DNA MEDIATED GENETIC TRANSFORMATION OF A HUMAN CANCEROUS CELL LINE CULTURED *IN VITRO*

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DNA MEDIATED genetic transformation is fairly established in many microbial systems including *Pneumococcus* (McCarty, Taylor and Avery, 1946), *B. subtilis* (Spizizen, 1958), *E. coli.* (Boivin, Vendrely and Lehoult, 1945), *Haemophilus* (Alexander and Leidy, 1951), etc. But in mammalian systems the problem has not been adequately explored. Results on transformation studies in intact animals are generally negative (Bearn, 1959; Kok, 1959; Shoffner *et al.*, 1961; Svoboda and Haskova, 1959; Tigyi, Benedeczky and Lissak, 1959). With ducks however some positive evidence has been recorded (Benoit *et al.*, 1957; Novikov, Chepinoga and Lyubarskoya, 1961). Genetic transformation has also been claimed in case of mammalian cells cultured both *in vitro* (Bradley *et al.*, 1962; Kraus, 1961; Szybalska and Szybalski, 1962) and *in vivo* (Podgejetskaja, 1964), although there are some cases of ambiguity in the interpretation of results on transformation (Bradley, Roosa and Law, 1962). This ambiguity or failure has so far been variously accounted for by the presence of insufficient number of recipient cells (Florsheim, 1962), high frequency of spontaneous mutation (Mathis and Fisher, 1962), the non-specific protective action of DNA (Bradley *et al.*, 1962) and lack of penetration of exogenous DNA (Yoon, 1964; Yoon and Sabo, 1964). That there are even cases in which the reported results could not be confirmed further complicate interpretation of data on mammalian transformation (Szybalska and Szybalski, 1962). In the present work a study has been made on DNA mediated genetic transformation of a clonal cell line of human uvular carcinoma isolated in this laboratory with reference to 6-azathymine (purchased from M/s. Sigma Chemical Company, U.S.A.) resistance marker.

MATERIALS AND METHODS

Cell line

A. Parental cell line is a clone obtained from a primary culture of human uvular carcinoma and subsequently maintained in liquid suspension culture.

B. Resistant cell line is an isolate in a single step at 40·0 mM/ml. level of 6-azathymine. In developing resistance, the sensitive cell line was cultivated in solid media (Majumdