \Delta\tau} =$$

$$= \frac{[\Delta S_i^{+}/\Delta t] \ . \ \Delta\tau}{\sum_{i=1}^{k} [\Delta S_i^{+}/\Delta t] \ . \ \Delta\tau} = \tag{43}$$

$$= \frac{[\Delta S_i^{+}/\Delta t] \ . \ \Delta t}{\sum_{i=1}^{k} \Delta S_i^{+}} \tag{44}$$

where k represents the total number of age groups considered. The numerator in this expression is equivalent to the one in expression (42) for $\phi(t)$, and the number of susceptible individuals at any given age group, S(t), as defined previously in this chapter, can be estimated by the total number of individuals which eventually will become

infected. If the oldest age-group can be assumed old enough so that most of the susceptible women (S) have been infected, then $\sum_{i=1}^{k} \Delta S_i^+$ should approximate S. The comparison of the expression for $\phi(t)dt$ derived from the model with the data of Ng et al. (1970) for clinical detection of HSV-2 primary infection, is presented in figure 6.

1.6.5 Age-specific Incidence Rates of Cervical Cancer Related and unrelated to HSV-2

The empirical fitting of a lognormal function to $P_s(t)$ and the estimation of the k_j values allows the calculation of age-specific prevalences of HSV-2 antibodies for different populations. Equation 45 (Aitchison and Brown, 1957):

$$P_j(t) = k_j \; P_s(t) = \frac{k_j}{s\sqrt{2\pi}} \int_0^t \frac{e^{-\frac{1}{2}\left(\frac{\ln t - \bar{x}}{s}\right)^2}}{t} \, dt \qquad (45)$$

was used to calculate age-specific prevalences of HSV-2 antibodies in each of the populations included in this study. In this expression $\bar{x}$ and s^2 are the mean and variance of ln t values. The values for $k_j \approx x_{i_{max}}$ were estimated by the fraction of positives in control women age-matched to cancer cases. The association of the $P_j(t)$ values with age-specific incidence rates y_i obtained from tables (Waterhouse et al., 1976) were analyzed by linear regression analysis. This procedure was followed with each of the other 13 5-year age-groups considered in the study. The printouts from a computer program with the entire collection of age-specific data are presented in appendix 3. The

Figure 6

Comparison between the relative age-specific incidence rate of HSV-2 primary infections ($\phi(t)$) predicted by the model (continuous line) with age-specific incidence rates estimated from the data of first clinical occurrence of herpes genitalis obtained by Ng et al., (1970) (broken line).

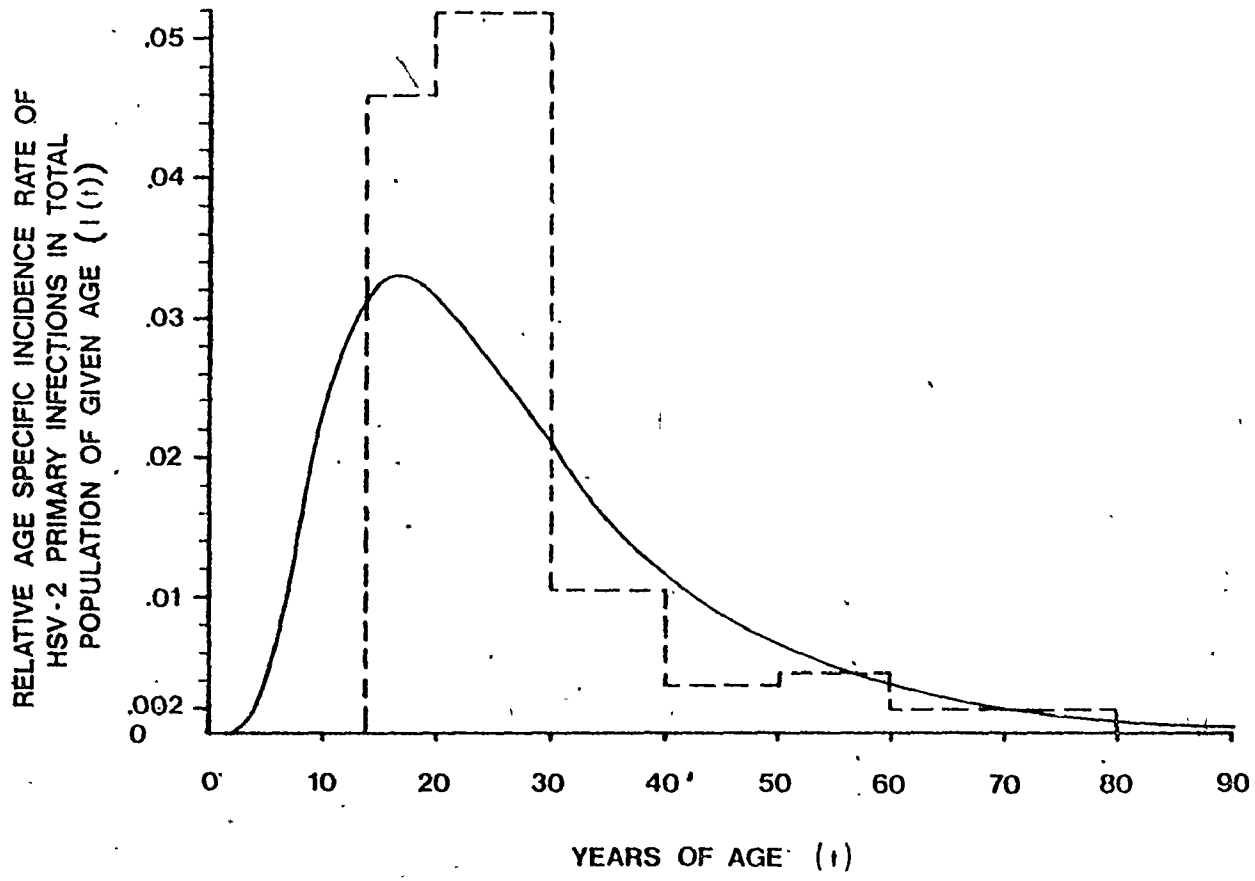

RELATIVE AGE SPECIFIC INCIDENCE RATE OF HSV-2 PRIMARY INFECTIONS IN TOTAL POPULATION OF GIVEN AGE (I(t))
.05
.04
.03
.02
.01
.002
0
0
10
20
30
40
50
60
70
80
90
YEARS OF AGE (t)

TABLE 3

PARAMETRIC VALUES FROM REGRESSION ANALYSIS OF AGE-SPECIFIC DATA

Age Group	i	a_i	b_i	SDb_i[a]	RR_i	r	p[b]
15-20	1	0.0651	5.6545	5.433	87.89	0.222	>0.05
20-25	2	1.238	15.9952	12.041	15.23	0.283	>0.05
25-30	3	4.2277	26.7564	15.309	7.33	0.373	>0.05
30-35	4	4.1650	73.8475	21.535	18.73	0.731	<0.001
35-40	5	6.4444	116.6443	31.756	19.10	0.783	<0.001
40-45	6	11.6872	128.6872	34.191	12.03	0.804	<0.001
45-50	7	13.3794	137.6209	43.089	9.95	0.681	<0.001
50-55	8	17.5720	153.7660	44.322	9.75	0.740	<0.001
55-50	9	13.7286	152.5733	42.142	12.11	0.772	<0.001
60-65	10	11.1944	184.7722	52.389	17.51	0.752	<0.001
65-70	11	9.9355	170.5709	46.084	18.17	0.789	<0.001
70-75	12	7.9146	157.8732	41.481	20.95	0.811	<0.001
75-80	13=k	3.4143	169.0840	45.930	50.52	0.785	<0.001
TOTAL		106.8520	1494.1233				

[a] SDb_i represents the standard deviations of the regression slopes (b_i).

[b] p is the probability with which the actual value of the correlation coefficient estimated by r could be equal to zero.

values obtained for the correlation coefficients (r), the intercepts a_i, the slopes b_i and for the relative risks RR_i, are presented in table 3. This last parameter was computed replacing in equation (18) the age-specific parameters a_i and b_i:

$$RR_i = \frac{a_i + b_i}{a_i} \quad . \tag{46}$$

Two aspects can be considered: the correlation coefficient is statistically significant beyond 30 years of age and the relative risk seems to have a "U"-shaped age distribution with a minimum around 50 years.

1.6.6 Analysis of the Incidence of Cervical Cancer Unrelated to HSV-2.

Most of the cervical cancer cases reflected by $y_{u_i} = a_i$ seem to occur before 80 years of age. The distribution of the a_i' values defined as:

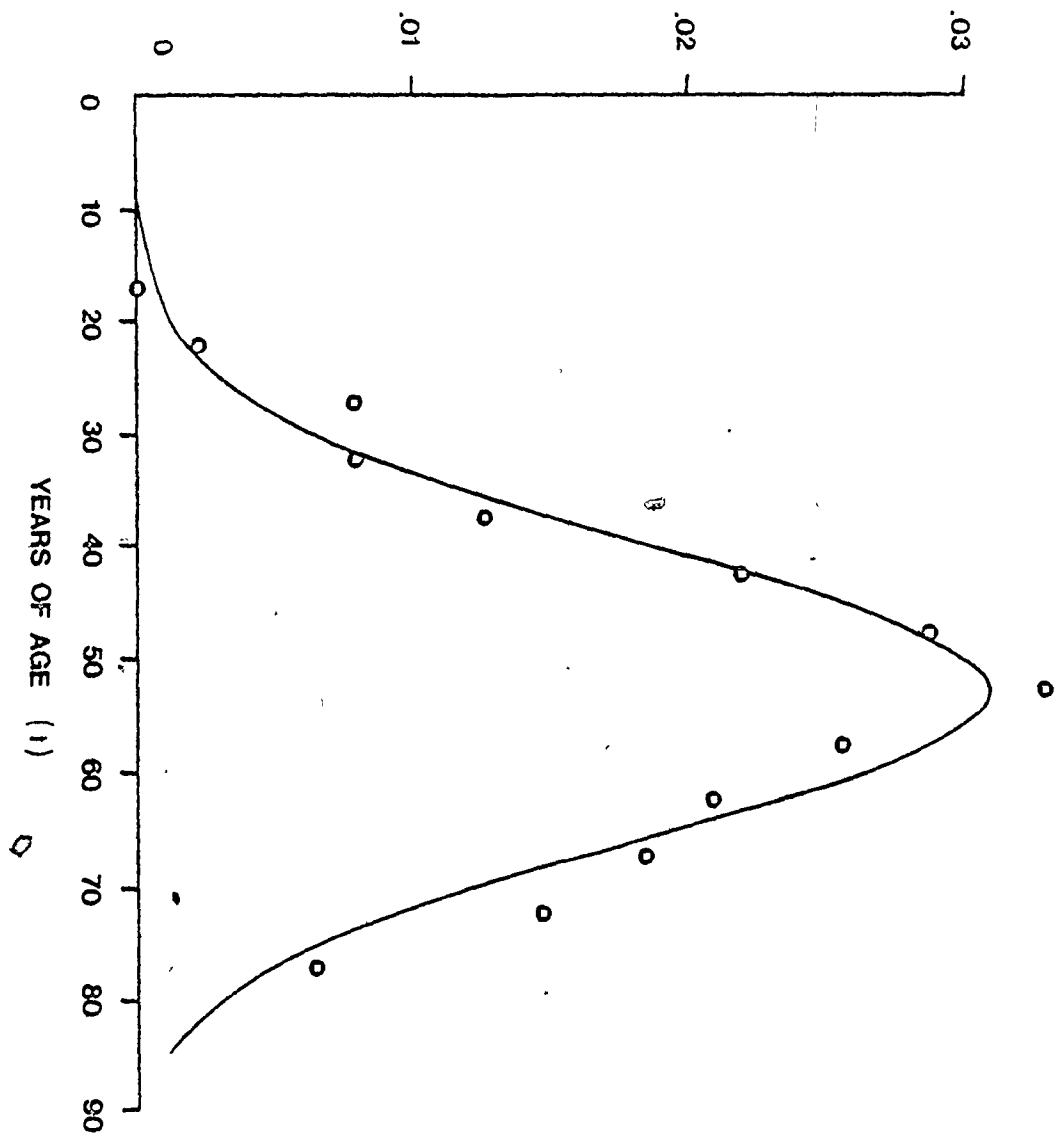
$$a_i' = \frac{a_i}{\sum_{i=1}^{k} a_i} \tag{47}$$

where k represents the age group with the upper boudary at age 80, should represent an estimate of the density probability associated with the age at which an invasive cervical cancer is detected. As such, density distributions can be directly fitted to the age distribution of a_i'. Figure 7 shows the least square fit normal distribution, with parameters $\bar{x}_a$ = 53.0 yrs and s_a = 12.7 yrs, which successfully

Figure 7

Normal distribution providing the best fit to the data estimating the relative age-specific incidence rates of cervical cancer unrelated to HSV-2 (a_i').

RELATIVE AGE SPECIFIC INCIDENCE RATE OF CERVICAL CANCER UNRELATED TO HSV-2 ($o'(t)$)



describes the empirical data.

1.6.7 Analysis of the Incidence of Cervical Cancer Related to HSV-2. Evaluation of $\psi(u)$.

The fitting of a function to the age distribution of b_i was not approached in the same empirical way. Expression (38):

$$\lambda(t) = \int_0^t \phi(v) \cdot \psi(t-v)dv$$

represents the density distribution of the age at developing cervical cancer when the tumor has been caused by the virus. $\phi(v)$ represents the density distribution of the age of primary infection and $\psi(t-v)$ is the density distribution of the transforming times (time elapsing from infection to development of detectable invasive cervical cancer). According to the model developed, the density of the age distribution of b_i represents an empirical approximation to $\lambda(t)$. $\phi(v)$ is known and, as seen before, a lognormal distribution has been successfully fitted to it. These two conditions provide enough information for the analysis of $\psi(u)$. The estimate of $\psi(u)$ can be approached by assigning different distributions to it; then, using the expression indicated above, the $\lambda(t)$ values can be determined, and the distribution assigned to $\psi(u)$ which provides the best fit of $\lambda(t)$ to the density of the b_i data can be determined.

The degree of fitness of $\lambda(t)$ to such data can be evaluated by calculating the squared deviations between the expected and observed values. One of the practical problems encountered in this analysis is that b_i represents a truncated distribution. Since its

complete shape cannot be determined from the data, and in order to allow the use of the convolution formula (which provides data for complete distributions) to estimate $\lambda(t)$, the values of b_i were transformed in the same way as described for a_i:

$$b_i' = \frac{b_i}{\sum_{i=1}^{k} b_i} \quad . \tag{48}$$

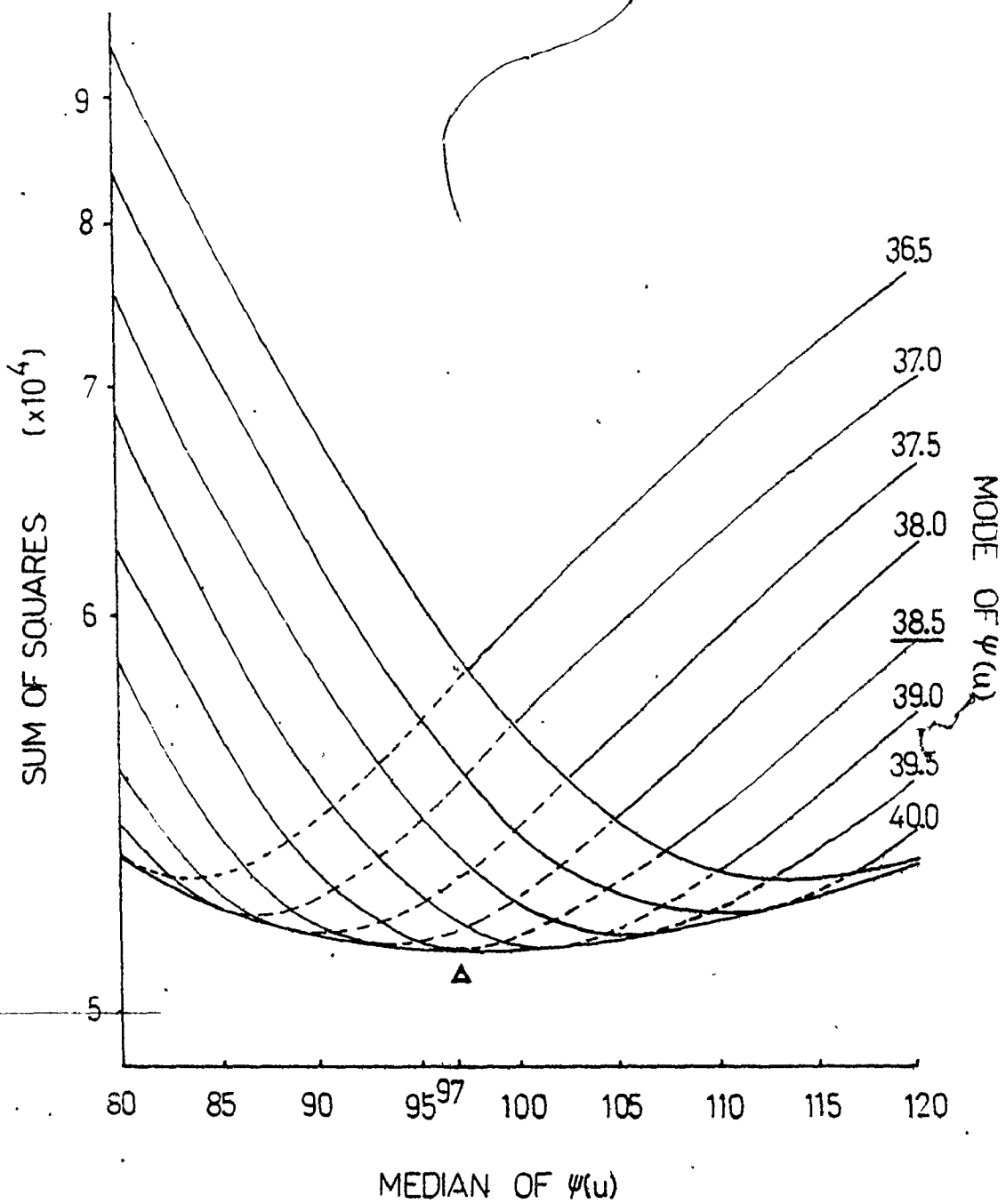
The values for $\lambda(t)$ were obtained from equation (38) and were transformed in a similar way:

$$\lambda'(t) = \frac{\lambda(t)}{\int_0^{80} \lambda(t)dt} \tag{49}$$

A computer program was written to evaluate the square deviations between $\lambda'(t)$ and the empirically derived b_i' values. It was immediately apparent that lognormal distributions of $\psi(u)$ provided smaller sum of squares than normal distributions. Fig. 8 shows a part of the surface produced by the square deviate values (vertical) as a function of the median (horizontal) and the mode of lognormal distributions assigned to $\psi(u)$. It can be seen that the lognormal distribution of $\psi(u)$ providing the best fit (least sum of square deviations) of $\lambda'(t)$ to the data had the mode at 38.5 years and the median at 97.0 years.

Figure 8

Contours of sum of squares between b_i' values and estimated $\lambda'(t)$ functions. The $\lambda'(t)$ functions were evaluated by the convolution of $\phi(v)$ and $\psi(t-v)$ after assigning different lognormal distributions to $\psi(t-v)$. The sum of squares (vertical axis) are shown as a function of the median (horizontal axis) and mode (imaginary axis perpendicular to the paper plan) or each lognormal distribution assigned to $\psi(t-v)$.


SUM OF SQUARES (x10^4)
9
8
7
6
5
36.5
37.0
37.5
38.0
38.5
39.0
39.5
40.0
MODE OF ψ(u)
80
85
90
95
97
100
105
110
115
120
MEDIAN OF ψ(u)

To estimate the boundary limits of the parameters of $\psi(u)$ providing the least squares the following procedure was followed: since the standard deviations of the b_i values are known (see table 3) the 95% confidence limits for these values $\left(t_{(\varepsilon=.05,\nu=11)} \cdot SDb_i\right)$ were computed and the sum of square deviates between these 95% limit values and the b_i means was determined. This value was found to be equal to 3.34×10^{-2} which is about 65 times larger than the minimum sum of squares value provided by a lognormal estimate of $\psi(u)$ using the convolution equation (see figure 8). Now, if 3.34×10^{-2} is taken as the limit of statistical significance for the sum of squares, a boundary can be drawn between significant and non-significant values. Due to the overall shape of the function of sum of squares the limits of the mode and the upper limit of the median are difficult to evaluate. But this is possible for the lower limit of the median. As indicated in table 4, the limit of significance for the median can be placed at 23 years of age. Provided that the rest of the assumptions used to develop the model hold true, with 97.5% confidence it can be determined that half of the individuals developing cervical cancer associated with HSV-2 will develop the tumor 23 or more years after the primary infection with HSV-2.

Figure 9 shows the $\lambda(t)$ distribution obtained and its fit to b_i' data. Figure 10 shows the lognormal distribution estimated for $\psi(u)$.

The estimation of $\psi(u)$ is important because of two reasons. First, it provides some insight into the possible role of the virus

TABLE 4

97:5% CONFIDENCE LOWER BOUNDARY FOR THE MEDIAN OF $\psi(u)$.[a]

MEDIAN OF $\psi(u)$ (in years of age)	MODE OF $\psi(u)$ (in years of age) 10	12	14	16	18	20	22
20	4.46	4.39	4.52	5.09	6.58	-	-
21	4.17	4.02	4.00	4.29	5.17	7.43	-
22	3.92	3.71	3.60	3.70	4.18	5.55	-
23	3.70	3.46	3.29	3.25	3.47	4.25	6.44
24	3.52	3.25	3.03	2.91	2.97	3.37	4.64
25	3.36	3.07	2.82	2.64	2.59	2.77	3.46
26	3.22	2.92	2.65	2.43	2.30	2.34	2.69
27	3.09	2.78	2.50	2.26	2.08	2.02	2.18
28	2.98	2.67	2.37	2.11	1.91	1.79	1.80
29	2.88	2.56	2.26	1.99	1.77	1.61	1.55
30	2.79	2.47	2.17	1.89	1.65	1.46	1.35

[a]The values in the table represent the sum of the square deviates ($\times 10^2$) between the b_i' values derived from the empirical data and the $\lambda'(t)$ values calculated with the convolution equation. The boundary limit of significance is placed at 3.34×10^{-2}, this being the value of sum of squares given by the 95% confidence limits of the b_i estimates.

Figure 9

Density distribution providing the best fit to the data estimating the relative age-specific incidence rates of cervical cancer related to HSV-2 (b_i'). This distribution ($\lambda(t)$) is the result of the convolution of two lognormal density distributions, one fitted to the age-distribution of primary infection with HSV-2 ($\phi(v)$) and another one assigned to the distribution of the time elapsing from infection to diagnosis of invasive cervical cancer ($\psi(u)$). The lognormal distribution assigned to ($\psi(u)$) is the one providing the least sum of square deviates between $\lambda'(t)$ and the b_i' data.

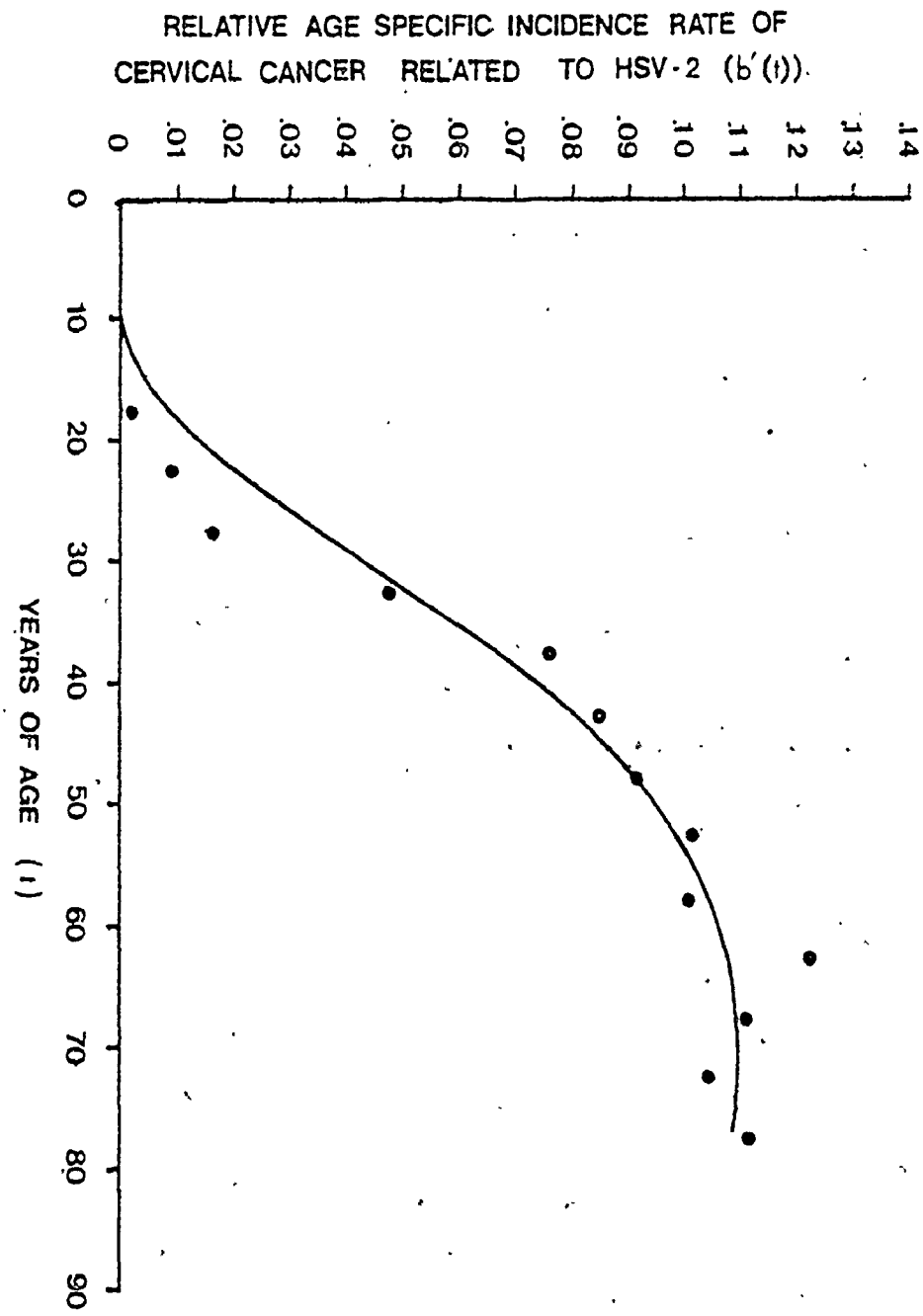
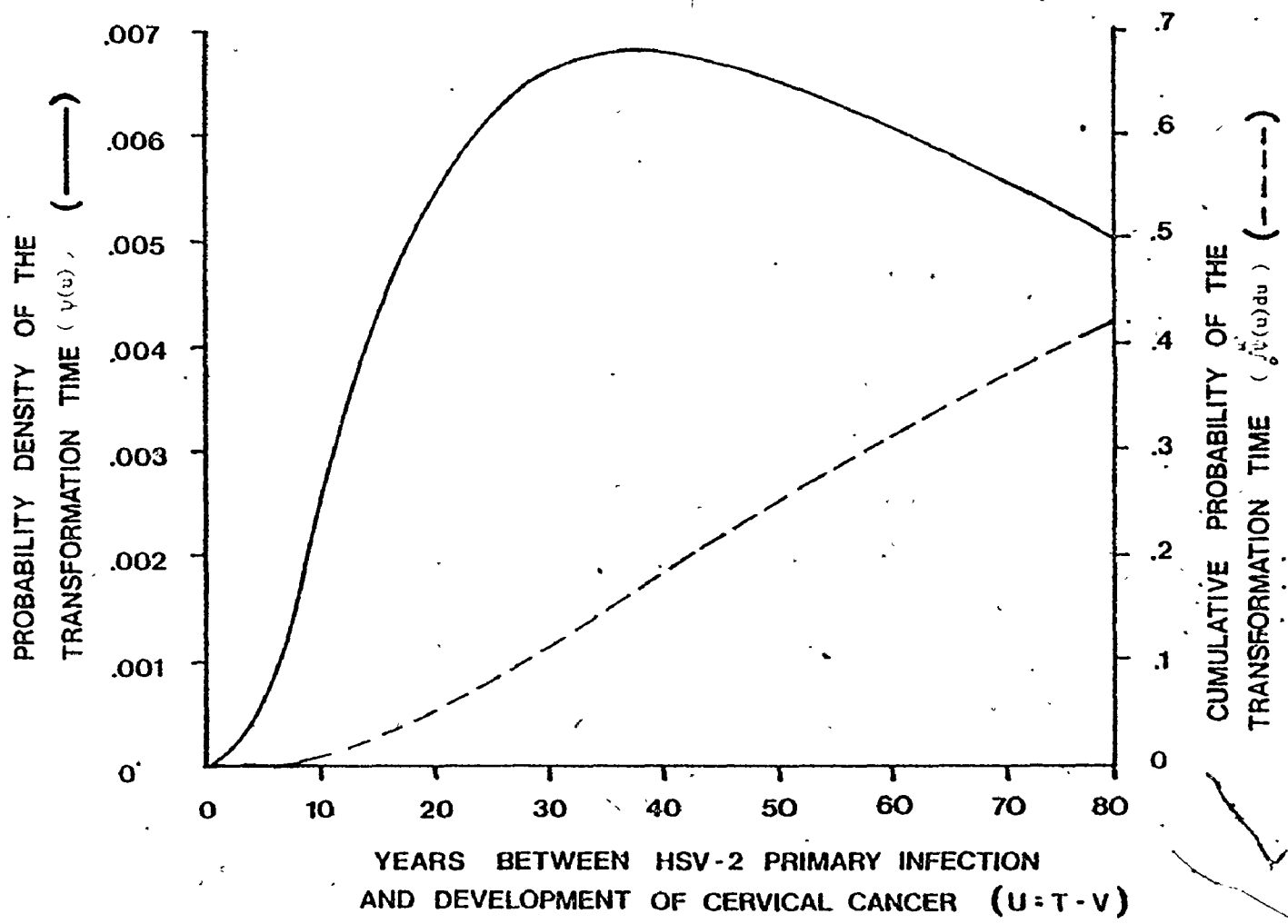

RELATIVE AGE SPECIFIC INCIDENCE RATE OF
CERVICAL CANCER RELATED TO HSV-2 (b′(t))
0
.01
.02
.03
.04
.05
.06
.07
.08
.09
.10
.11
.12
.13
.14
0
10
20
30
40
50
60
70
80
90
YEARS OF AGE (t)

Figure 10

Density (continuous line) and cumulative (broken line) distributions of the time elapsing from HSV-2 primary infection to the diagnosis of invasive cervical cancer $\psi(u)$. The function shown here corresponds to the lognormal distribution providing the least sum of square deviates between $\lambda'(t)$ and the b_i' data.


PROBABILITY DENSITY OF THE TRANSFORMATION TIME (ψ(u) , ———)
CUMULATIVE PROBABILITY OF THE TRANSFORMATION TIME (∫₀ᵘ ψ(u)du) (- - - -)
.007
.006
.005
.004
.003
.002
.001
0
.7
.6
.5
.4
.3
.2
.1
0
0
10
20
30
40
50
60
70
80
YEARS BETWEEN HSV-2 PRIMARY INFECTION
AND DEVELOPMENT OF CERVICAL CANCER (U = T - V)

62

theoretical aspects. Second, it provides information about the efficiency of transformation by the virus. In a population of 10^5 infected individuals, in the absence of any other cause of death, and considering the parametric values providing the least squares fit:

$$\sum_{i=1}^{k} b_i \times 10^5 = 1149$$

$$\int_0^{80} \psi(u)\,du = 0.42$$

and the total number of people who were committed to develop cervical cancer, had they lived long enough, was in total:

$$\frac{1149}{0.42} = 2736.$$

Since it was assumed that they all belong to a population of 10^5 infected individuals, it can be estimated that the probability of a woman having cells committed to develop into cervical cancer related to HSV-2 after being infected is:

$$P_b = \frac{2736}{10^5} \approx 0{,}027$$

and the probability that this will happen before 80 years of age:

$$P_b' = \frac{1149}{10^5} = 0.011 .$$

If it is assumed that these cases are caused by the virus, it has to be concluded that the efficiency of the transforming event in-

duced by HSV-2 remains quite low. On the other hand, if the same kind of calculations are carried out for a_i, in a population of 10^5 non-infected individuals:

$$\sum_{i=1}^{k} a_i \times 10^5 = 107$$

$$\int_0^{80} Y_u(t)dt \approx 1$$

and the probability of developing cancer not associated with HSV-2 is then:

$$P_a = \frac{10^7}{10^5} \approx 0.001 .$$

The overall relative risk is:

$$RR = \frac{P_a + P_b}{P_a} = \frac{0.028}{0.001} = 28$$

and considered up to age 80:

$$RR' = \frac{P_a + P_b'}{P_a} = \frac{0.012}{0.001} = 12 .$$

Also, among the invasive cervical cancers appearing before 80 years of age, about 92 percent will be associated with HSV-2.

$$\frac{P_b' \times 100}{P_a + P_b'} \approx 92 .$$

Even if the transforming efficiency of HSV-2 may be low, its potential responsibility in the pathogenesis of cervical cancer remains considerably large.

1.7 Conclusions

From the evidence summarized in the first part of the chapter and from the analysis presented, it can be concluded that there exists some kind of association between HSV-2 occurrence and invasive cervical cancer in humans. The quantitative data indicating this connection is satisfactorily explained by a model assuming the existence of at least two etiologic factors in the development of the tumor. One factor is closely linked to HSV-2 infection at the level of the individual and another factor is independent in its participation from the viral infection. The model developed allowed the segregation of the incidences related to each of these factors and the age distributions of these incidences were shown to differ from one another. The factor related to the virus is relatively more important at the two extremes of life and would be responsible for most tumors developing at old age.

The age-distribution of the transforming times elapsing from infection to the development of a clinically detectable invasive tumor associated with HSV-2 infection suggests that the virus transforms cells with low efficiency. If the virus is the actual oncogenic factor, this result agrees with experimental studies of transformation by inactivated virus or viral DNA (reviewed in section 1-2). Even more interesting is the finding that the transforming times are relatively

ger tha tim DNA

Available data for antibodies against Epstein-Barr Virus and incidence of Burkitt lymphoma in the same population suggest a mean around 4 years for periods spanning from primary infection with EBV to the clinical detection of Burkitt lymphoma (De-Thé et al., 1978, De-Thé, 1979). The results presented here indicated that HSV-2 has a comparatively long latency of tumor induction, about 10 times longer than EBV. This suggests the possible important role of virus latency and reinfection in the induction of the tumor. On the other hand, since the primary contact of the virus occurs long before the transforming effect, this result is also compatible with the idea that HSV-2 may be triggering a sequence of events required for transformation in a similar way to chemical carcinogens. In this respect, an inducer effect of the viral infection requiring the later participation of a promotor for the triggering of transformation, cannot be discarded.

Rotkin (1972) postulated an epidemiologic model of cervical cancer in which neoplasia starts early in life as a result of factors encountered at the onset of sexual life. After a certain time a second co-carcinogenic event would develop which would trigger the final stages of the malignant transformation. The results of the theoretical approach already discussed suggest that one of the factors acting early in life may be closely related to the primary infection with HSV-2.

In view of the fact that some cervical cancers seem not be related to HSV-2 and since the possiblity exists that HSV-2 may be med a hit-and-run mec ism

different from what is currently accepted for other tumor viruses, the development of efficient molecular markers for viral genes present in the transformed cells seems of utmost importance. The attempts to develop one of such markers constitute the rest of this thesis.

CHAPTER 2

A SELECTIVE ASSAY FOR HERPES SIMPLEX VIRUSES EXPRESSING THYMIDINE KINASE

2.1 Introduction

This chapter presents the development of a technique for selecting herpes simplex viruses expressing the viral thymidine kinase (TK^+) from a population in which most of the viruses do not express the enzyme. This method was developed as a technical requirement for the experiments to be described in following chapters. Most of the results reported in this chapter of the thesis are already published (Campione-Piccardo *et al.*, 1979).

Among the enzymes induced by herpes simplex virus (HSV) there is a deoxypyrimidine kinase which phosphorylates both thymidine and deoxycytidine (Jamieson *et al.*, 1974; Kit, 1976). This enzyme, here referred to as thymidine kinase (TK), is coded by the virus genome (Dubbs and Kit, 1964; Summers *et al.*, 1975), and the genetic information for it can be transferred to cells deficient in cytosol TK (Bacchetti and Graham, 1977; McAuslan *et al.*, 1974; Minson *et al.*, 1978; Munyon *et al.*, 1971; Pellicer *et al.*, 1978; Wigler *et al.*, 1977, 1978).
The transfer of the virus TK gene to mammalian cells deficient in TK (TK^-), or biochemical transformation, represents a useful system to study the interaction between the DNAs of the virus and of the host cell. In addition, observations derived from this system may provide knowledge relevant to oncogenic transformation.

A number of TK^- mutants of HSV, some of which behave as chain termination mutants, have been isolated (Summers *et al.*, 1975). These TK^- mutants render the HSV TK system potentially useful for a variety

of studies, including the measurement of spontaneous and induced mutation rates of virus genes, the isolation of recombinant viruses, the rescue of virus genes from transformed cells, and possibly the identification of nonsense suppressor mutants of mammalian cells capable of overcoming the chain termination defect of the virus genes. The exploration of these problems has been hampered by the lack of a quantitative and sensitive technique for selecting TK^+ revertants or recombinants from a population of predominantly TK^- viruses. W.C. Summers and coworkers (Summers et al., 1975; Summers and Summers, 1977) have described two approaches for either selection or quantitation of TK^+ viruses. The new technique described here permits the direct measurement of TK^+ viruses and facilitates the selection of clones of TK^+ virus.

2.2 Materials and Methods

2.2.1 Cells

The human TK^+ cell line R970-5 (Rhim et al., 1975) and its 5-bromodeoxyuridine (BUdR)-resistant TK^- mutant, line 143, were a gift from C. Croce and K. Huebner (Wistar Institute, Philadelphia, Pa.). Vero cells were purchased from Microbiological Associates, Walkersville, Md. In experiments where cell growth was measured, the cells were counted in a hemacytometer and viability was assessed by trypan blue exclusion.

2.2.2 Media

All of the cell lines were grown in α-minimum essential medium supplemented with 0.075% sodium bicarbonate, 10 mM N-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES) (Sigma Chemical Co., St. Louis, Mo.), 100 U of penicillin and 100 μg of streptomycin per ml, 2 mM L-glutamine, and 5% heat-inactivated fetal bovine serum. This

will be designated here as normal medium. The methotrexate-containing medium used in this study was a modification of MTAGG medium (Davis et al., 1974); it contained 1.2 X 10^{-6} M methotrexate (Lederle Products, Montreal, Quebec, Canada), 1.6 X 10^{-5} M thymidine (Sigma), 5 X 10^{-5} M adenosine and guanosine (Sigma), and 1 X 10^{-4} M glycine. This medium will be designated here simply as methotrexate medium. To select for TK^- viruses, the normal medium was supplemented with 50 μg of BUdR per ml.

2.2.3 Viruses

The HSV type 1 TK^+ strains used in this study were KOS (Smith, 1964) and clone 101 (Dubbs and Kit, 1964). The HSV type 1 TK^- strains were B2006 (Dubbs and Kit, 1964) and TK^- 21 (Summers et al., 1975). Clone 101 (cl-101) and TK^- 21 were kindly provided by W.P. Summers (Yale University, New Haven, Conn.), and B2006 was a gift from R. Hughes (Roswell Park Memorial Institute, Buffalo, N.Y.). Strain HSV-1 d_2 is a mutant with a partial deletion engineered within the TK gene (Smiley, 1980) spanning 800 base pairs of the sequence used for TK expression. Unless otherwise indicated, the TK^+ viruses were routinely passaged in Vero cells at a multiplicity of infection of 0.1 PFU per cell. Unless otherwise indicated, TK^- viruses were grown in 143 TK^- cells in the presence of 50 μg of BUdR per ml. None of the viruses induced large syncitia; the cytopathic changes they induced were comparable to those produced by fresh isolates (Ejercito et al., 1968).

The virus preparations were assayed by plaque formation using cell monolayers grown in 60-mm plastic tissue culture plates (Corning,

New York, N.Y.), or in Linbro plates with 24 wells of 2 cm^2 each, (Flow Laboratories, Hamden, Conn.). After the plates were inoculated with appropriately diluted virus or infected cells, the cultures were held at 37°C for 1 h. Then the cells were overlaid either with 1% agar (Bacto-agar; Difco Laboratories, Detroit, Mich.) in normal medium containing 0.01% protamine sulfate, or with normal medium containing 0.2% human immune serum globulin (Connaught Laboratories Ltd., Willowdale, Ontario, Canada). The cultures were incubated at 37°C in 5% CO_2 in air for 4 to 5 days, after which the overlays were removed and the cells were fixed for 10 min with ethanol-acetic acid (3:1, vol/vol) and stained with 0.1% crystal violet. When Linbro plates were used the plaques were often detected directly in the fresh monolayer using an inverted microscope and low magnification. Virus titers were expressed as PFU per milliliter. The 50% tissue culture infectious dose was determined by inoculating 0.1 ml of serial dilutions of virus into microtiter wells containing 3×10^4 cells in 0.1 ml. Six wells were inoculated for each 10-fold dilution. After 4 or 5 days of incubation, the cells were fixed and stained as described above, and the 50% tissue culture infectious dose was calculated by the method of Reed and Muench (Reed and Muench, 1938).

2.2.4 Incorporation of [^{3}H]dThd.

Thymidine kinase activity was assessed indirectly by measuring the uptake of [^{3}H]thymidine ([^{3}H]dThd; 20 Ci/mmol; New England Nuclear Corp., Boston, Mass.) into acid-precipitable counts. Infected TK^- cells were either labeled with 1 μCi/ml for 24 h from the time of infection, or pulse-labeled with 2 μCi/ml for 60 min beginning at 7 h after infection. The cultures were lysed with 1% sodium dodecyl sulfate and precipitated

with ice-cold 5% trichloroacetic acid. The precipitates were collected by filtration on glass-fiber filters and counted in a toluene-based scintillation fluid.

2.3 Results

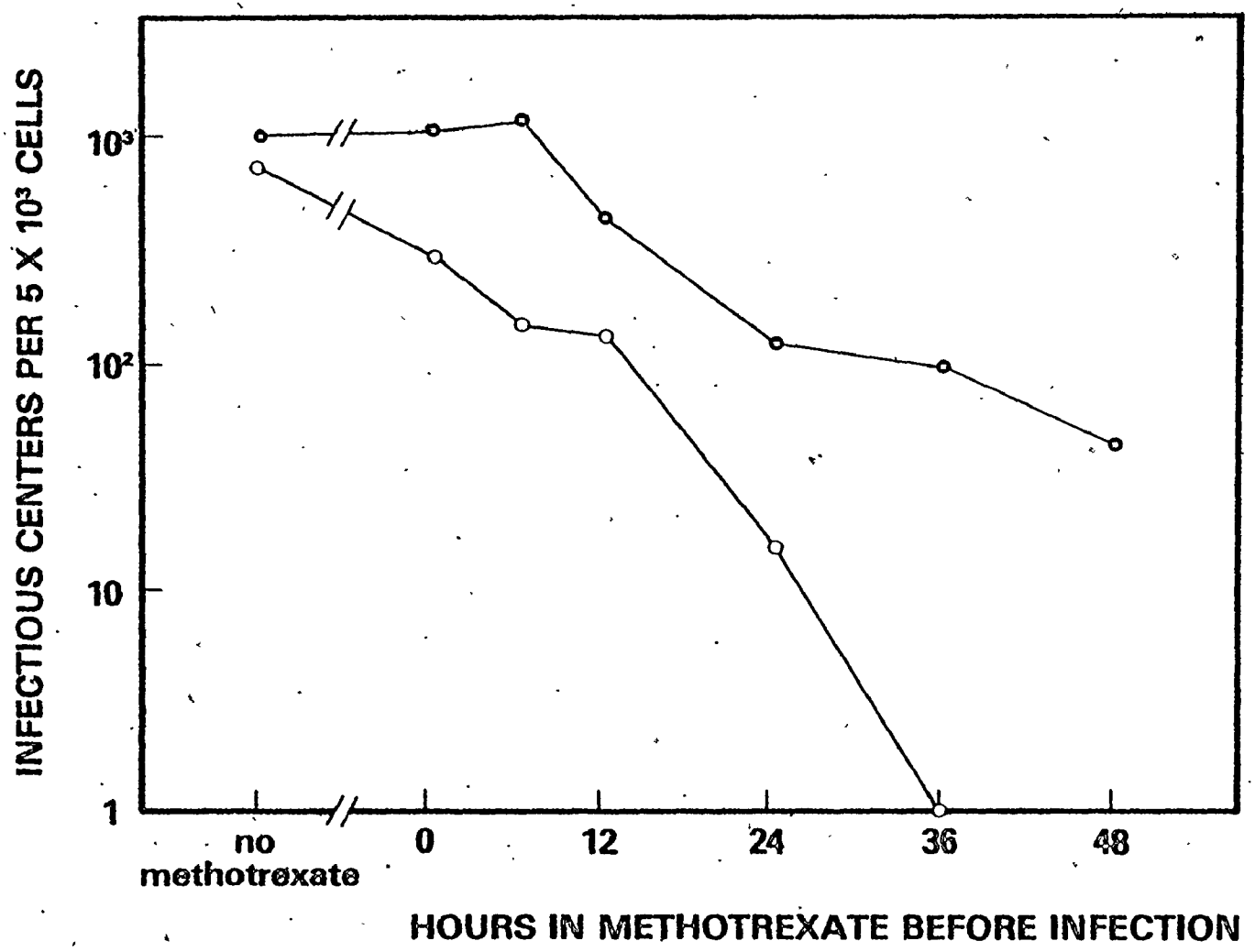
2.3.1 Development of the Technique

Attempts were made to select for TK^+ viruses on the basis of reduced virus replication associated with lower levels of *de novo* pyrimidine synthesis caused by serum starvation (Jamieson *et al.*, 1974) or methotrexate (Rueckert and Mueller, 1960). The initial studies made use of TK^+ cells, but the plaquing efficiencies of TK^+ and TK^- viruses were found to be similar under both conditions (data not shown). The potential of selecting TK^+ viruses using TK^- cells incubated in the presence of methotrexate was then examined. Since time is required for the depletion of preexisting deoxypyrimidine-nucleotide pools after the addition of methotrexate (Rueckert and Mueller, 1960), the influence of time after the addition of methotrexate on the ability of TK^- cells to register as infectious centers when infected with TK^+ or TK^- virus was examined. It was found that preincubation of cells in methotrexate produced a significant difference in the yields of infectious centers by TK^+ and TK^- viruses. By preincubating the cells in methotrexate medium for 36 h, the difference in infectious centers induced by the TK^+ and TK^- viruses could be increased by about 2 $\log_{10}$; however, with increased time there was a significant decrease in the number of cells registering as infectious centers after infection with a TK^+ virus. These results are shown in figure 11.

The limited selectivity of these approaches prompted me to attempt

Figure 11

Effect of preincubation in methotrexate on the yield of infectious centers by TK^- cells infected with HSV. Duplicate aliquots of a suspension of 143 (TK^-) cells were incubated at 37°C in methotrexate medium for different periods. Then the cells were infected (MOI = 1) with HSV-1 cl-101 (TK^+) (●——●) or HSV-1 B2006 (TK^-) (o——o). The infected cells were washed, resuspended in methotrexate medium containing 0.2% human immune-serum globulin and plated on monolayers of Vero cells. The first point in the graphs represents the yield from monolayers which were not incubated in methotrexate.


INFECTIOUS CENTERS PER 5 X 10³ CELLS
10³
10²
10
1
no methotrexate
0
12
24
36
48
HOURS IN METHOTREXATE BEFORE INFECTION

to assay TK^+ and TK^- viruses directly on TK^- cells, which were then incubated in medium with methotrexate. The success of this approach was dependent upon the survival of the TK^- cells in the methotrexate medium. This parameter was studied under different conditions of cell growth (Fig. 12). The results show the expected decrease in cell survival with increasing time of incubation in the presence of the inhibitor; serum starvation per se or in addition to methotrexate reduced cell survival slightly more. More relevant for the development of the selective technique for TK^+ viruses is that the survival of the cells in methotrexate medium remained essentially unaffected during the first 48 h of exposure to the drug. This finding suggested that the addition of a second inoculum of uninfected cells to the plates might provide a suitable substrate for plaque formation by TK^+ viruses.

Monolayers of TK^- cells were infected with serial 10-fold dilutions of TK^+ or TK^- viruses and overlaid with liquid medium containing methotrexate and immune serum globulin. After 48 h, the medium was removed, and fresh inocula of TK^- cells suspended in the same kind of medium were added to the plates, which were then further incubated for 36 to 48 h before fixing and staining.

Figures 13 and 14 show pictures of stained monolayers in Linbro plates with results obtained titrating viruses with different frequencies of reversion. In figure 13 it can be seen that the condition of the monolayer receiving an MOI of TK^- PFU's above 1, hinders the detection of TK^+ plaques. This limits the resolution of the technique to a 5-6 $\log_{10}$ difference in concentration between the TK^+ and the TK^- virus in the mixtures. A "prozone" effect is clearly distinguishable in figure 13.

Figure 12

Effect of methotrexate and serum on survival of 143 (TK^-) cells. 143 (TK^-) cells were seeded in 50-mm dishes at 10^5 per dish in regular medium. After 48 h of incubation, the medium was replaced with complete medium (O), medium containing methotrexate (●), medium without serum (Δ), and serum-free medium containing methotrexate (▲). Triplicate cultures were monodispersed with trypsin, and the number of viable cells was counted in a hemacytometer.

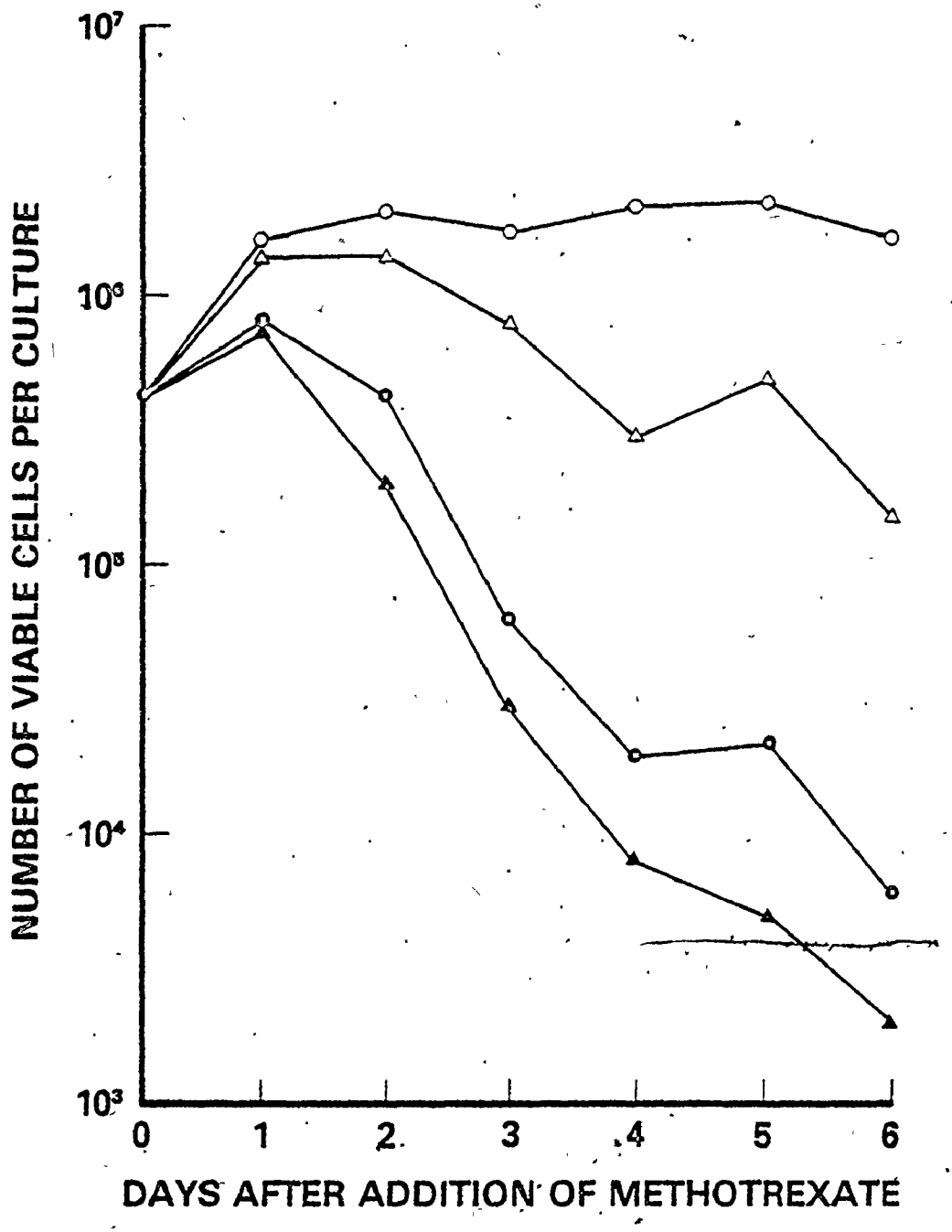

NUMBER OF VIABLE CELLS PER CULTURE
10⁷
10⁶
10⁵
10⁴
10³
0
1
2
3
4
5
6
DAYS AFTER ADDITION OF METHOTREXATE

Figure 13

Titration of the TK^+ fraction in a preparation of HSV-1 TK^-21. The two first rows (A & B) show the result of the control titration in 143 cells in normal medium. The dilutions of the virus in these rows go from 10^{-3} to 10^{-8}. One of the plaques in dilution 10^{-8} is shown before fixing in figure 15a. In the two rows on the bottom (C & D) the cells were incubated in methotrexate medium and the monolayers were replaced as indicated in the text. In these rows the dilution of the virus ranges from 10^{-1} to 10^{-6}.

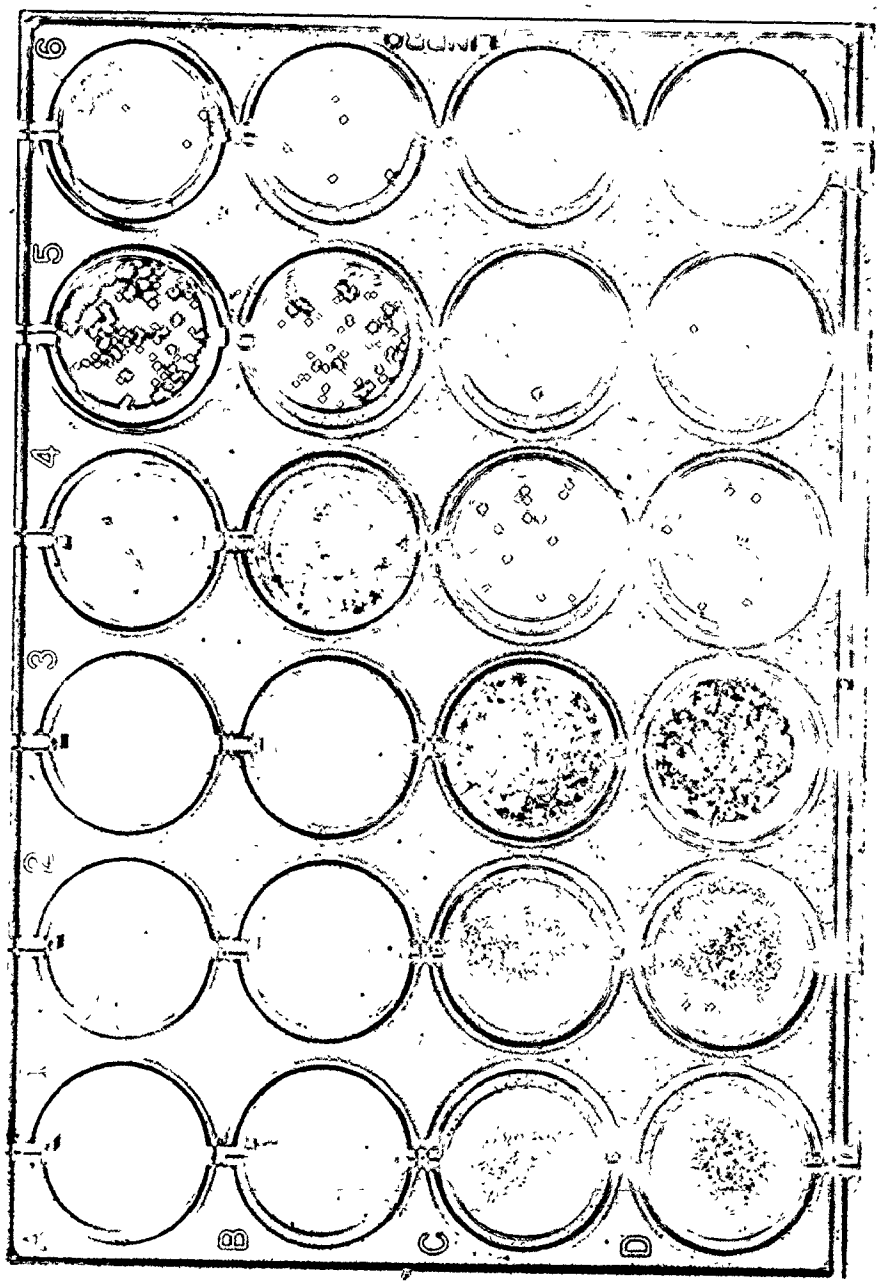

1
2
3
4
5
6
B
C
D

Figure 14

Titration of the TK^+ fraction in an uncloned preparation of HSV-1 B2006. For a description of this figure refer to the legend for figure 13. TK^+ plaques (▼) can be seen in the wells corresponding to dilution 10^{-2}. Figure 15b shows one of these plaques before fixing the monolayer. Figure 15c shows another field in the same monolayer.

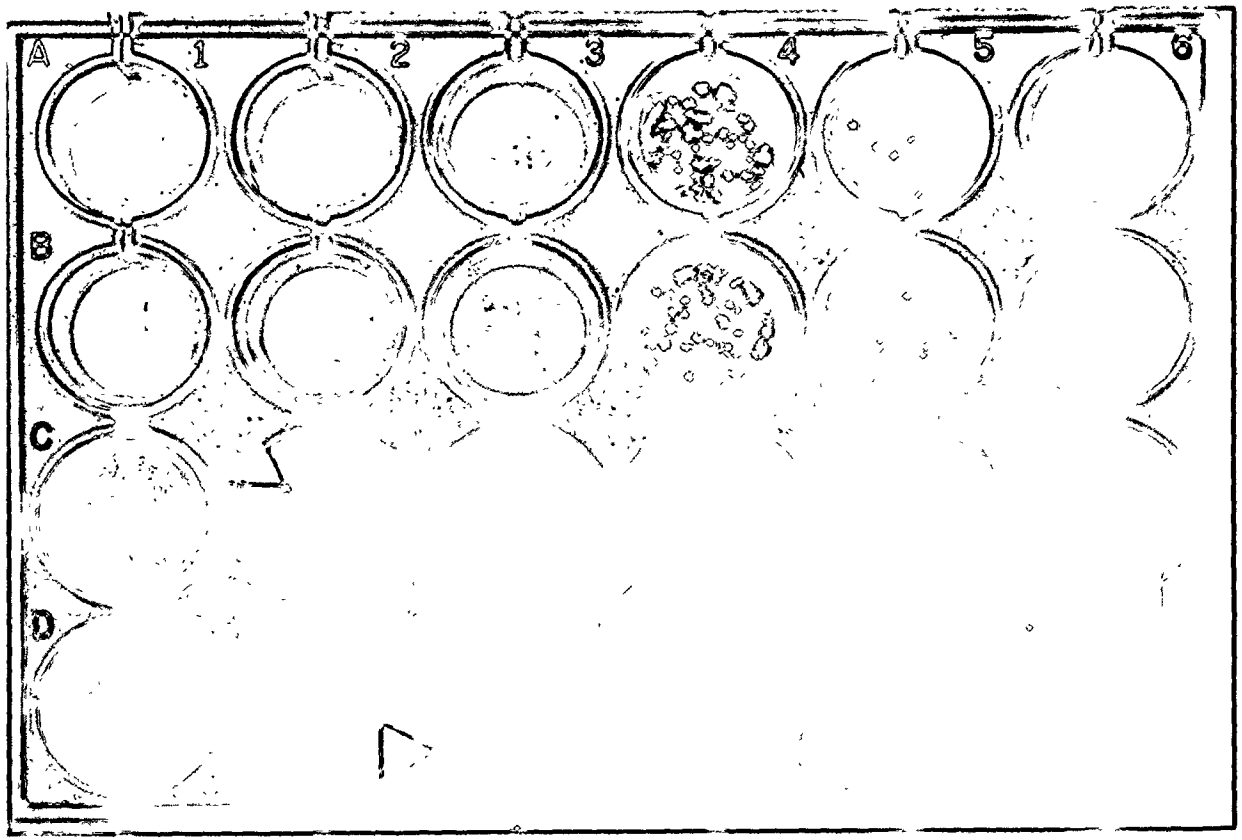
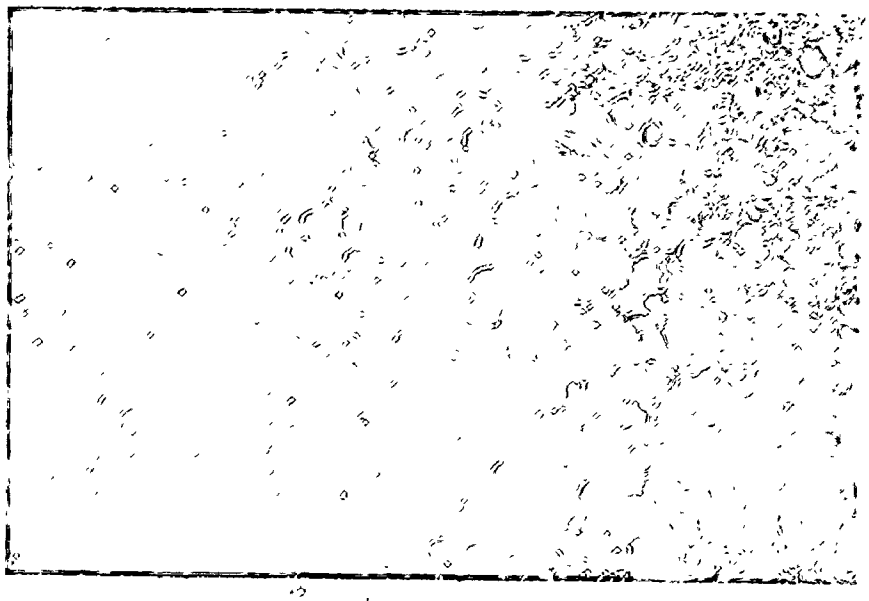
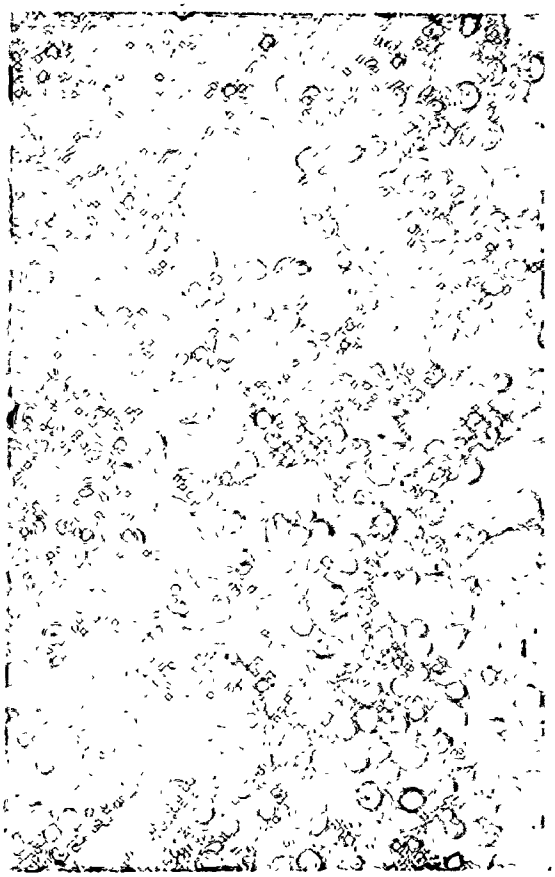

A
B
C
D
1
2
3
4
5
6

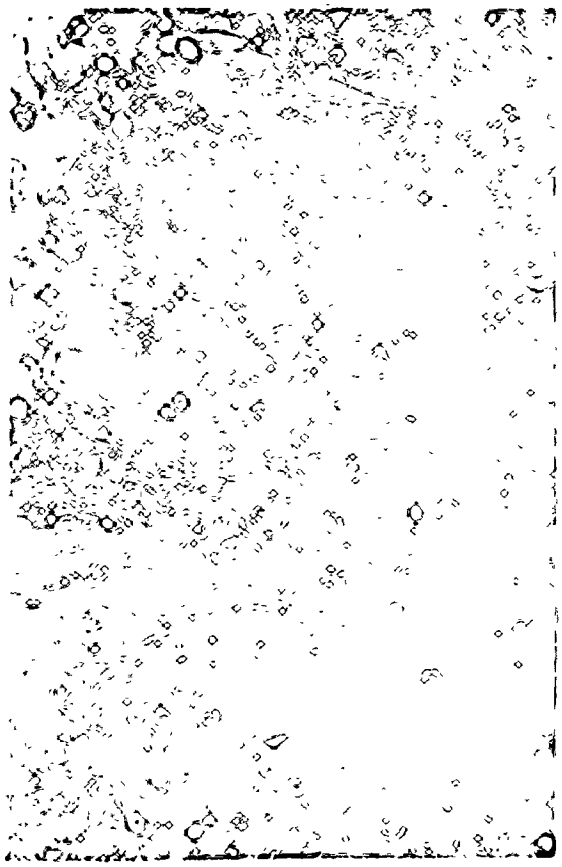
Figure 15

Plaques formed by HSV-1 in monolayers of 143 cells. A plaque formed by HSV-1 B2006 in 143 cells in normal medium (100 X) is shown in figure 15a. A plaque formed by a TK^+ revertant of HSV-1 B2006 in 143 cells in methotrexate (100 X) is shown in figure 15b. This plaque corresponds to one of the plaques indicated (▽) in figure 14. Small cell-agregates induced by TK^- viruses in high concentration (MOI ~ 1) in 143 cells in methotrexate are shown in figure 15c. This field corresponds to another aspect of the same monolayer shown in figure 15b.





b



c

This reproducible phenomenon was attributed to the fast disruption of the original monolayers receiving a TK^+ multiplicity above 0.01. This would cause the detachment of most of the original cells prior to the addition of the fresh monolayer which would explain why at higher concentration of original inoculum the final cells survived better than in wells where the inoculum was further diluted.

The results of representative experiments using this technique are shown in Table 5. The plaquing efficiency of the TK^+ viruses, KOS and cl-101, were similar in normal and in methotrexate medium, whereas the TK^- virus stock produced plaques at an efficiency of only 1×10^{-5} to 5.4×10^{-5} in methotrexate as compared to normal medium. The plaques induced by the TK^+ viruses in methotrexate medium were found to be slightly larger than those formed in monolayers incubated in normal medium. Microscopic examinations revealed the TK^+ plaques to consist of an empty center and a wide edge of rounded cells. TK^- viruses were found to produce small foci of rounded cells at 24 h after infection; however, these areas did not enlarge, and no plaques were visible by day 3 of incubation.

Figure 15a shows a plaque formed by a TK^- virus in methotrexate-free control monolayer of 143 cells. Figure 15b shows a plaque of similar age produced by a TK^+ virus in a 143 cell-monolayer incubated in methotrexate medium (TK^- MOI=1), and Figure 15c shows small cellular aggregates produced by the TK^- virus in the same monolayer shown in figure 15b.

Table 5

Plaquing Efficiencies of HSV Type 1 TK^+ and TK^- Viruses on 143 TK^- Cells in Medium Containing Methotrexate

Expt.	Virus[a]	Titers (PFU/ml)		Plaquing efficiency[b] in methotrexate
		Normal medium	Methotrexate medium[c]	
1	KOS	2.3×10^7	2.4×10^7	1.04
	B2006	5.0×10^7	5.0×10^2	1×10^{-5}
2	B2006	9.7×10^8	5.0×10^4	5.2×10^{-5}
	B2006	1.6×10^9	8.6×10^4	5.4×10^{-5}
3	cl-101	5.0×10^8	4.7×10^8	0.94
	B2006	4.6×10^7	2.0×10^3	4.4×10^{-5}

[a]The TK^- virus (B2006) was grown for three passages in 143 TK^- cells in medium containing 50 μg of BUdR per ml. The two TK^+ viruses were grown in Vero cells in normal medium.

[b]The plaquing efficiency was calculated by dividing PFU per milliliter in methotrexate medium by PFU per milliliter in normal medium.

[c]After infection the monolayers of cells were overlaid with the corresponding medium supplemented with 0.2% human immune serum globulin. At 48 h after infection, a new inoculum of cells was added to the cultures growing in medium containing methotrexate.

2.3.2 Detection of TK^+ Virus in Mixed Populations

The use of this assay to measure the TK^+ fraction in a mixed population of predominantly TK^- viruses requires that the presence of the larger TK^- fraction does not affect the plaquing efficiency of the TK^+ viruses. To assess this, a reconstruction experiment was performed in which different mixtures of known amounts of TK^+ and TK^- viruses were titrated in TK^- cells using the normal plaque assay and the methotrexate medium overlay (Table 6). It can be seen that the plaquing efficiency of the TK^+ was not significantly affected over a wide variation in the proportion of TK^+ to TK^- viruses. Within countable range and independently from the total input of HSV, there was a linear dose-response relation between the input of TK^+ viruses per plate and the dilution of the mixture (data not shown).

It was noted that at high multiplicities of infection with TK^- virus, there was a diffuse destruction of the monolayers which precluded the detection of TK^+ virus. The formation of plaques by TK^+ virus in the system was possible provided that the total input (TK^+ plus TK^- virus) did not exceed 1 PFU per cell. Since each monolayer contained about 2.5×10^6 cells, the limit of resolution for the detection of TK^+ virus is thus between 10^{-5} and 10^{-6}.

2.3.3 Plaquing Efficiency of TK^- Viruses

A small but consistent number of plaques were produced when preparations of TK^- viruses were assayed in the TK^- cells incubated with methotrexate (Table 5). These plaques could represent leakiness (i.e., genotypically TK^- viruses which produced plaques in the system) or could be caused by a low frequency of TK^+ virus in the TK^- virus

Table 6

Plaquing Efficiencies of Mixed Populations of TK^+ and TK^- Viruses on 143 TK^- Cells in Normal and Methotrexate Media

Estimated titers in virus mixtures (PFU/ml)		Observed titers of virus mixtures (PFU/ml)[a]	
TK^-	TK^+	Normal medium	Methotrexate medium
2.5×10^8	0	2.6×10^8	$<1.0 \times 10^3$
2.5×10^8	5.0×10^6	2.5×10^8	3.8×10^6
2.5×10^8	5.0×10^5	2.8×10^8	2.8×10^5
2.5×10^8	5.0×10^4	2.8×10^8	3.6×10^4
2.5×10^8	5.0×10^3	3.0×10^8	4.0×10^3
2.5×10^8	5.0×10^2	2.2×10^8	$<1.0 \times 10^3$
0	5.0×10^6	4.9×10^6	4.9×10^6

[a]Mixtures of TK^+ and TK^- viruses were assayed on 143 TK cells in the presence of normal and methotrexate media. Two 60-mm plates were inoculated with 0.5 ml of 10-fold serial dilutions of the virus mixtures. At 48 h after infection a new inoculum of cells was added to the plates containing methotrexate medium, as described in the text.

stocks. To distinguish between these possibilities, monolayers of TK^- cells were infected with an appropriately diluted stock of the B2006 strain of virus, and the cells were incubated in medium containing methotrexate and 0.2% human immune serum globulin. Twenty well-isolated plaques were identified microscopically 48 h after addition of the second layer of 143 cells, and 10 µl of cells and fluid were aspirated from the area of each plaque. The aspirated material was used to inoculate flasks containing monolayers of 143 cells, which were then incubated in normal medium. Cytopathogenic changes characteristic of HSV developed in 15 of the 20 inoculated cultures. Viruses harvested from these cultures were assayed for their ability to induce the incorporation of [^{3}H]dThd. The incorporation induced by the different clones ranged from values slightly higher than the incorporation of [^{3}H]dThd into uninfected cells to those similar to stocks of known TK^+ HSV-1 (cl-101) (see Table 7).

Since the clone selection was from plaques which developed under antibody-containing liquid medium, this variability in induction of [^{3}H]dThd uptake was thought to be due to varying mixtures of TK^+ and TK^- viruses in the stocks of the clones. This was confirmed by plaque assaying the stocks of three selected clones under normal agar, under agar containing BUdR, and by the assay described above using TK^- cells and methotrexate. The results of these assays permitted estimates to be made of TK^- and TK^+ viruses present in each stock (Table 7). The three putative TK^+ clones appeared to be contaminated, although to a different extent, with TK^- viruses, and the levels of [^{3}H]dThd incorporation roughly correlate with the estimated TK^+ fraction in the stocks.

Table 7

Estimated Frequencies of TK^+ Viruses in the Progeny of Viruses Selected from Plaques Produced by B2006 in TK^- Cells in Methotrexate

Virus	[^{3}H]dThd uptake induced in TK^- cells[a] (cpm/5 X 10^4 cells)	Titer (PFU/ml X 10^{-7})[b]	Percent of TK^+ virus estimated:		
			In BUdR[c]	In TK^- cells and methotrexate[d]	By testing subclones for TK activity[e]
Cl-101	5,360 ± 182	1.91	100	100	100 (14/14)
B2006	495 ± 28	8.27	0	0	0 (0/13)
C-5	1,083 ± 236	0.18	36	18	12 (3/25)
C-6	2,235 ± 153	0.23	46	46	53 (8/15)
C-11	3,702 ± 186	0.68	91	100	82 (14/17)

[a]Monolayers of 143 cells in 60-mm plates were inoculated with 0.2 ml of undiluted virus stock, and after adsorption of the virus, each culture was incubated for 24 h at 37°C in the presence of 1 μCi [^{3}H]dThd. The average counts per minute (cpm) and standard deviation of [^{3}H]dThd incorporated into acid-precipitable material were computed from three separate experiments.

[b]Titer of virus stocks was determined by plaque assays in 143 cells using normal medium and 0.2% immune serum globulin.

[c]The titers of virus in 143 cells incubated in the presence of 50 μg of BUdR per ml were determined and divided by the titers in normal medium. The fraction of TK^+ virus was estimated from the calculated percentage of TK^- virus; the average percent of three experiments is shown.

[d]Percent TK^+ calculated by dividing titers in 143 cells and methotrexate by titers in normal medium. The average percents of three experiments are shown.

Table 7 (Footnotes cont'd)

[e]The subclones shown in Fig. 18 were considered TK^+ or TK^- according to the amounts of [^{3}H]dThd incorporated into infected cells. The number of TK^+ viruses over the total number tested is shown in parentheses, and the percents of TK^+ were calculated from these values.

Further confirmation of the mixed nature of the clones was obtained by infecting monolayers of 143 cells with the viruses from the first cloning and picking subclones from plaques which developed under agar overlayers. These subclones were found to fall into two distinct groups with respect to their ability to induce the incorporation of [^{3}H]dThd (Fig. 16). The levels of incorporation of the two groups were similar to those induced by TK^+ and TK^- viruses, and the frequencies of TK^+ subclones obtained from the parent populations were similar to those calculated using the other methods of estimating TK^+ virus (see Table 7).

The results of these experiments strongly suggest that the plaques observed are not due to leakiness of the assay system but rather that they are caused by TK^+ viruses.

2.3.4 Reversion Frequencies* of TK^- Virus Strains

The TK^- virus strains used in developing the assay had not been plaque purified, and although they were passaged three times in TK^- cells in the presence of BUdR, it may be that TK^+ viruses were present in the

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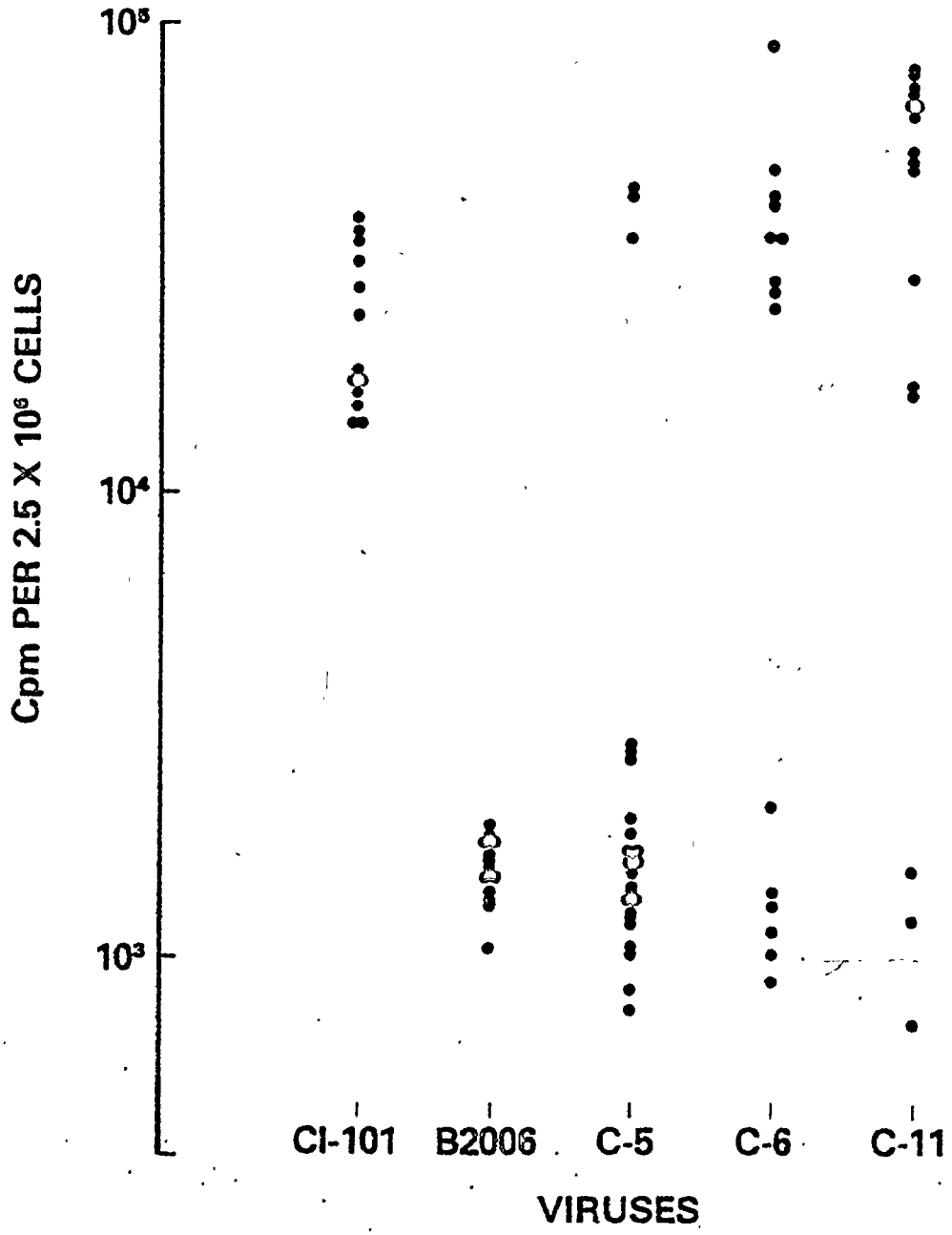
*Throughout this thesis the terms reversion frequency, frequency of reversion and frequency of revertants will be used interchangeably to describe the fraction of revertants within defined viral populations. No attempt is made to relate any of these terms with the reversion rate of a virus. The reversion rate is a biological constant of each system, and the frequency of revertants, albeit related to the reversion rate, depends largely on the specific reversion events occurring during the early stages in the growth of a clone. As a consequence the reversion frequency may substantially vary from one population to another even under standardized conditions of growth.

Figure 16

Uptake of [^{3}H]dThd by 143 cells infected by subclones of various viruses. cl-101, B2006, and clones C-5, C-6, and C-11 of B2006 were plaqued in 143 cells under normal agar overlay. Plaques were picked randomly from plates inoculated with each of the above, and progeny virus was grown in 143 cells incubated in normal medium. The virus stocks of the progeny virus were titrated (50% tissue culture infectious dose), and fresh monolayers of 143 cells were infected with comparable amounts of each virus stock. For each subclone, triplicate cultures were inoculated, and 7 h after infection the cultures were exposed to 2 μCi of [^{3}H]dThd for 1 h. The amounts of [^{3}H]dThd incorporated into acid-precipitable material were determined, and the average of the triplicate counts was plotted.


10^5
10^4
10^3
Cpm PER 2.5 X 10^6 CELLS
CI-101
B2006
C-5
C-6
C-11
VIRUSES

preparations. Thus, the plaquing efficiencies in methotrexate shown in Table 5 cannot be considered as estimates of the reversion frequencies of the TK^- mutants.

To estimate reversion frequencies, clones were obtained from B2006 and TK^- 21. The clones were plaque purified three times from well-isolated plaques formed in 143 cells under normal agar overlay. The final plaques were passaged twice in 143 cells under normal medium, and a total progeny yield of close to 10^8 PFU was obtained. Each clone represented an independent plaque picked in the first step of purification. These clones were then titrated in 143 cells under normal medium and under methotrexate using the technique described above. The results obtained for seven clones are shown in Table 8. No plaques were observed in the clones derived from B2006, suggesting that the reversion frequency of this mutant is below the level of detection of the technique. The clones from TK^- 21 showed a reproducible value (about 10^{-4}) which agrees with previous estimates of 10^{-4} to 10^{-6} (Summers et al., 1975).

The presence of TK^+ virus in stocks of B2006 which had at one time been plaque purified suggests that this TK^- mutant can revert. To prove this, plaque-purified B2006 was passed in TK^- cells under methotrexate medium. The harvested virus from each passage was assayed for TK^+ virus by the technique described above. After one passage in TK^- cells under methotrexate, TK^+ plaques were detected (Table 8), and virus progeny grown from virus picked from the plaques induced the incorporation of [^{3}H]dThd at levels comparable to TK^+ virus (data not shown).

The following experiments were undertaken to obtain more precise estimates of the reversion frequencies of the B2006 strain. Plaque purified subclones of TK^- 21 and B2006 viruses were grown up to a total

Table 8

Plaquing Efficiencies of Purified Clones from TK^- Viruses on 143 TK^- Cells in Methotrexate Medium

Virus[a]	Plaquing efficiency in methotrexate[b]	
	Stock virus[a]	Passaged virus[c]
B2006/A-11	$<1.2 \times 10^{-5}$	4.2×10^{-5}
B2006/B-11	$<6.8 \times 10^{-6}$	3.0×10^{-4}
B2006/C-11	$<2.9 \times 10^{-5}$	2.9×10^{-5}
TK^- 21/D-11	1.3×10^{-4}	2.0×10^{-3}
TK^- 21/E-11	1.0×10^{-4}	4.3×10^{-3}
TK^- 21/F-11	5.2×10^{-5}	1.8×10^{-3}
TK^- 21/G-11	1.7×10^{-4}	2.4×10^{-3}

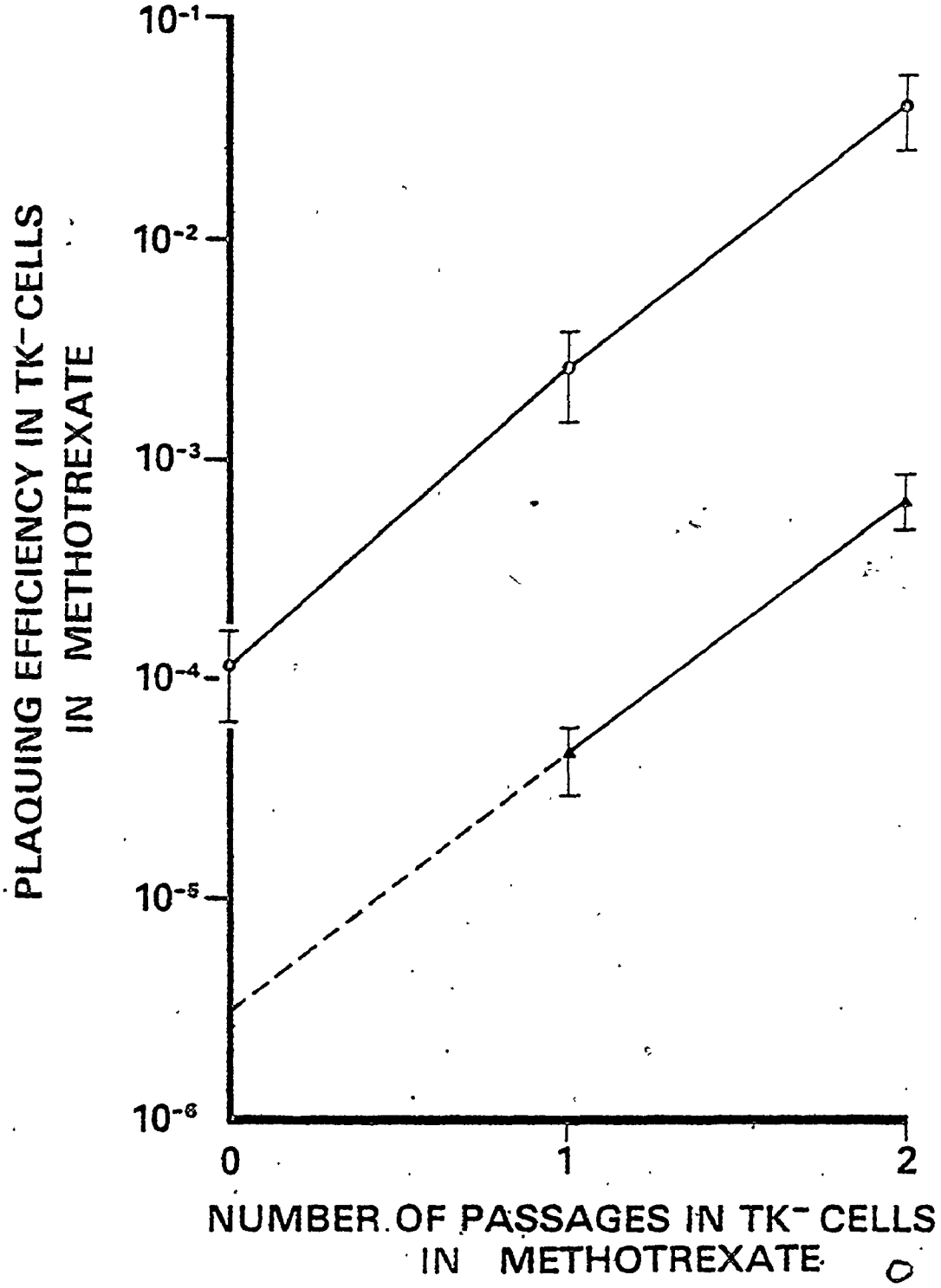
[a]Both 2006 and TK^- 21 viruses were plated on 143 cells under normal agar medium. Well-isolated plaques were picked. This procedure was repeated twice. Each virus was grown on 143 cells in normal medium up to a total viral progeny of about 10^8 PFU (stock virus).

[b]Measured as described in legend of Table 5.

[c]143 TK^- monolayers were infected with stock virus at a multiplicity of 0.5 PFU/cell and incubated in methotrexate medium for 48 h. The plaquing efficiency of the resulting virus was then determined in methotrexate versus normal medium.

Figure 17

Reversion frequency of B2006. Two enrichment steps were performed with plaque purified subclones of TK^-21 and B2006 by passaging in 143 (TK^-) cells in methotrexate medium. The infected monolayers were harvested 48 hrs after infection and the frequencies of TK^+ PFU's were determined as described in Section 2.2.3. The extrapolation to zero enrichment step in B2006 gives an estimate of its reversion frequency (dotted line).


PLAQUING EFFICIENCY IN TK⁻ CELLS
IN METHOTREXATE
10⁻¹
10⁻²
10⁻³
10⁻⁴
10⁻⁵
10⁻⁶
0
1
2
NUMBER OF PASSAGES IN TK⁻ CELLS
IN METHOTREXATE

yield of about 10^8 PFU and subsequently passaged twice in 143 cells (TK^-) in methotrexate medium. The high reverting mutant TK^- 21 was used as a control. The results of triplicate titrations of the two mutants are presented in figure 17. Within the range analyzed, the effect of the two enrichment steps on both mutants is linear and parallel. The extrapolation of the B2006 line to zero enrichment step (dotted line) provides an estimate of the reversion frequency of this mutant (2.0×10^{-6}).

HSV-1 d2 was analyzed in a similar way and no TK^+ plaques were detected.

2.4 Discussion

The technique described here for titrating TK^+ virus in TK^- virus populations is based on the availability of TK^- mutant cells and on the ability to block *de novo* synthesis of deoxypyrimidines by the addition of methotrexate. Under these conditions the only source of thymidilates is provided by the phosphorylation of thymidine carried out by the virus-coded thymidine kinase. Thus, only TK^+ virus will replicate, whereas TK^- virus will be capable of only limited growth. Since the survival of TK^- cells under the selective conditions used is also limited, a fresh inoculum of cells has to be added after 2 days to allow sufficient time for the plaques to develop. The presence of methotrexate and the addition of fresh TK^- cells halfway through the development of plaques did not significantly affect the plaquing efficiency of TK^+ virus, but there was a greater than 5-log reduction in the number of plaques caused by TK^- virus. The data obtained from the reconstruction experiments and from the analysis of viral progenies from detected TK^+ plaques indicate that this is an efficient and specific system to assay for TK^+ viruses in virus stocks with large TK^- fractions. The lower limit of resolution corresponds to a frequency of TK^+ virus between 10^{-5} and 10^{-6}.

Others have described techniques for the isolation and quantitation of TK^+ HSV in predominantly TK^- stocks. A one-step growth of HSV in TK^- cells, followed by a quantitation of the TK^- fraction as well as total virus yield, has been used to estimate the reversion frequencies of HSV TK^- mutants (Summers et al., 1975). This approach is valuable for the purpose of selecting TK^+ viruses, but it lacks both the sensitivity and the accuracy needed for careful quantitation. A sensitive technique has been published for the detection of viral TK expression in infected and transformed cells (Summers and Summers, 1977) which takes advantage of the ability of the viral TK to phosphorylate radiolabeled iododeoxycytidine. This technique can accurately quantitate TK^+ viruses, but its use for selecting and cloning TK^+ virus would appear to require a replicate-plating step. The method described in this chapter can be used for both quantitation and cloning of TK^+ viruses. In fact, the sensitivity of this technique should be sufficient to determine the spontaneous or induced mutation frequencies of TK^- virus, especially if the frequencies occur in the order of 10^{-3} to 10^{-6} (Esparza et al., 1974). The technique may also provide a more direct approach to the quantitation of marker-rescue recombinants of TK mutants than the one already used (Stow et al., 1978).

In the experiments reported in this chapter the selection procedure was used to analyze the reversion frequency of two TK^- strains of HSV type 1. Preparations of viruses which had not been plaque purified for several passages and which had been grown under BUdR were found to contain a small fraction of TK^+ contaminants. The analysis of

plaque-purified clones of these viruses showed lower frequencies of TK^+ revertants for B2006 than those found in the unpurified stock grown in BUdR. The plaquing efficiencies obtained with the plaque-purified clones appear to be more accurate estimates of the true reversion frequencies of these viruses, and it can be concluded that these frequencies are in fact different for the two TK^- mutants analyzed in this study.

Previous reports (Subak-Sharpe et al., 1974) have considered B2006 to be a possible deletion mutant in the TK locus. However, TK^+ viruses have been found in stocks of B2006 (R. Hughes, personal communication). The virus does not induce the synthesis of a polypeptide presumed to be the TK enzyme (Summers et al., 1975), and since it does not complement other TK^- mutants, the mutation was thought not to be located in a trans-acting regulatory gene (Jamieson and Subak-Sharpe, 1978). Although we could not find TK^+ virus after a single passage of recently plaque-purified virus, TK^+ virus could be detected after a second passage under selective pressure, and the reversion frequency of B2006 appears to be close to 10^{-6}; the occurrence of reversion, suggests that this virus strain is not a deletion mutant. The strain TK^- 21 induces the synthesis of a 24,000-dalton polypeptide instead of the 45,000-dalton polypeptide thought to represent the complete viral TK. Thus, TK^- 21 has been tentatively considered a nonsense mutant caused by a point mutation in the TK structural gene (Summers et al., 1975). The reversion frequency for this virus was reported to be between 10^{-4} and 10^{-6} (Summers et al., 1975). I found similar frequencies of reversion using the technique reported above . The

different reversion frequencies found for both viruses seem to indicate that the defects of B2006 and TK^- 21 causing the TK^- phenotype are qualitatively different.

The use of the technique described above also indicates that the deletion mutant HSV-1 d2 may be unable to revert to a TK^+ phenotype. This is not unexpected since the deletion engineered within the TK gene of this mutant encompasses a large part of the structural gene coding for the thymidine kinase.

Finally, the method might be useful in attempts to rescue viral genes from transformed cells. Such attempts have been successful with other DNA viruses at an estimated frequency between 10^{-3} and 10^{-6} (Gluzman et al., 1977b). Results of applications in the HSV system will be shown in the subsequent chapter of this thesis.

CHAPTER 3

ATTEMPTS TO RESCUE VIRAL TK GENES PRESENT IN CELLS BIOCHEMICALLY TRANSFORMED BY HERPES SIMPLEX VIRUSES

3.1 Introduction

This chapter presents the results obtained with a system designed to optimize the chances of retrieval of viral genes from cells transformed by HSV-2. These experiments were undertaken to evaluate the feasibility of such an approach for the detection of the endogenous viral DNA present in transformed cells. Most of this chapter reproduces a paper which has been submitted for publication (Campione-Piccardo and Rawls, 1981).

Seroepidemiologic studies have established an association between herpes simplex virus type 2 (HSV-2) and squamous cell carcinoma of the cervix (reviewed by Rawls and Campione-Piccardo, 1980). The model discussed in chapter 1 confirmed this association but strongly suggested that other factors may be capable of causing cervical cancer in the absence of HSV-2 infection. The factor associated with HSV-2 infection remains the most important but, the fact that a fraction of the cancer cases may occur independently from HSV-2 infection, stresses the need for molecular markers to evaluate the actual role of HSV-2 in the pathogenesis of human cervical cancer.

Although reports have described the detection of viral antigens (Royston and Aurelian, 1970b; Nahmias et al., 1975; Notter et al., 1978; Dreesman et al., 1980) and viral mRNA (Jones et al., 1978; McDougall et al., 1980) in cancer cells, great difficulty has been encountered in detecting HSV-2 DNA in these cells (Zur Hausen et al., 1974; Pagano, 1975). High rates of intermolecular recombination appear to be a property of the herpesviruses. It is not uncommon to obtain recombi-

nants at a frequency higher than 10^{-2} in cells coinfected at high multiplicities with HSV-1 and HSV-2 (Esparza et al., 1974). In addition, marker rescue experiments have yielded high frequencies of recombinants between replicating intact viral DNA and cotransfected fragments of DNA (Stow et al., 1978). These observations suggest that it might be possible to rescue HSV-2 genes at high frequency from cells transformed by HSV-2. The present study was undertaken to examine the feasibility of this approach.

The system chosen attempts the rescue of the thymidine kinase gene from human cells biochemically transformed with HSV-2 DNA and uses as rescuing virus a TK^- strain of HSV-1. The choice of this system was originally influenced by a number of considerations. First, the cells chosen for this study are human cells which have been extensively characterized (Bacchetti and Graham, 1979). their viral DNA content is known (S. Bacchetti, personal communication) and they contain and express the viral thymidine kinase gene. Second, well characterized TK^- viral strains are available which bear specific mutations within the same viral gene known to be present in the transformed cells. Third, the reversion frequencies of the mutations in some of these strains have been accurately determined in the experiments reported in chapter 2. Fourth, the technique described in chapter 2 may be sensitive enough for the selection of putative recombinants, and this selection is determined by the expression of the same gene whose rescue is being attempted, and finally, the possibility of using as a rescuer an intertypic strain provides means of unequivocally identifying the rescue-products from a possible background of revertants.

Unlike the recently reported success in retrieving HSV-1 DNA sequences from rat cells morphologically transformed with temperature sensitive mutants or sheared viral DNA (Park et al., 1980), in the experiments reported in this chapter I was unable to detect the rescue of the viral TK gene. The analysis of the results indicate that in this system the occurrence of a rescuing event has a frequency below 7×10^{-7}. Similar results were obtained using biochemically transformed mouse L TK^- cells infected with homotypic and heterotypic rescuer strains.

3.2 Materials and Methods

The materials and methods to be considered here are only those which have not been already described in the equivalent section of the preceding chapter.

3.2.1 Cells and Viruses

The lines AC-1, AC-4 and AC-5 are separately derived clones of 143 cells biochemically transformed with sheared DNA from HSV-2 strain 219 (Bacchetti and Graham, 1977). These cells were kindly provided by Dr. S. Bacchetti (McMaster University), were maintained and passaged in methotrexate medium (Bacchetti and Graham, 1979) and were used at early passages (below passage #20). L TK^- (Kit et al., 1963) and Hela Bu cells (Kit et al., 1966) were also provided by S. Bacchetti (McMaster University). 148 C-1, a clone of L TK^- cells biochemically transformed with a plasmid (pTK-1) (Graham et al., 1980) containing a BamHI fragment carrying the viral TK gene of HSV-1 KOS, was provided by R. McKinnon (McMaster University). HSV-2 strain 219 is a TK^+ strain originally isolated from a cervical lesion (Seth et al., 1974).

3.2.2 Detection of TK^+ Viruses

The quantitation of the TK^+ fraction in the different virus preparations was carried out using the technique described in Chapter 2 (Campione-Piccardo *et al.*, 1979).

3.2.3 Extraction of Viral DNA from Cell-Released Virions

Vero cell monolayers were infected at a multiplicity of 0.1 PFU per cell. Three days later the cells were resuspended in the medium, spun at 1500 rpm for 10 minutes at 4°C, and the supernates were further spun at 30,000 rpm for 60 minutes at 4°C in a B-60 International ultracentrifuge. The pellet was washed once in Tris-HCl 10 mM, EDTA 50 mM, pH 7.8 and resuspended in Tris-HCl 10 mM, EDTA 1 mM, pH 7.8; NaCl was added at a 0.1 M final concentration and the suspension was digested with ribonuclease A (Sigma Chemical Co., St. Louis, Mo.) 5 mg/ml, 30 minutes at 37°C, and with proteinase K (Boehringer Mannheim, Canada) 25 mg/ml, 20 minutes at 37°C. Sodium dodecyl sulphate and Sarkosyl (ICN, K and K Laboratories Inc., Cleveland, Ohio) were added at 0.5% (wt/vol) and 1.0% (vol/vol) final concentrations, respectively. After 1 hour incubation at 37°C the preparation was extracted 3 times with a 4% (vol/vol) solution of isoamyl alcohol in chloroform. The final aqueous phase was dialyzed overnight at 4°C against Tris-HCl 10 mM, EDTA 1 mM, pH 7.8. Then, sodium acetate was added to 0.3 M final concentration and the DNA was precipitated overnight with 5 volumes of absolute ethanol at -20°C. The DNA was then pelleted at 10,000 rpm (10 minutes at 4°C) in a Sorvall centrifuge. The pellet was immediately dissolved in Tris-HCl 10 mM, EDTA 1 mM, pH 7.8 and was kept frozen at -45°C.

3.2.4 Purification of Viral DNA from Total DNA of Infected Cells

The viral DNA was also purified from DNA extracts of infected cells (Bacchetti and Graham, 1977). Vero cells were infected at a multiplicity of infection of 0.1 and were kept in [^{3}H]dThd (1 mCi/ml) from the time of infection. At completion of the cytopathic effect, the cells were lysed with 0.2% (wt/vol) sodium dodecyl sulfate and digested overnight, at 37°C, with Pronase (Sigma) (0.5 mg/ml in 10 mM Tris-HCl, 10 mM EDTA, pH 7.8). The digestion was followed by 2 phenol extractions and one extraction in isoamyl alcohol/chloroform (1/24 vol/vol). Sodium Acetate was added to the final aqueous phase at 0.3M and the DNA was spooled during precipitation after the addition of 2 volumes of ethanol at -20°C. The DNA was resuspended in 10 mM Tris-HCl, EDTA 1 mM, pH 7.8. Purification of the viral DNA was achieved by two successive centrifugations in sodium iodide isopicnic gradients (Lohman et al., 1973; Anet and Strayer, 1969). The purity of the viral DNA preparations was monitored by analytical ultracentrifugation in gradients of cesium chloride. Fractions were collected from the top of the NaI, and the fractions with the DNA were identified by scintillation counting of 20 µl of each fraction spotted on filter paper and precipitated with trichloroacetic acid (5% wt/vol). The NaI was removed from the final fractions by extensive dialysis against 10 mM Tris-HCl, 1 mM EDTA, pH 7.8. The OD_{260}/OD_{235} index was found to be a sensitive indicator of the complete removal of NaI by the dialysis. An example of this viral DNA purification is shown in figures 18 and 19.

Figure 18

Separation of B2006 DNA from cellular DNA by isopicnic gradients of sodium iodide. The separation of the viral (ρ_{20} = 1.505) and cellular (ρ_{20} = 1.462) DNA's obtained in a NaI gradient, run with the total DNA from infected cell lysates is shown at the top of the figure. Equivalent results obtained after a rerun of the fractions identified as dna_1 are shown at the bottom. Vero cells were infected with HSV-1 B2006 as described in the text and were kept in the presence of [^{3}H]dThd (1 μCi/ml) from the time of infection. The fractions identified as dna_2 were pooled to be used in later studies. The graph at the very top indicates the change of density (ρ_{20}). The density values at which the cellular and the viral DNA band in the NaI gradients are slightly different than those predicted by the equation of Lohman *et al.*, (1973) but the identity of the DNA's was confirmed by simultaneous CsCl gradients where, as expected, the cellular DNA peaked at 1.700 gm/cm^3.

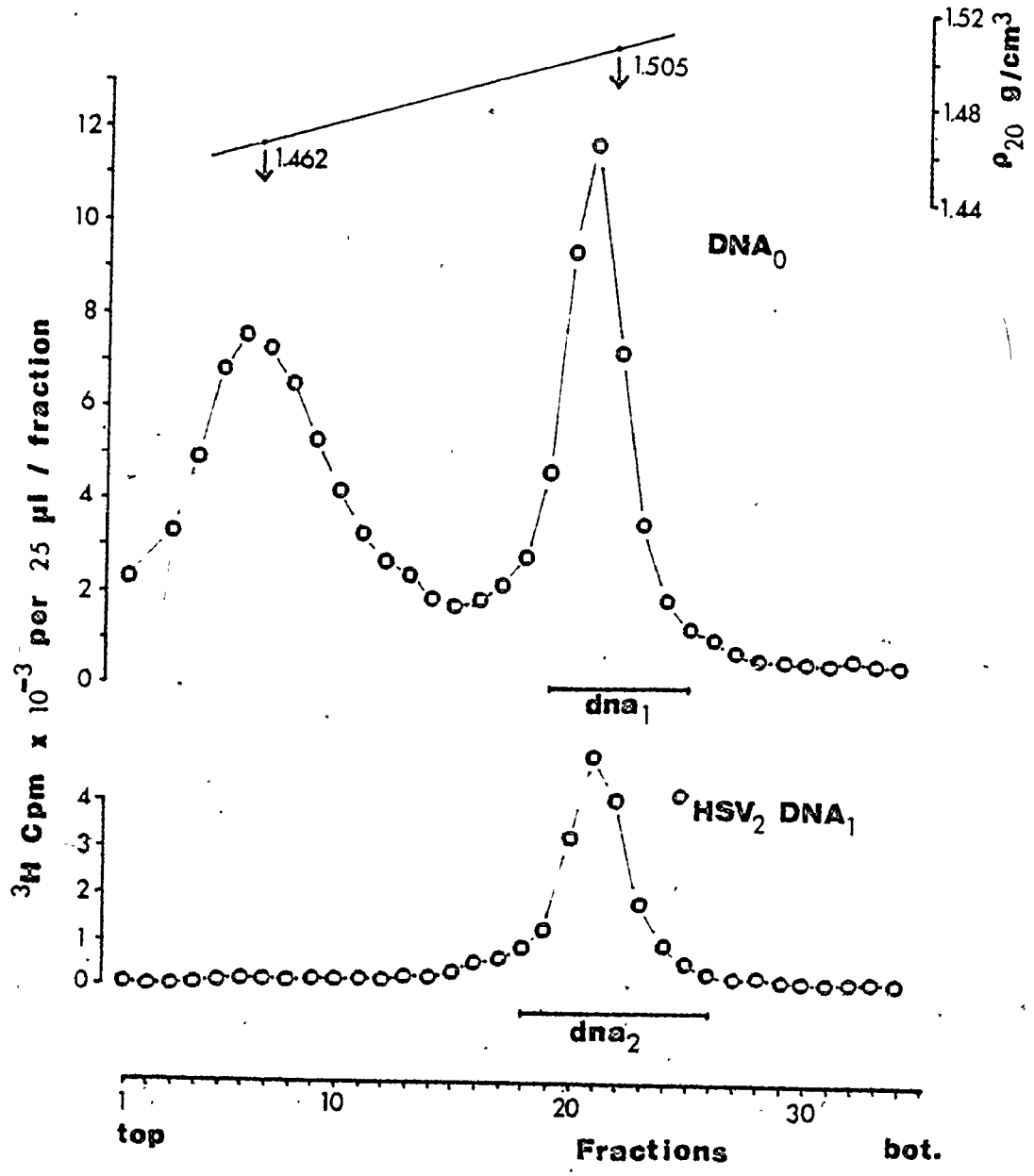
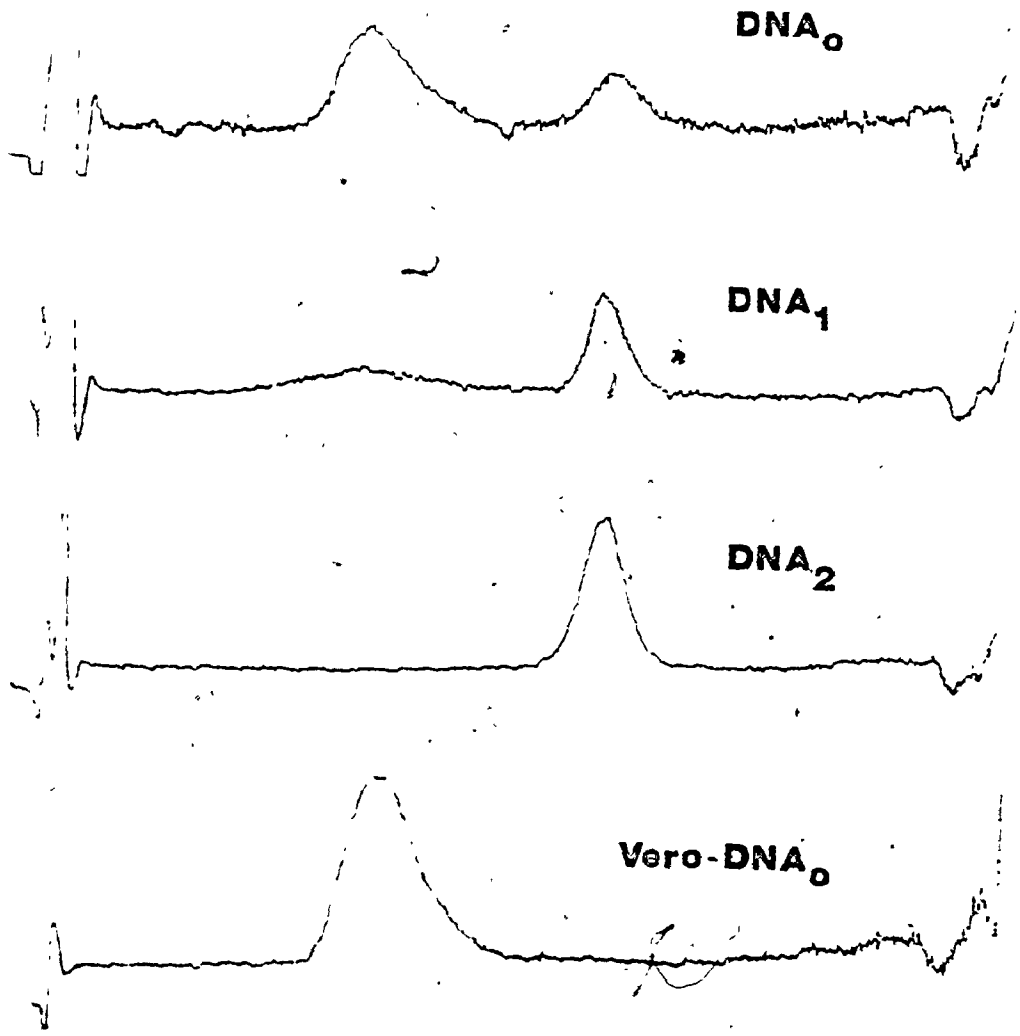

1.52
1.48
1.44
ρ_{20} g/cm^3
1.505
1.462
DNA$_0$
dna$_1$
^{3}H Cpm x 10^{-3} per 25 µl / fraction
HSV$_2$ DNA$_1$
dna$_2$
top
Fractions
bot.

Figure 19

Monitoring in the analytical ultracentrifuge of the purity of the viral DNA obtained from the NaI gradients shown in figure 18. DNA_0, DNA_1 and DNA_2 correspond to the equivalent fractions in figure 18. Vero DNA_0 is the DNA from a lysate of uninfected cells.


DNA$_0$
DNA$_1$
DNA$_2$
Vero-DNA$_0$

3.2.5 Transfection and Infectivity Assays of HSV-DNA

Transfections were performed using the technique described by Graham and Van der Eb, 1973 as modified by Graham *et al.*, 1974. The viral DNA was either dialysed or directly resuspended in Hepes buffered saline, pH 7.05 containing per ml 8.0 mg of NaCl, 0.37 mg of KCl, 0.125 mg of $Na_2HPO_4.2H_2O$, 1 mg of dextrose and 5 mg of N'-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (Hepes), pH 7.05. Unless otherwise specified, carrier DNA (calf thymus or salmon sperm DNA) was added to a final concentration of 20 μg/ml. $CaCl_2$ (0.125 M final concentration) was slowly added to the solution while air was being bubbled through the DNA mixture. After 20 to 30 minutes at room temperature the precipitates were added to subconfluent monolayers of Vero cells. 0.5 ml of the suspension containing a specified amount of viral DNA and 10 μg of the carrier DNA was added to the 4.5 ml of medium covering the cell monolayers in 60 mm tissue culture plates. After 4 hrs at 37°C the monolayers were washed and the medium was changed, and 24 hrs after transfection it was replaced with medium containing 0.2% (vol/vol) of human immune serum globulin (Connaught Laboratories Ltd., Willowdale, Ontario, Canada). An example of an infectivity assay carried out with this procedure is shown in figure 20. The amount of carrier DNA yielding maximum viral DNA infectivity was determined at 15-20 μg/ml (figure 21).

3.2.6 Digestion with Restriction Endonucleases and Separation of DNA Fragments

Restriction endonucleases EcoRI, HindIII, BamHI and XbaI were purchased from New England Biolabs (Beverley, Mass.). The digestions

Figure 20

Infectivity of B2006 DNA (clone B4). The plotted points represent average values from triplicate assays of the infectivity in Vero cells of B2006 DNA. The bars indicate one standard deviation above and below the mean.

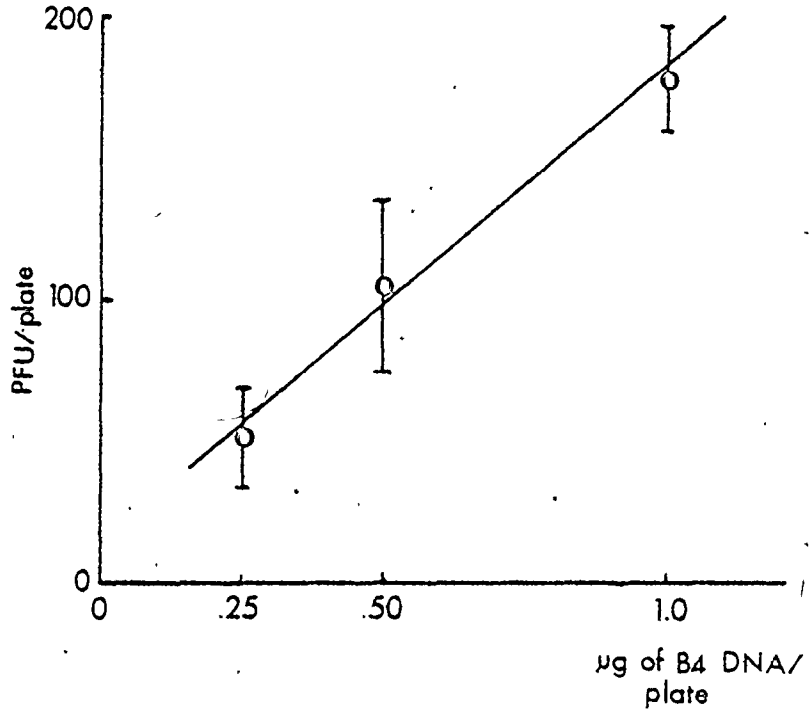
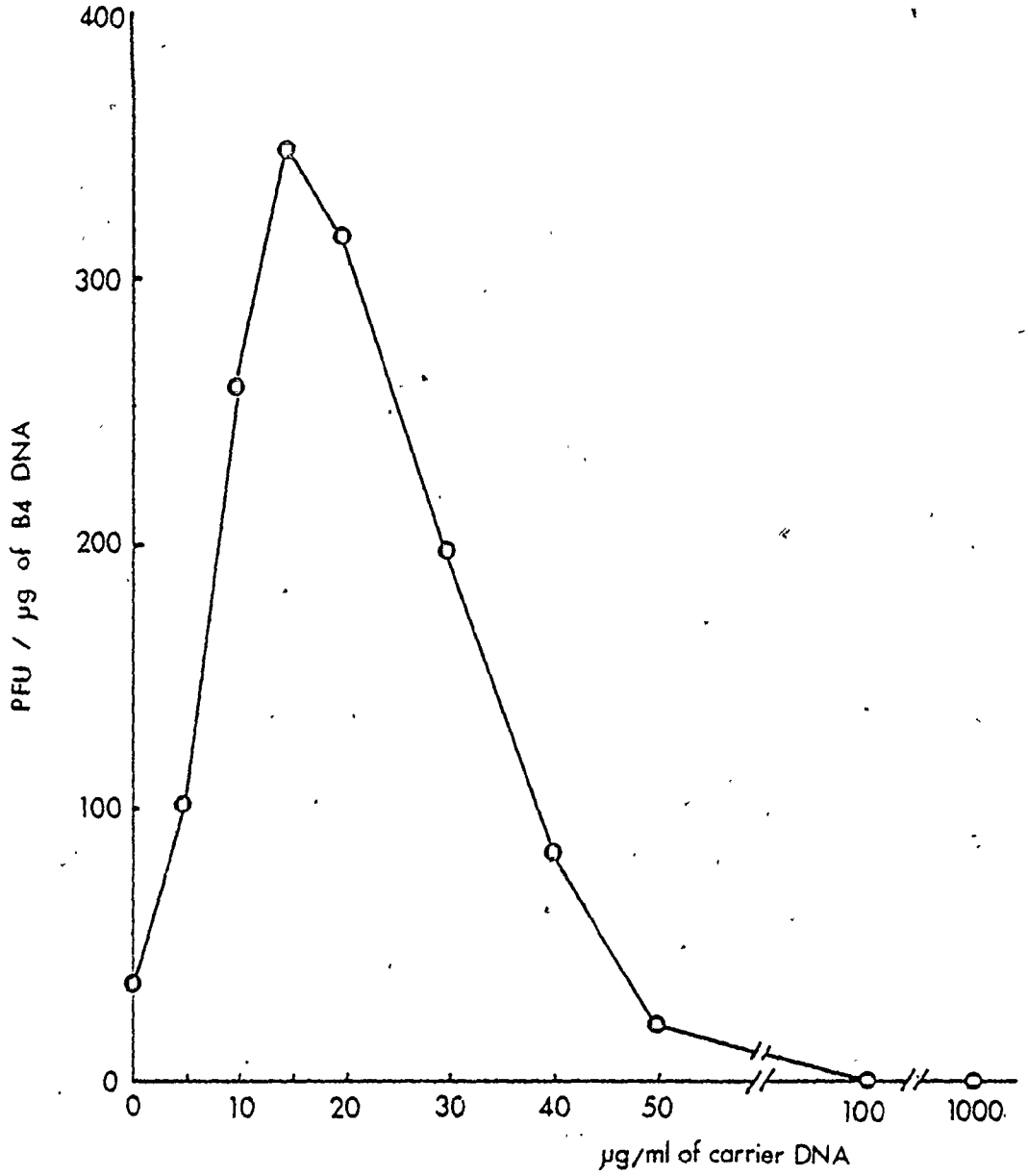

200
100
0
PFU/plate
0
.25
.50
1.0
μg of B4 DNA/
plate

Figure 21

Effect of the amount of carrier DNA on the infectivity of B2006 DNA (clone B4).


400
300
200
100
0
PFU / µg of B4 DNA
0
10
20
30
40
50
100
1000
µg/ml of carrier DNA

were carried out in the buffers specified for the titration of each enzyme, with 2 units of enzyme per μg of DNA during 3 hours at 37°C. The fragments were separated by horizontal slab gel electrophoresis using 0.5% (wt/vol) agarose in Tegtmeyer's buffer (Tegtmeyer and Macasaet, 1972) with 1 μg/ml of ethidium bromide. Direct visualization of the fragments was obtained by UV illumination and pictures were taken using type 57 Polaroid film (Polaroid Corp., Oak Brook, Ill.) and a red filter. For transfection in transformation experiments the digested DNA was directly resuspended in Hepes saline buffer, pH 7.05.

3.2.7 Thymidine Kinase Assays

The thymidine kinase assays were performed with the procedure used by Munyon et al., 1971. The standard assay was performed by suspending 2 x 10^6 infected cells in 50 μl of extraction buffer (Tris-HCl 10 mM, ATP 1 mM, 2-mercaptoethanol 1.4 mM, pH 8) and disrupting the cells by sonication at 4°C. Cell debris were removed by centrifugation and the supernate was then appropriately diluted in reaction buffer (Tris-maleate 0.1 M, KCl 25 mM, $MgCl_2$ 20 mM, 2-mercaptoethanol 1.4 mM, ATP 10 mM, pH 6.5). Each reaction was carried out for 1 hour at 37°C in a final volume of 100 μl with 1 μCi of tritiated thymidine ([^{3}H]dThd; 20 Ci/mmol; New England Nuclear Corp., Boston, Mass.). To stop the reaction, 10 μl of cold dThd (1.2 mg/ml) were added. The phosphorylated products of the kinase reaction were separated from the unreacted [^{3}H]dThd by chromatography on DEAE paper (DE81, Whatman Ltd., England) as described by Bresnick and Karjala (1964).

The TK of HSV-2 has been reported to be more thermolabile than the one expressed by HSV-1 (Ogino and Rapp, 1971; Lowry et al., 1971; Thouless and Skinner, 1971). During the present studies I found that this lability was dependent on the presence of ATP in the extraction buffer and that HSV-2 TK was preferentially inactivated during an extraction procedure carried out at room temperature if ATP was removed from the extraction buffer (figure 22). The activity of the enzyme extracted in the absence of ATP was expressed as a percentage of the activity obtained after extraction in the presence of ATP. This was found to yield reproducible values which allowed the differentiation of the TK activities expressed by both HSV serotypes (figure 23).

3.2.8 Neutralization of Thymidine Kinase Activity

Monospecific antiserum to HSV-1 thymidine kinase was prepared in rabbits and was provided by S. Girvitz (McMaster University). An extract was prepared from infected cells in extraction buffer containing ATP and 2-fold dilutions of the extract were assayed for enzyme activity. 10 µl of the dilution which corresponded to the middle of the linear portion of the dose-response curve were mixed with 10 µl of different 2-fold dilutions of the antiserum. After 1 hr at 37°C, 80 µl of reaction buffer containing 1 µCi of (^{3}H)dThd were added to each mixture which was then further incubated 1 hr at 37°C. The residual TK activity was then determined. Preimmune serum replaced the antiserum in control mixtures.

3.3 Results

3.3.1 Replication of HSV-1 in Biochemically Transformed Cells

The cell lines AC-1, AC-4 and AC-5 were biochemically trans-

Figure 22

Incorporation of [^{3}H]dThd into thymidilate induced by different dilutions of extracts obtained in the absence of ATP or with 1 mM ATP in the extraction buffer (●——●), (□——□). The circles correspond to extracts of cells infected with HSV-1 (C1-101), and the squares to extracts of cells infected with HSV-2 (219).

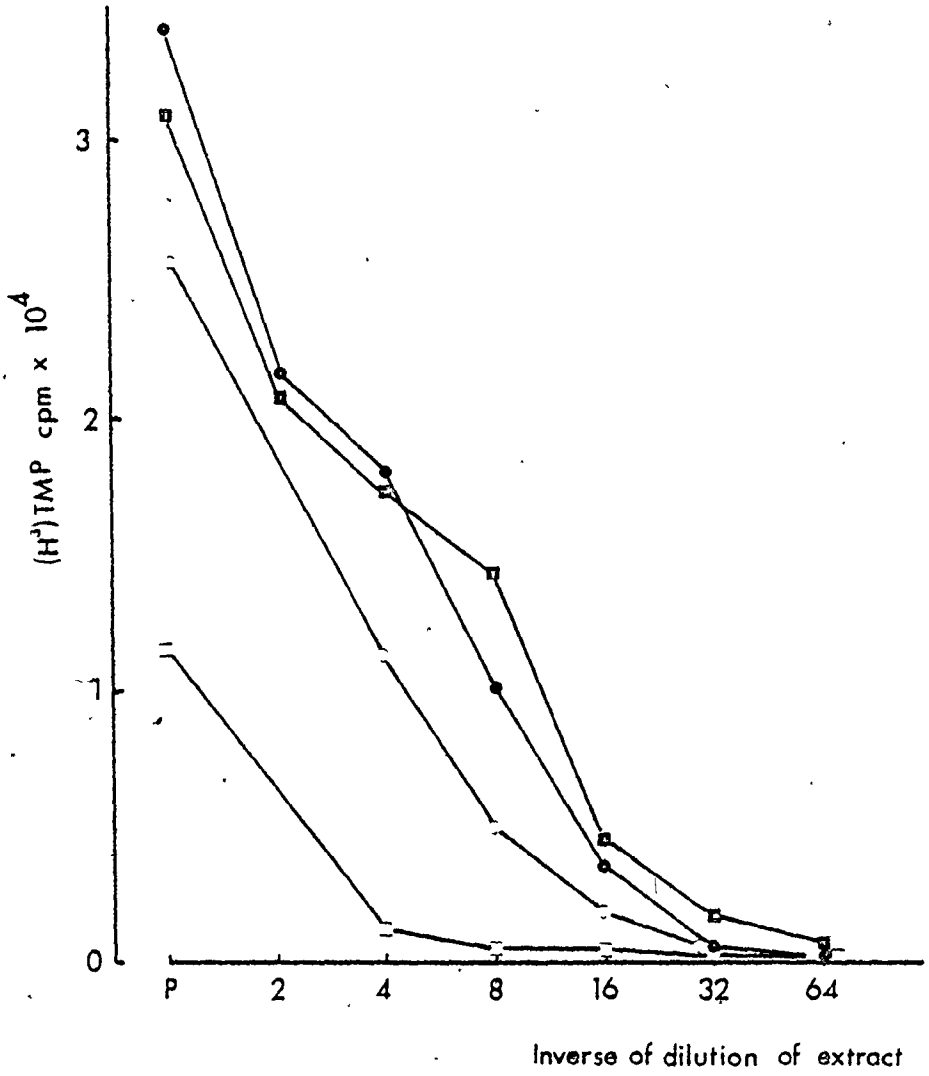
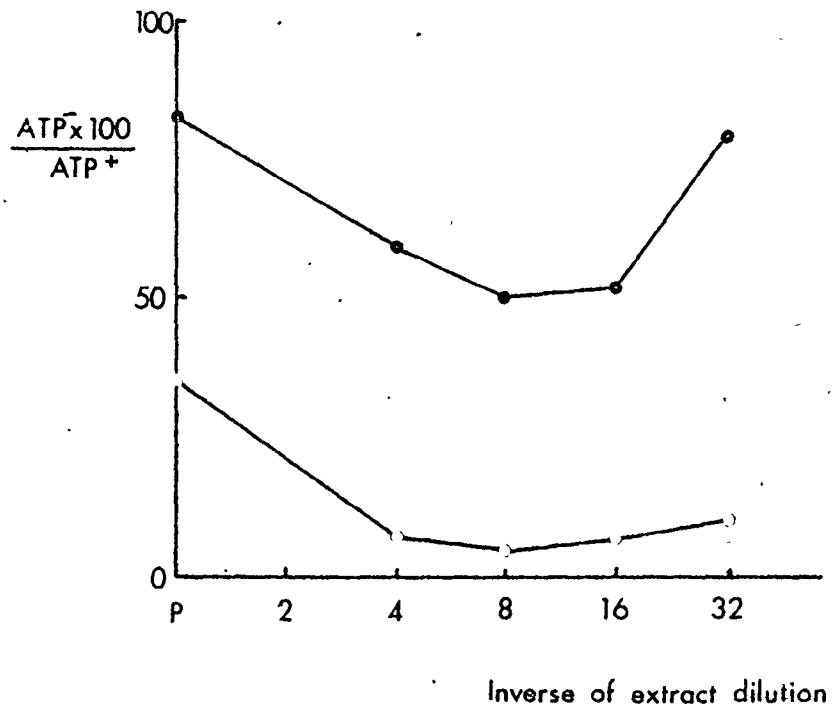

3
2
1
0
(H³)TMP cpm x 10⁴
P
2
4
8
16
32
64
Inverse of dilution of extract

Figure 23

Plot of the same data shown in figure 22 expressed as the percentage of TK activity present in the extracts obtained in the absence of ATP (ATP^-) with respect to the activity in extracts obtained with complete extraction buffer (ATP^+). HSV-1 (C1-101) (●——●), HSV-2 (219) (○——○).


100
50
0
ATP⁻ x 100 / ATP⁺
P
2
4
8
16
32
Inverse of extract dilution

formed by sheared HSV-2 DNA. These cells have been shown to contain HSV-sequences colinearly linked to nonviral DNA. Only one integration site has been detected in each cell line and the HSV-DNA content estimated for cells analyzed at early passages after transformation was found to be equal to or less than 1 genome equivalent (S. Bacchetti, personal communication).

Initially, the permissiveness of the cells for HSV-1 replication was established. Figure 24 shows the results of one step growth of TK^- mutant B2006 in different cell lines. The results clearly indicate the full permissiveness of AC-1 and AC-5 to infection with HSV-1. A similar result was obtained with AC-4 (data not shown).

3.3.2 Recovery of TK^+ Virus from Transformed Human Cells Infected with TK^- Mutants

The biochemically transformed cell lines, AC-1, AC-4 and AC-5, as well as the parental cell lines (both TK^+ and TK^-) and Vero cells were infected with the HSV-1 TK^- mutants B2006 and d2 with a multiplicity of 1. Assays for TK^+ virus were done immediately and after 1 or 2 enrichment steps in which the virus was replicated in 143 TK^- cells in methotrexate medium. The results for B2006 are shown in table 9. No TK^+ virus was found without enrichment; however, following enrichment TK^+ viruses appeard. Slightly higher yields of TK^+ virus were consistently observed when the virus was replicated in the TK transformed cell lines as compared to the nontransformed cell lines. Similar experiments carried out with HSV-1 d2 failed to yield any TK^+ virus. If rescue occurred with HSV-1 d2, the data suggest that it was an uncommon event (data not shown). Rescue attempts using as rescuer transfected DNA of HSV-1

Figure 24

One step growth of B2006 in different cell lines. Cell monolayers with about $2x10^6$ cells were infected at a multiplicity of infection of 1 with plaque purified B2006 virus. After 1 hr of adsorption at 37^oC the monolayers were repeatedly washed and fresh medium was added. At different times after infection the cells were resuspended in the medium and disrupted by sonication. After removal of cell debris the viral particles were pelleted by ultracentrifugation. They were resuspended in 1 ml of medium and virus titers were determined on Vero cell monolayers.

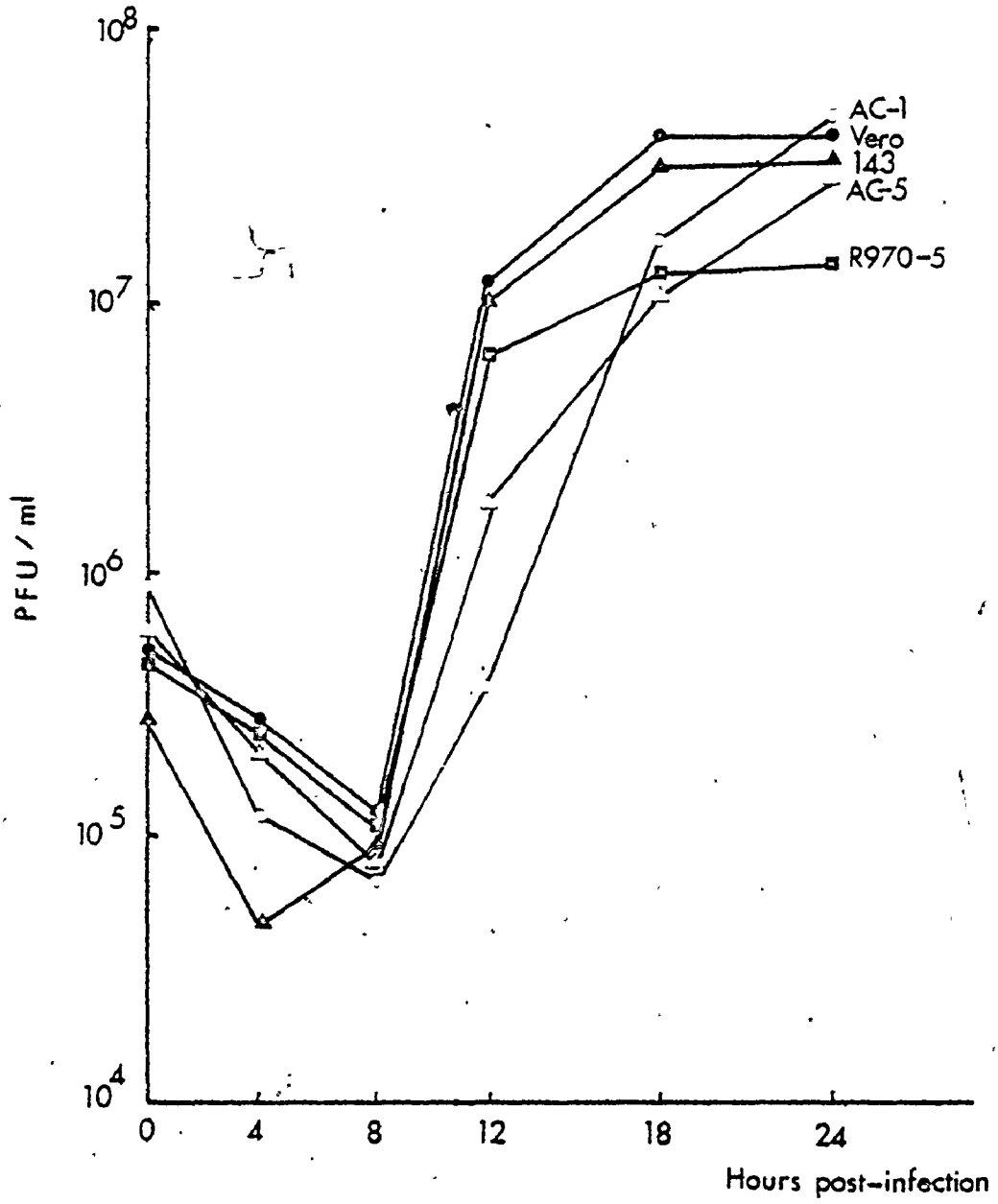

10^8
10^7
10^6
10^5
10^4
PFU / ml
AC-1
Vero
143
AC-5
R970-5
0
4
8
12
18
24
Hours post-infection

Table 9

Emergence of TK^+ Virus After Passage of TK^- Virus in TK Transformed Human Cells.[a]

	B2006 virus[b] Passages in TK^- cells in Mtx[d]			B2006 DNA[c] Passages in TK^- cells in Mtx[d]		
	0	1	2	0	1	2
Vero	$<1.4\times10^{-5}$	6.3×10^{-5}	2.1×10^{-4}	$<9.6\times10^{-6}$	1.9×10^{-5}	3.6×10^{-4}
R970-5	$<9.6\times10^{-6}$	6.8×10^{-5}	4.7×10^{-4}	ND	ND	ND
143	$<1.0\times10^{-5}$	1.4×10^{-5}	7.4×10^{-5}	$<1.5\times10^{-5}$	3.2×10^{-5}	4.4×10^{-4}
	$<1.1\times10^{-5}$	1.9×10^{-5}	7.0×10^{-5}	$<1.0\times10^{-6}$	5.6×10^{-5}	6.8×10^{-4}
AC-1	$<9.5\times10^{-6}$	1.1×10^{-5}	4.6×10^{-3}	1.1×10^{-5}	1.9×10^{-5}	2.6×10^{-4}
	$<1.4\times10^{-5}$	1.1×10^{-4}	1.4×10^{-3}	1.0×10^{-5}	6.8×10^{-5}	4.7×10^{-4}
AC-4	$<1.7\times10^{-5}$	2.3×10^{-5}	8.4×10^{-4}	ND	ND	ND
AC-5	$<9.3\times10^{-6}$	1.2×10^{-5}	2.2×10^{-3}	$<9.1\times10^{-6}$	1.8×10^{-5}	3.0×10^{-4}
	$<9.0\times10^{-6}$	1.4×10^{-5}	8.2×10^{-4}	$<1.0\times10^{-5}$	2.8×10^{-5}	9.9×10^{-4}

Table 9 (Footnotes)

[a]The values in the table represent the frequencies of TK^+ PFU in each of the viral preparations. They reflect the efficiencies of plaquing of the virus in TK^- cells in methotrexate and were determined as described in Chapter 2.

[b]The cells were infected at a multiplicity of infection of 1.0 and were harvested upon completion of the CPE.

[c]Infectious B2006 DNA was used to transfect the different cell lines. The specific infectivity of the B2006 DNA measured in Vero cells was 200 PFU/μg. 10 μg of this DNA were used to transfect about $2x10^7$ cells. The viruses were harvested when CPE was complete. The enrichment steps were carried out as described below.

[d]Enrichment steps were performed in TK^- cells (143) in methotrexate medium (Mtx). The first column refers to the original passage in each cell lines. The 2nd and 3rd column show the results of titrations after 1 and 2 enrichmont steps. Both enrichment steps were performed by infecting the TK^- cell at a multiplicity of infection of 1.

B2006 yielded results similar to those obtained with cells infected with viral particles (table 9).

3.3.3 Transformation of Mouse L Cells and Recovery of TK^+ Virus from Progenies of HSV TK^- Cells Transformed by Homotypic and Heterotypic Strains

This section reports the preparation of mouse L cells biochemically transformed with specific fragments of HSV-1 and HSV-2 DNA. The purpose of these experiments was to prepare freshly transformed cells to be used in attempts to retrieve the viral TK gene using homotypic and heterotypic strains of HSV. Subconfluent monolayers of L TK^- cells were transfected with HSV-1 KOS DNA digested with HindIII endonuclease or with HSV-2 219 DNA digested with XbaI endonuclease. The transfections were carried out as described in Section 3.2.5 except that at 24 hours post transfection methotrexate medium was added. This medium was changed twice a week and TK^+ colonies were directly visualized between 10 and 20 days after transfection. Independently from the DNA used, the efficiency of transformation in L TK^- cells was about 5-6 colonies per μg of DNA per $4x10^6$ cells. No transformed colonies were detected in HeLa Bu or 143 TK^- cells in simultaneous experiments. The same negative result was obtained in L TK^- cells transfected with carrier DNA alone. Well isolated colonies, each from an independently transfected monolayer, were individually trypsinized and grown to a total yield of about 10^8 cells before being used in the attempts to rescue the viral TK gene. Table 10 shows the results of these attempts in 5 lines of transformed mouse L cells. These rescue experiments were performed following the same protocol outlined in Section 3.3.2.

Table 10

Emergence of TK^+ Virus After Passage of TK^- Virus in TK Transformed L Mouse Cells.[a]

Cells	Viral DNA[b]	HSV-1 B2006	HSV-1 d2[c]
LTK^-	-	6.0×10^{-4}	$<3.7 \times 10^{-5}$
		3.5×10^{-4}	$<9.3 \times 10^{-6}$
148-Cl[d]	PTK-1 (HSV-1 KOS)[e]	7.3×10^{-4}	$<4.3 \times 10^{-5}$
		3.7×10^{-4}	$<1.0 \times 10^{-5}$
LKH-1	HSV-1 KOS/HindIII	6.0×10^{-4}	$<3.2 \times 10^{-5}$
		4.6×10^{-4}	$<1.7 \times 10^{-5}$
L2X-1	HSV-2 219/XbaI	9.2×10^{-4}	$<5.0 \times 10^{-5}$
		ND	ND
L2X-2	HSV-2 219/XbaI	ND	$<2.4 \times 10^{-5}$
		ND	$<9.7 \times 10^{-6}$
L2X-3	HSV-2 219/XbaI	4.6×10^{-4}	ND
		$<1.2 \times 10^{-5}$	ND

[a]The values in columns 3 and 4 show the efficiencies of plaquing in 143 TK^- cells in methotrexate of the viral preparations after 2 enrichment passages for TK^+ viruses in 143 TK^- cells in methotrexate medium. The duplicates correspond to different series of enrichment steps.

[b]This column indicates the viral strain whose DNA was used to obtain the corresponding transformed cell line.

[c]The 2 enrichment steps for TK^+ viruses lower the titers of the virus to values between 10^{-4} and 10^{-5}. Since these titers may not allow the detection of low frequencies of TK^+ viruses, before the final titration the preparations of HSV-1 d2 were passaged once (at MOI $\approx$ 0.01) on monolayers of Vero cells.

Table 10 (Footnotes cont'd)

[d]Cells 148-C1 transformed with pTK-1 DNA were provided by R. McKinnon (McMaster University).

[e]pTK-1 is a recombinant plasmid containing the BamHI fragment carrying the TK gene of HSV-1 strain KOS (Graham *et al.*, 1980).

3.3.4 Analysis of TK^+ Viruses Isolated from the Rescue Experiments

The possibility existed that a rescue frequency close to the reversion frequency of B2006 might have yielded mixtures of revertants and rescue products. A series of 10 TK^+ strains independently picked from the titrations of the enrichment steps were analyzed for parentage. All these strains originated independently in monolayers of human transformed cells, separately infected with plaque purified clones of HSV-1 B2006. The stability of the TK activity after extraction in the absence and presence of ATP for 3 of the strains is shown in figure 25. The TK activity of all 3 strains had the stability of HSV-1. Similar results obtained with the other 7 strains are shown in table 11, thus, all 10 strains had the HSV-1 phenotype with respect to enzyme stability.

The influence of different dilutions of antiserum to HSV-1 TK on the enzymatic activity of HSV-1, HSV-2 and 3 of the strains is shown in figure 26. The TK activity of all 3 strains was neutralized to a degree observed with HSV-1. Analysis of the other 7 strains revealed that the enzymes of these viruses were antigenically similar to HSV-1 (table 12).

The TK gene of HSV-1 resides in the N fragment of EcoRI and in the P fragment (3.4 Kbp) of BamHI cleaved HSV-1 DNA. The EcoRI and BamHI DNA fragments carrying the TK gene in HSV-1 and HSV-2 have different sizes; besides, a BamHI cleavage site cleaving inside the TK gene is present only in HSV-2 DNA (Reyes *et al.*, 1980). Therefore, DNA of the 10 putative recombinant strains was examined by cleavage with these endonucleases to detect possible additions or deletions in the regions coding for TK. Cleavage with EcoRI and BamHI of the DNA from 3 of the strains and from B2006 are shown in figure 27. Figure 28 shows the

Figure 25

Lability of TK activity in extracts obtained in the absence of ATP. Extracts of infected cells were prepared in the absence and the presence of ATP (1 mM). The percentage of TK activity present after extraction in the absence of ATP was determined in two fold serial dilutions of the extracts. The results obtained with HSV-1 C1-101 (●——●) and HSV-2 219 (o——o) are shown for reference. The results obtained with three putative recombinants are also shown. RnAC1A1 (▲), RnAC4A2 (□), RnAC5A3 (◇).

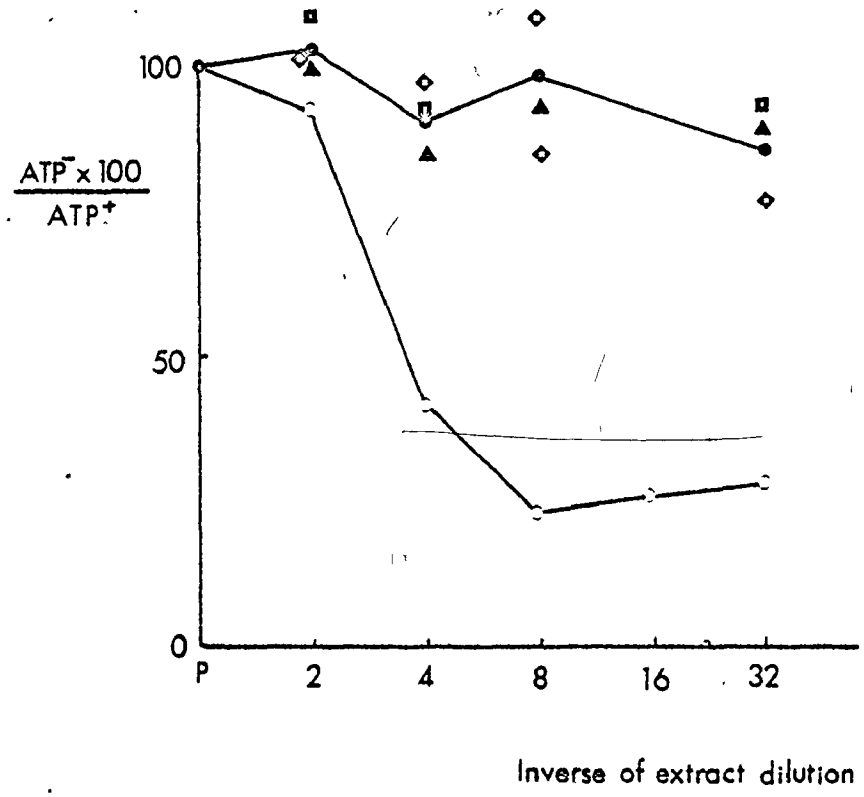

100
50
0
ATP⁻ x 100 / ATP⁺
P
2
4
8
16
32
Inverse of extract dilution

Table 11

Stability in the Absence of ATP of the TK Activity in Extracts of TK^- Cells Infected with Putative Recombinants.[a]

Virus	ATP^+	ATP^-	% Activity[b]
C1101	9071	9271	102.2
219	5133	965	18.8
RnAC1B4	8882	7976	89.8
RnAC1B5	12403	12763	102.9
RnAC1B6	11527	8518	93.9
RnAC5B7	7281	5592	76.8
RnAC5B8	12086	9355	77.4
RnAC5C9	10885	8044	73.9
RnAC5C10	5868	6126	104.4

[a]Columns 2 and 3 show, in counts per minute, the [^{3}H]ThdR transformed into [^{3}H]TMP by the viral TK present in each reaction. The background activity detected in uninfected cells has been subtracted from the original values to yield the numbers shown in the table.

[b] $\% \text{ activity} = \frac{ATP^+ \times 100}{ATP^-}$

Figure 26

Neutralization of viral thymidine kinase activity by a monospecific anti-HSV-1 TK antiserum. The thymidine kinase activity in extracts of infected TK^- cells was titrated and dilutions corresponing to the middle of the linear dose were mixed in equal amounts with two-fold dilutions of an anti-TK (HSV-1) serum. After 1 hr incubation at 37°C a given volume of reaction buffer containing [^{3}H]dThd and ATP was added as indicated in the text. After 1 hr at 37°C the products of the kinase reaction were chromotgraphed on DEAE paper and the percentage of cpm in each reaction, with respect to controls with equivalent dilutions of preimmune sera, were determined. HSV-2 (219) (□—□) and HSV-1 (C1-101) (o---o) are shown as references. The results given by three putative rescued recombinants are also shown: RnAC1A1 (●), RnAC4A2 (▲), RnAC5A3 (□). The vertical bars indicated the standard deviations in triplicate estimates obtained with the reference viruses.

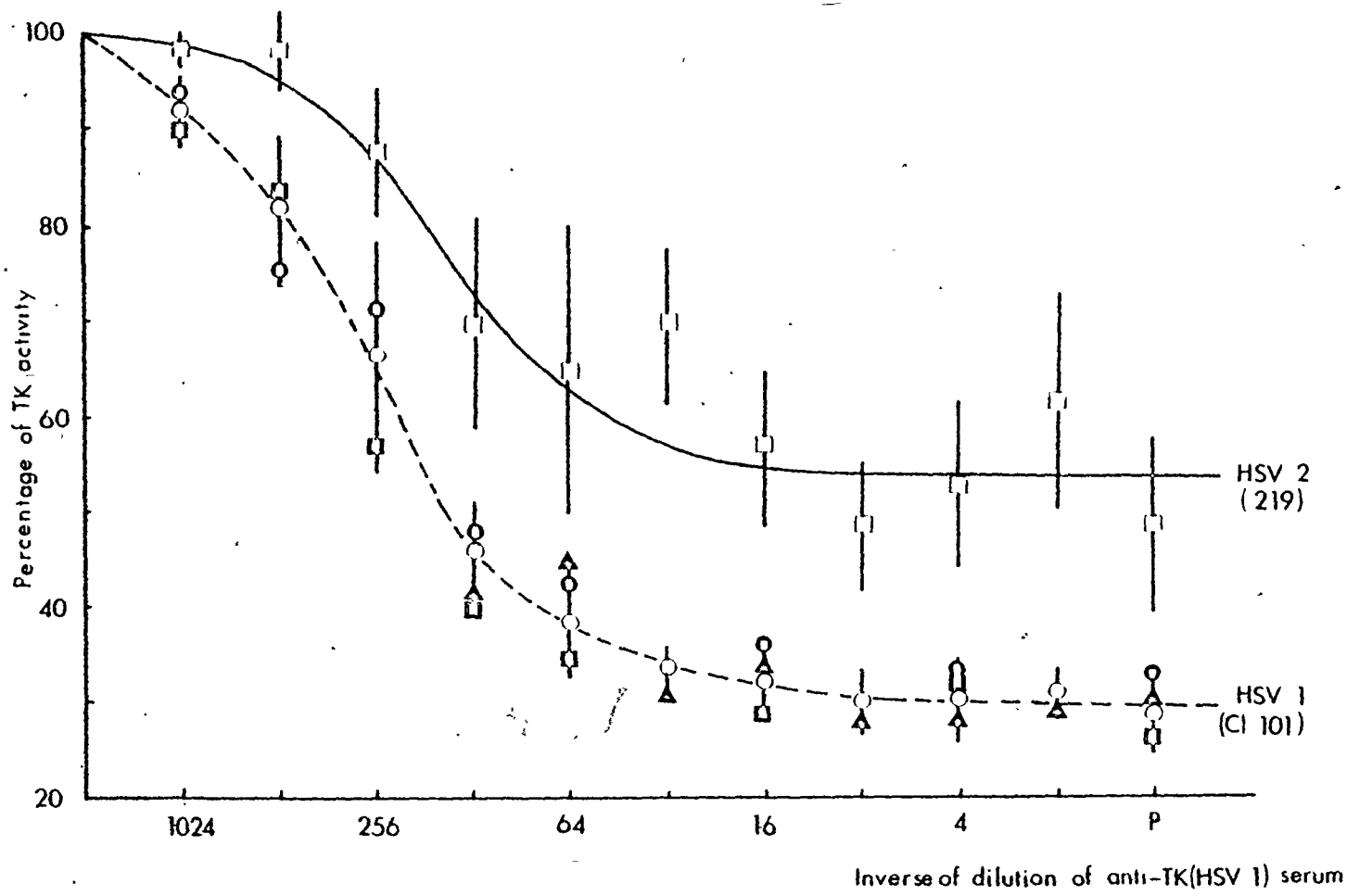

100
80
60
40
20
Percentage of TK activity
1024
256
64
16
4
P
Inverse of dilution of anti-TK(HSV 1) serum
HSV 2
(219)
HSV 1
(Cl 101)

Table 12

In Vitro Neutralization of the TK Activity in Extracts of TK$^-$ Cells Infected with Putative Recombinants, with a Type-specific Anti-TK Serum (aTK-1).[a]

Virus	Control[b]	Serum[c] aTK-1(¼)	% Activity[d]
C1101	12128	3508	28.1
219	13647	7533	55.2
RnAC1B4	15081	3966	26.3
RnAC1B5	16997	4487	26.4
RnAC1B6	18831	5781	30.7
RnAC5B7	12667	4041	31.9
RnAC5B8	13630	2808	20.6
RnAC5C9	17126	5223	30.5
RnAC5C10	19735	6414	32.5

[a]Column 2 and 3 show the amount (counts per minute) of [^{3}H]ThdR transformed into [^{3}H]TMP by the viral thymidine kinase activity present in each preparation.

[b]The control represents the activity present in mixtures of infected cell extracts with a 1/4 dilution of preimmune sera.

[c]This column shows the residual TK activity present in the extracts after neutralizations with a 1/4 dilution of an HSV-1 (KOS) TK antiserum (aTK-1). This serum was provided by S. Girvitz (McMaster University).

[d] $\% \text{ activity} = \frac{(\text{aTK-1}) \times 100}{(\text{CONTROL})}$

Figure 27

Agarose (0.5%, wt/vol) gel electrophoresis of HSV-DNA's digested with EcoRI (1-4) and BamHI (5-8) restriction endonucleases. (1 & 5) RnAC1A1, (2 & 6) RnAC4A2, (3 & 7) RnAC5A3, (4 & 8) B2006. The arrows indicate the position of the fragments of HSV-1 DNA carrying the TK gene.

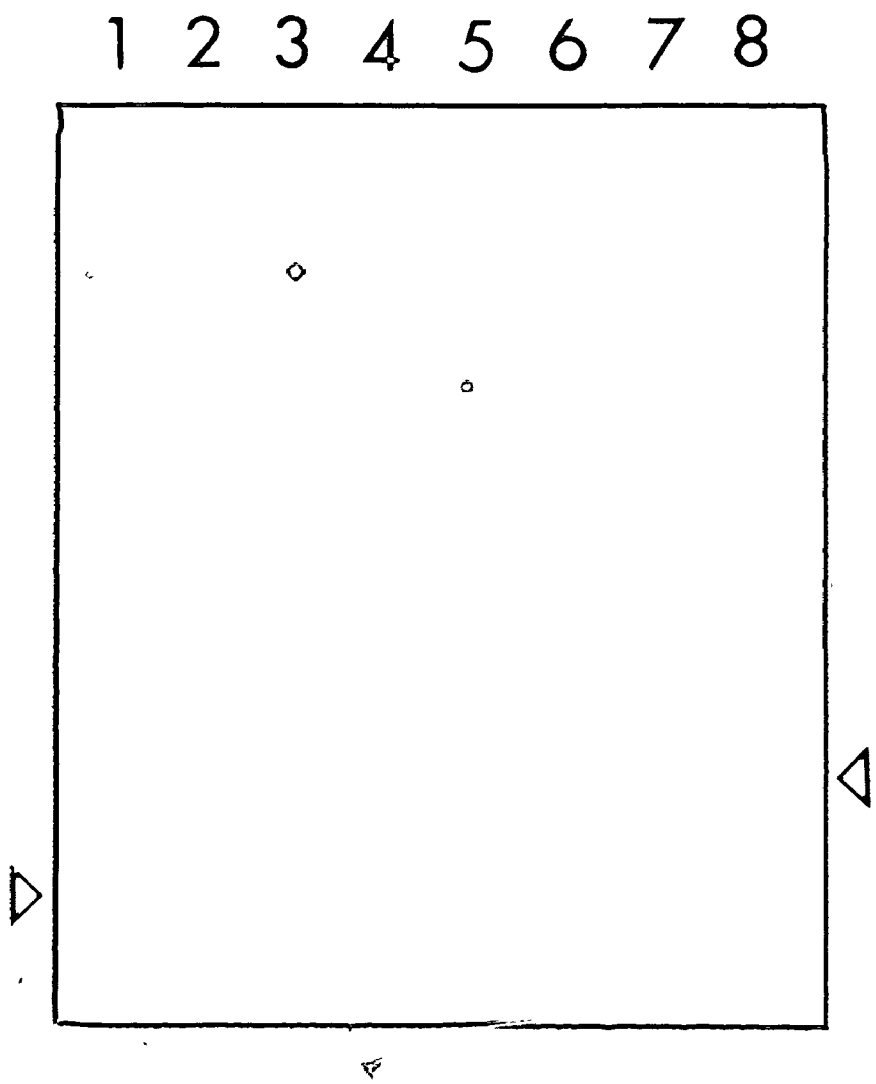
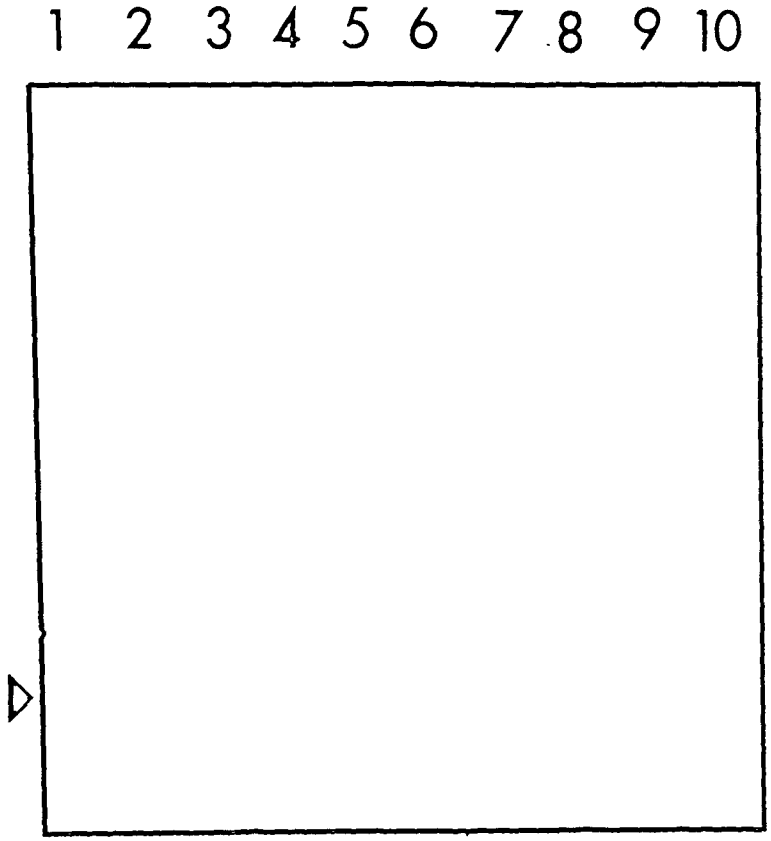

1 2 3 4 5 6 7 8

Figure 28

Agarose (0.5%, wt/vol) gel electrophoresis of HSV-DNA's digested with BamHI restriction endonuclease. (1) RnAC1B4, (2) RnAC1B5, (3) RnAC1B6, (4) HSV-1 KOS, (5) HSV-2 219, (6) RnAC5B7, (7) RnAC5B8, (8) HSV-1 B2006, (9) RnAC5C9, (10) RnAC5C10. The arrow indicate the position of the 3.4 kbp fragment in HSV-1 DNA carrying the viral TK gene.


1 2 3 4 5 6 7 8 9 10

BamHI cleavage patterns of the remaining 7 strains, together with HSV-1 B2006, HSV-1 KOS and HSV-2 strain 219. All putative recombinants appear to have the cleavage pattern characteristic of the parental B2006 strain.

3.3.5 Estimates of the Frequency of Rescue

Since no TK^+ virus was obtained with the deletion mutant, HSV-1 d2, the frequency of rescue should be below $1x10^{-7}$. This value is reached assuming a 100 fold increase in the frequency of TK^+ viruses at the second enrichment step (see figure 17), which yielded a frequency below $1x10^{-5}$. A further estimation of the upper limit of occurrence of the rescue event can be derived assuming that the TK^+ viruses analyzed represented a random sequence of trials with constant probability p (Bernoulli sequence) (Lindley, 1965). If the rescue event is then assigned a frequency f_s and the frequency of reversion of the rescuer virus f_v is known, the probability p of picking a rescue product in each trial is given by

$$p = f_s/(f_s + f_v) \quad . \tag{50}$$

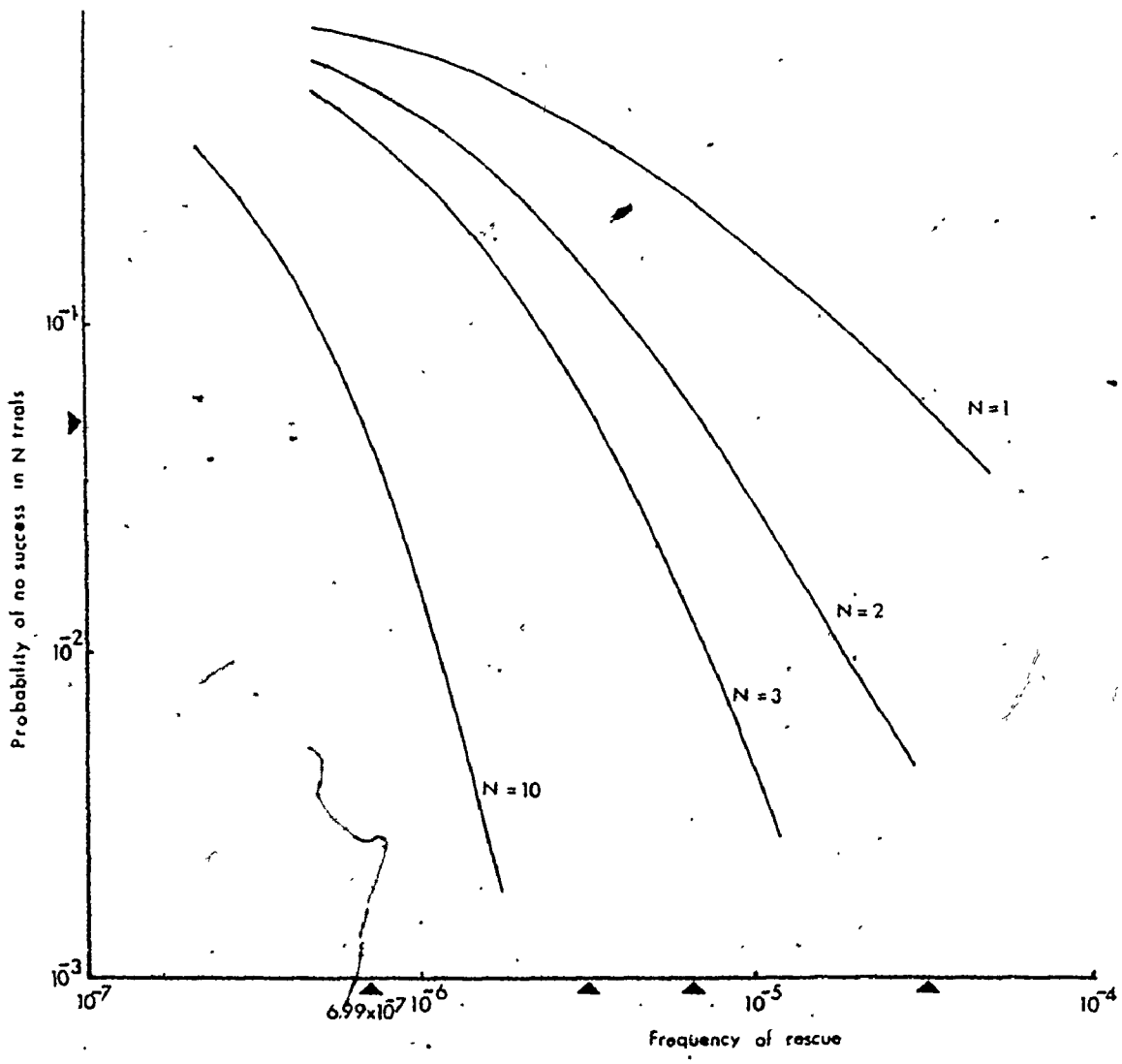
For each value assigned to f_s a binomial distribution can be used to estimate the probability of a given number of successes r in a certain number of trials n:

$$P\binom{r}{n} = (n!/(n-r)!r!)\ p^r\ (1-p)^{n-r} \ , \quad r = 0,1,\ldots,n \quad . \tag{51}$$

Theoretical curves relating $P\binom{r}{n}$ to f_s for different values of n and r=0, are shown in figure 29. If $P\binom{r}{n}$ is fixed at an acceptable probability level, i.e.: $P\binom{r}{n} = 0.05$, then for given values of n and r

Figure 29

Theoretical curves linking the probability of no success in n trials to the frequency of recombinants with rescued TK gene in viral progenies. Different values were assigned to the frequency of the rescuing event. For each of these values the probability of picking a rescue product in the TK^+ progeny was calculated as indicated in the text. These probabilities were used to determine the corresponding probabilities of no success in n trials as predicted by the binomial distribution. The abscissa value for a given probability of no success in n trials gives the corresponding value for the frequency of rescue. This value represents the upper limit of occurrence for the rescuing event.


Probability of no success in N trials
10^{-1}
10^{-2}
10^{-3}
10^{-7}
6.99×10^{-7}
10^{-6}
10^{-5}
10^{-4}
Frequency of rescue
N = 1
N = 2
N = 3
N = 10

it is possible to estimate the maximum value of f_s. If the ten viruses analyzed are considered together, then n = 10, r = 0 and, as shown in figure 29, for $P(^{0}_{10}) = 0.05$, $f_s \leq 6.99 \times 10^{-7}$. A similar result is obtained assuming that an additional trial would yield a recombinant (r = 1, n = 11). Then, the upper 95% ($\varepsilon = 0.05$) confidence limit of p is given by (Lindley, 1965):

$$p \leq 1/(1+(n-r)/r.F_{\varepsilon}) = 0.2587.$$

F_{ε} being the value of the F distribution for $\varepsilon = 0.05$ and for [2r, 2(n-r)] degrees of freedom. Then, for $f_v = 2\times10^{-6}$, $f_s \leq f_v/(1-p) = 6.98\times10^{-7}$.

3.4 Discussion

The analysis of TK activity in 10 independent strains of TK^+ virus picked from the progeny of B2006 replicated in human transformed cells indicated that all had properties of HSV-1 TK. In addition, the restriction endonuclease profiles of the viruses were those expected from revertants. These observations strongly suggest that rescue, if it occurred, had a frequency of not more than 7×10^{-7}. The transfection with the viral DNA of the rescuer strain failed to increase the yield of TK^+ virus. A similar result was obtained using a nonreverting TK^- mutant bearing a partial deletion within the TK gene (HSV-1 d2).

The frequency of viral gene retrieval detected in these studies is considerably lower than frequencies reported in other virus-cell systems. Frequencies ranging from 10^{-2} to 10^{-5} have been reported for SV40 and SV40-transformed cells (Gluzman et al., 1977b, Vogel, 1980). Frequencies of close to 10^{-3} were obtained in experiments attempting to rescue HSV-DNA sequences from human ganglia (Brown et al., 1979). In

other systems no effort seems to have been made to determine the frequency of the rescuing events, but in every case it seems clear that the genomic exchange between endogenous and exogenous viral sequences occurred at frequencies higher than the upper limit determined in these studies. Several factors may have contributed to the low frequency of gene retrieval in the systems used in this study. First, the intertypic recombination frequency seems to vary along the HSV genome (Spear and Roizman, 1980), and even if intertypic intragenic TK recombinants have been obtained, the viral TK gene may correspond to a genomic stretch where intertypic crossovers may occur with difficulty. Second, the presence of small pieces of viral endogenous DNA colinearly linked to DNA of higher molecular weight demands two closely spaced crossovers for the occurrence of rescue. Since a single crossover is enough to generate regular recombinants this may explain the difference in frequency between rescue and recombination events. Third, the selection assay used in this study is based on the induction of a functional enzyme, and some rescue products, failing to yield a functional intertypic product, may have been undetected. Fourth, the choice of rescuer TK^- virus with low reversion may have affected the chances of the rescue events. The deletion in HSV-1 d2 may have substantially reduced these chances even if the virus possesses DNA stretches homologous to both extremes of the sequences required for the expression of the TK gene. Fifth, if the endogenous viral sequences are integrated in the cellular genome, an important factor in the yield of rescue products is the possibility of direct interaction between both genomes. Since HSV blocks the cellular macromol-

ecular biosynthesis at an early state of its replication, this may explain the differences in rescue efficiencies between HSV and other viruses. Sixth, rescue experiments using ts mutants can apply a selective pressure on the growth of rescue products from the early stages of the rescuing event. In the system used here this was not possible because the salvage pathway mediated by the endogenous viral TK enzyme in the biochemically transformed cells would have bypassed the methotrexate blockage of the de novo synthesis of thymidilate. Finally, the multiplicity of infection which is known to influence the yield of regular recombinants, or the moment in the cell cycle which may modulate the expression of cellular enzymes involved in the replication of DNA, may have also influenced the low yield of recombinants.

The persistence in cells of a complete or almost complete viral genome and the feasibility of its excision and autonomous replication seem to be common denominators in all the systems showing relatively high frequency of viral gene retrieval. The permissive transformed cell lines used in experiments of rescue with SV40, contained most of the sequences of a viral genome defective for a function related to the initiation of viral DNA synthesis (Gluzman et al., 1977a). The endogenous viral genome is chromosomally associated and an excision step has been considered as a possible prerequisite for the genetic exchange (Gluzman et al., 1977b, Vogel, 1980). The normal A-gene product provided by the rescuer virus may provide the conditions for the active replication of the endogenous DNA. This may explain the low frequency of rescue detected using a tsA mutant. Retrieval of viral sequences has also been reported for mouse cells superinfected with type C viruses (Stephenson et al., 1974; Barbacid et al., 1978) and similar

findings have been published for avian RNA tumor viruses (Weiss et al., 1973). The cells involved in these studies are known to contain endogenous information similar to the viral genomes (Weiss et al., 1971; Aaronson and Stephenson, 1973; Levy, 1973) and to chronically produce low levels of endogenous virus (Aaronson and Dunn, 1974; Crittenden et al., 1974). The successful rescue of viral genes from cells carrying Epstein-Barr virus genomes has also been reported (Fresen et al., 1979). Earlier reports have indicated the synthesis of viral particles in the same cells (Gerber, 1972; Hampar et al., 1971) suggesting the presence of endogenous viral genomes able to replicate autonomously. The isolation of HSV from ganglia obtained from patients with recurrent HSV infections has been well documented (Warren et al., 1977; 1978a, 1978b). This indicates the presence in these tissues of complete HSV genomes. The rescue of HSV DNA sequences from ganglia failing to spontaneously release viruses has been claimed (Brown et al., 1979), but the possibility that the whole viral DNA was present in these cells was not discarded.

The results presented in this chapter differ from those reported by Park et al., 1980, who rescued HSV-genes from cells transformed by sheared DNA and temperature sensitive mutants. There are several possible explanations for the divergent results. First, the differences could be due to different lengths of the endogenous viral genomes in both kinds of transformed cells. Park and coworkers seem to have detected recombinational events occurring over long distances along the HSV genome, distances which are much longer than the TK gene itself;

and the human cells used in my study may carry the viral TK in shorter stretches of viral sequences. Second, the biochemically transformed human cells used here were found to be fully permissive for HSV replication, while the morphologically transformed rat cells used by Park and coworkers were shown to be only partially permissive for superinfecting HSV (McNab, 1975). The frequency of HSV gene retrieval in completely permissive cells may be less efficient than in partially permissive cells since cellular functions allowing better chances for genome exchange may be rapidly terminated in the lytic cycle. Third, the state of the viral genome in the biochemically transformed human cells and the morphologically transformed rat cells used by Parker and coworkers may be different. The state of the viral genome in the morphologically transformed rat cells has not been reported; however, most of the HSV DNA sequences are probably present in the resident viral genomes since some rescued viruses had DNA restriction endonuclease profiles indistinguishable from the original transforming viral genomes. It is conceivable that these HSV DNA sequences may have persisted in an episomic or integrated state and that they were induced to replicate upon superinfection with the mutant rescuer virus. In the human cells used in the experiments reported in this chapter the viral TK gene is carried by a subgenomic fragment of viral DNA integrated into nonviral DNA (S. Bacchetti, personal communication). If, as suggested by other systems, autonomous replication of the endogenous viral sequences is required for rescue, the discrepancy of the results reported in this chapter with those published for other similar systems, may depend upon the particular viral genomic sequences present in the cells. This

interpretation also suggests that HSV gene retrieval may not be an efficient system for detection of HSV-DNA sequences in transformed cells unless the gene to be rescued is linked to DNA sequences with sites recognized for excision and initiation of viral DNA replication.

From the results reported in this chapter it can be concluded that the high yield of HSV rescue-recombinants detected in morphological transformed rat cells by Park et al. (1980) cannot be extended to other systems. Also, rescue experiments may be a valid approach for the detection of latent HSV (Brown et al., 1979) but they do not seem to represent a sensitive way for the detection of small viral DNA present in transformed cells.

APPENDICES

Appendix 1

Mathematical Expressions*

1) Expressions (6) and (10) are directly derived from the assumptions implied by each of the situations considered in the text.

Both situations assume that the fraction of positives among the cases not related to HSV-2 will be equal to the fraction of positives in the control population (x). Thus, the incidence rate of cases not related to HSV-2 but with antibodies for the virus will be $y_u.x$.

The first situation also assumes that all the cases related to HSV-2 will be positive, and then, y_v will represent the incidence rate of positive cases related to the virus; as a consequence, the total fraction of positive cases can then be predicted to be:

$$z = \frac{y_u.x + y_v}{y} \quad . \qquad (6)$$

or replacing for the parametric symbols:

$$z = \frac{ax + bx}{a + bx} \qquad (7)$$

The second situation assumes that the fraction of positives

*The equations in the appendix are assigned the same numbers used in the text.

among the cases related to HSV-2 will also be equal to the value in the control population (x). Then, the incidence rate of cases related to HSV-2 and with antibodies for the virus will be $y_v.x$, and as a consequence the total fraction of positive cases (z) can be predicted to be:

$$z = \frac{y_u.x + y_v.x}{y} \qquad (10)$$

2) Expression (8) can be derived from (7) in the following way:

$$z = (ax + bx)/y$$

$$z = [(a + b)x]/y \qquad (7)$$

from (1):

$$x = \frac{y - a}{b}$$

Replacing above:

$$z = [(a + b)\frac{y-a}{b}]/y$$

$$z = \frac{(a + b)(y - a)}{by}$$

$$z = \frac{ay - a^2 + by - ba}{by}$$

$$z = \frac{a}{b} - \frac{a^2}{by} + 1 - \frac{a}{y}$$

$$z - 1 - \frac{a}{b} = -\frac{a^2}{by} - \frac{a}{y}$$

$$1 + \frac{a}{b} - z = \frac{a^2}{by} + \frac{a}{y}$$

$$\frac{b + a - bz}{b} = \frac{a^2y + aby}{by^2}$$

$$a + b - bz = \frac{a^2 + ab}{y}$$

$$y = \frac{a(a + b)}{a + b - bz} \tag{8}$$

3) Expression (9) is also implied by (7):

From (8):

$$\frac{1}{y} = \frac{a + b - bz}{a(a + b)}$$

$$\frac{1}{y} = \frac{1}{a + b} + \frac{b}{a(a + b)} - \frac{bz}{a(a + b)}$$

$$\frac{1}{y} = \frac{a(a + b) + b(a + b)}{a(a + b)^2} - \frac{b}{a(a + b)} \cdot z$$

$$\frac{1}{y} = \frac{a + b}{a(a + b)} - \frac{b}{a(a + b)} \cdot z$$

$$\frac{1}{y} = \frac{1}{a} - \frac{b}{a(a + b)} \cdot z \tag{9}$$

if $\alpha = \frac{1}{a}$, $\beta = \frac{b}{a(a + b)}$

$$\frac{1}{y} = \alpha - \beta . z$$

and $a = 1/\alpha$

also:

$$\beta a(a + b) = b$$

$$\beta a^2 + \beta ab = b$$

$$\beta a^2 = b - \beta ab$$

$$\beta a^2 = b(1 - \beta a)$$

and

$$b = \frac{\beta a^2}{1 - \beta a}$$

4) Expression (13) can be derived from (7) and (1):

$$z = (ax + bx)/y \qquad (7)$$

$$yz = ax + bx$$

from (1):

$$b = \frac{y - a}{x}$$

replacing above:

$$yz = ax + y - a$$

rearranging:

$$yz - y = ax - a$$

$$y(z - 1) = a(x - 1)$$

changing signs:

$$y(1 - z) = a(1 - x)$$

and

$$a = y \cdot \frac{(1 - z)}{(1 - x)} \tag{13}$$

5) From the equations presented above it is possible to derive an expression for $z - x = f(y)$:

From (1):

$$x = \frac{y}{b} - \frac{a}{b}$$

Subtracting this from 12a:

$$z - x = \frac{a}{b} - \frac{a^2}{by} + 1 - \frac{a}{y} - \left(\frac{y}{b} - \frac{a}{b}\right)$$

$$z - x = \frac{2a}{b} + 1 - \frac{a^2}{by} - \frac{a}{y} - \frac{y}{b}$$

$$z - x = \frac{2a}{b} + 1 - \left(\frac{a^2by + ab^2y + by^3}{b^2y^2}\right)$$

$$z - x = \frac{2a + b}{b} - \frac{a(a + b)}{b} \cdot \frac{1}{y} - \frac{1}{b} \cdot y \, .$$

From the first derivative of this function it can be deduced that it has an extreme value at $y = y_E$:

$$\frac{d}{dy}(z - x) = D_y[\frac{2a + b}{b}] - D_y[\frac{a^2 + ab}{by}] - D_y[\frac{y}{b}]$$

$$\frac{d}{dy}(z - x) = (-1) \cdot \left(- \frac{a^2 + ab}{b}\right) \cdot \frac{1}{y^2} - \frac{1}{b}$$

$$\frac{d}{dy}(z - x) = \frac{a^2 + ab}{b} \cdot \frac{1}{y^2} - \frac{1}{b} .$$

At y_E :

$$\frac{d}{dy}(z - x)\Big|_{y_E} = 0$$

and

$$\frac{a^2 + ab}{b} \cdot \frac{1}{y_E^2} = \frac{1}{b}$$

$$y_E^2 = a^2 + ab$$

$$y_E = (a^2 + ab)^{\frac{1}{2}} \tag{15}$$

The second derivative is:

$$\frac{d^2}{dy}(z - x) = -\frac{(a^2 + ab)}{b} \cdot \frac{2}{y^3}$$

and at y_E, replacing (15):

$$\frac{d^2(z - x)}{dy}\Big|_{y_E} = - \frac{2}{ab(a + b)}$$

which is less than zero, confirming that the extreme value at y_E is a maximum value.

6) Expression (18), relating the relative risk only to parameters a and b, can be derived from (1) and (7).

The Relative Risk (RR) of developing cervical cancer, associated with HSV-2 infection is the ratio of probabilities of developing the tumor among infected (P_{C+}) and noninfected (P_{C-}) individuals.

$$RR = P_{C+}/P_{C-} .$$

These probabilities can be estimated from x, z and y. If C is the total number of cancer patients and T the total size of the population:

$$P_{C+} = \frac{C.z}{T.x} = \frac{yz}{x}$$

$$P_{C-} = \frac{C(1-z)}{T(1-x)} = \frac{y(1-z)}{(1-x)}$$

and

$$RR = \frac{z(1 - x)}{x(1 - z)} .$$

Replacing (7) above:

$$RR = \frac{\dfrac{(a + b)x\ (1 - x)}{a + bx}}{x\left[1 - \dfrac{(a + b)x}{a + bx}\right]}$$

$$RR = \frac{(a + b)x\ (1 - x)}{x[a + bx - (ax + bx)]}$$

$$RR = \frac{ax + bx - ax^2 - bx^2}{ax - ax^2}$$

$$RR = \frac{a(x - x^2) + b(x - x^2)}{a(x - x^2)}$$

$$RR = \frac{a + b}{a} \tag{18}$$

Appendix 2

Procedure Followed to Estimate $P_s(t)$ from Available Data for Age-specific Prevalence of HSV-2 Antibodies

Assuming that the number of infected individuals in the age group yielding the largest fraction of women with antibodies actually represents S_j, the values of the total of individuals analyzed for each age group were corrected to estimate the total number of susceptible individuals in each age-group sample.

This was done in the following way: If for a given population j different age groups i have been studied and S^+_{ij} are the number of positive individuals reported for each age group i and T_{ij} is the total number of individuals tested for each age group, then the fraction:

$$F_{ij} = S^+_{ij}/T_{ij} \qquad \text{(A3-1)}$$

can be considered as an estimate of $P_j(t)$. These values cannot be pooled together unless estimates are derived for $P_s(t)$ which, as discussed in the text, can be assumed to be independent from the population considered.

Now, the maximum value for F_{ij}, $F_{ij_{max}}$, can be assumed to correspond to:

$$F_{ij_{max}} \approx \frac{S_{ij}}{T_{ij}} \doteq x_j \qquad \text{(A3-2)}$$

and

$$S_{ij} = T_j \cdot F_{ij_{max}} \quad . \qquad \text{(A3-3)}$$

This last expression was used to compute the S_{ij} values for each age group in each population included in the study. Then the S^+_{ij} and S_{ij} values were assumed to distribute homogenously within each given age group and were reassorted in seven 10-year intervals.

The assumption, discussed in the text, that S^+_{ij}/S_{ij} can represent estimates of $P_s(t)$ independent from the population studied allowed the data from different populations to be pooled together before being used as the input to a computer program performing a weighted probit analysis.

Appendix 3

Age Specific Data for Cancer Incidence (y) and prevalence of HSV-2 Antibodies in Normal Women (x).

These computer printouts show the entire collection of age-specific data used in the study.

The points included in this analysis correspond to the same populations indicated in Table 1 except that two points, Uganda and Johannesburg, were omitted in this part of the study due to the lack of reliable data for age-specific cancer incidence rates.

AREAS FOR (%).

		AGE 17.5	AGE 22.5
1	CHICAGO	.0540749466 0.3	.0821349463 2.2
2	ATLANTA	.1051457295 4.9	.1596873955 15.4
3	HOUSTON (W)	.0690957651 1.7	.1049374313 1.8
4	HOUSTON (B)	.1562165124 4.9	.2372498447 15.4
5	CHARLESTON	.0510707829 0.1	.0775624492 1.6
6	MONTREAL	.0630874377 0.2	.0958124373 0.9
7	TORONTO	.1051457295 0.	.1596873955 10.6
8	COPENHAGEN	.1411956939 0.	.2144373597 2.2
9	PRAGUE	.0630874377 0.	.0958124373 1.4
10	PECS	.0270374733 0.	.0410624731 0.
11	TURKU	.0540749466 0.1	.0821349463 0.4
12	YUGOSLAVIA	.0720999286 0.3	.1094999283 0.9
13	NORWAY	.0841165836 0.3	.1277499164 2.
14	ISRAEL	.0210291459 0.	.0319374791 0.2
15	OSAKA	.0450624555 0.	.0684374552 0.
16	NEW ZELAND	.0690957651 0.	.1049374313 0.7
17	TAIWAN	.0690957651 0.	.1049374313 0.8
18	TEGUCIGALPA	.1381915303 0.1	.2098748626 1.1
19	IBADAN	.0330458007 0.	.0501874672 0.
20	INDIA	.1081498932 0.1	.1642498925 1.2
21	MANITOBA	0.030041637 0.	.0456249701 10.6
22	SWEDEN	.0811124199 0.3	.1231874194 2.1
23	WEST INDIES	.2313206049 0.	.3513122701 3.
24	CALI	.2042831316	0.310249797

		AGE 27.5	AGE 32.5	AGE 37.5
1	X	.1056670599	.1241090448	.1380874903
	Y	6.1	14.	14.3
2	X	.2054637276	.2413231426	.2685034533
	Y	19.2	35.8	56.2
3	X	0.135019021	.1585837794	.1764451264
	Y	6.5	11.9	17.9
4	X	.3052603953	.3585372404	.3989194163
	Y	19.2	35.8	56.2
5	X	.0997966677	.1172140978	0.130415963
	Y	7.5	13.	17.
6	X	.1232782366	.1447938856	0.161102072
	Y	4.7	15.9	20.9
7	X	.2054637276	.2413231426	.2685034533
	Y	26.7	29.7	33.9
8	X	.2759084342	.3240625056	.3605617801
	Y	11.1	39.9	69.8
9	X	.1232782366	.1447938856	0.161102072
	Y	9.6	17.	31.8
10	X	0.05283353	.0620545224	.0690437451
	Y	2.2	11.8	30.3
11	X	.1056670599	.1241090448	.1380874902
	Y	2.3	7.7	19.3
12	X	.1408894132	.1654787262	.1841166537
	Y	9.4	14.3	25.9
13	X	.1643709821	.1930585141	.2148027526
	Y	8.2	21.3	38.7
14	X	.0410927455	.0482646285	.0537006907
	Y	0.5	2.3	4.8
15	X	.0880559833	0.103424204	.1150729085
	Y	0.2	2.9	14.3
16	X	0.135019021	.1585837794	.1764451264
	Y	3.	9.	13.
17	X	0.135019021	.1585837794	.1764451264
	Y	3.6	7.9	16.6
18	X	0.270038042	.3171675588	.3528902529
	Y	5.7	12.7	28.3
19	X	.0645743144	.0758444162	.0843867996
	Y	0.5	3.2	16.5
20	X	.2113341198	.2482180895	.2761749805
	Y	4.2	13.7	29.7
21	X	.0587039222	.0689494693	.0767152724
	Y	26.7	29.7	33.9
22	X	.1585005899	.1861635671	.2071312354
	Y	6.9	22.6	38.1
23	X	.4520202007	.5309109137	.5907075972
	Y	10.7	32.2	52.7
	X		[illegible]	0.52

		AGE 42.5	AGE 47.5	AGE 52.5
1	X	.1485234403	.1562704907	.1620190467
	Y	15.2	20.7	27.2
2	X	.2887955784	.3038592876	.3150370353
	Y	45.2	34.5	68.2
3	X	.1897799515	.1996789604	.2070243375
	Y	31.6	22.6	37.3
4	X	.4290677165	.4514480844	.4680550238
	Y	45.2	34.5	68.2
5	X	.1402721381	.1475887968	.1530179886
	Y	18.6	24.5	25.6
6	X	0.173277347	.1823155725	.1890222212
	Y	25.5	33.8	38.2
7	X	.2887955784	.3038592876	.3150370353
	Y	40.2	38.7	37.5
8	X	.3878112053	.4080396147	.4230497331
	Y	90.9	94.8	81.7
9	X	0.173277347	.1823155725	.1890222212
	Y	47.7	53.1	62.3
10	X	.0742617202	.0781352454	.0810095234
	Y	46.7	49.5	56.2
11	X	.1485234403	.1562704907	.1620190467
	Y	28.6	43.9	45.3
12	X	.1980312538	.2083606542	.2160253956
	Y	36.8	46.9	52.9
13	X	.2310364627	0.24308743	.2520296282
	Y	48.	57.1	45.4
14	X	.0577591157	.0607718575	.0630074071
	Y	9.6	14.	17.7
15	X	.1237695336	0.130225409	.1350158723
	Y	30.	42.5	47.
16	X	.1897799515	.1996789604	.2070243375
	Y	19.3	21.7	30.1
17	X	.1897799515	.1996789604	.2070243375
	Y	28.5	51.	50.6
18	X	0.379559903	.3993579208	.4140486749
	Y	49.	61.4	63.6
19	X	.0907643246	.0954986332	.0990116397
	Y	23.8	47.4	79.8
20	X	.3970468806	.3125409815	.3240380934
	Y	50.8	66.4	72.7
21	X	.0825130224	.0868169393	.0900105815
	Y	40.2	38.7	37.5
22	X	.2227851605	.2344057361	.2430385701
	Y	51.6	48.3	45.1
23	X	.6353502725		
	Y	81.9		

		AGE 57.5	AGE 62.5	AGE 67.5
1	X	.1662957029	.1694910801	.1718912045
	Y	24.3	27.2	29.1
2	X	.3233527556	0.329565989	.3342328981
	Y	63.1	92.5	78.2
3	X	.2124889537	.2165719356	.2196387616
	Y	26.4	26.9	36.1
4	X	.4804098083	.4896408979	.4985745915
	Y	63.1	92.5	78.2
5	X	.1570570527	.1600749089	.1623416934
	Y	28.	26.6	30.1
6	X	.1940116534	.1977395934	.2005397389
	Y	42.1	44.7	40.
7	X	.3233527556	0.329565989	.3342328981
	Y	34.3	38.7	30.2
8	X	.4342165575	.4425600424	.4488270246
	Y	72.5	62.3	53.6
9	X	.1940116534	.1977395934	.2005397389
	Y	52.7	59.6	62.2
10	X	.0831478514	0.08474554	.0859456024
	Y	27.8	38.2	34.4
11	X	.1662957029	.1694910801	.1718912048
	Y	36.2	38.2	29.8
12	X	.2217276038	.2259881067	0.2291863273
	Y	58.3	53.4	49.4
13	X	.2586822045	.2636537912	.2673863185
	Y	42.8	34.6	32.7
14	X	.0646705511	.0659131978	.0668465796
	Y	9.9	12.6	10.8
15	X	.1385797524	.1412425667	.1432426706
	Y	53.	65.6	60.3
16	X	.2124889537	.2165719356	.2196387616
	Y	26.7	26.9	33.9
17	X	.2124869537	.2165719356	.2196387616
	Y	72.6	59.1	73.
18	X	.4249779074	.4331438712	.4392775233
	Y	66.4	90.	95.
19	X	.1016251518	.1035778823	.1050446251
	Y	89.9	98.1	79.9
20	X	.3325914058	.3389821601	.3437824095
	Y	53.9	80.7	57.9
21	X	.0923865016	.0941617111	.0954951138
	Y	34.3	38.7	30.2
22	X	.2494435543	.2542366201	.2578368071
	Y	42.7	33.8	29.3
23	X	.7113760623	.7250451758	.7352123759
	Y	[illegible]	[illegible]	[illegible]

	AGE 72.5	AGE 77.5
1	.1737044764	.1750826712
	26.	23.9
2	.3377587042	.3404385273
	64.4	73.3
3	.2219557199	.2237167465
	37.4	31.1
4	.5018129319	.5057943825
	64.4	73.3
5	.1640542277	.1653559561
	30.9	33.6
6	.2026552225	.2042631164
	48.1	34.8
7	.3377587042	.3404385273
	29.3	39.5
8	.4595616884	.4571603081
	51.3	36.9
9	.2026552225	.2042631164
	60.4	47.5
10	.0868522382	.0875413256
	37.	36.5
11	.1737044764	.1750826712
	35.6	32.6
12	.2316059686	.2334435616
	37.3	31.6
13	.2702069633	.2723508219
	30.7	24.3
14	.0675517408	.0680877055
	13.8	10.1
15	.1447537304	0.1459032326
	59.2	47.7
16	.2219557199	.2237167465
	35.5	33.8
17	.2219557199	.2237167465
	45.	69.6
18	.4439114397	.4474334931
	87.3	75.8
19	.1061527356	.1069949657
	44.8	51.3
20	.3474089523	.3501653424
	50.	44.4
21	.0965024869	.0972681507
	29.3	39.5
22	.2605967146	.2626240068
	32.	23.3
23	.7430691491	.7489647602
	121.6	139.8
24	.6562169109	.6614234246
	171.	184.2

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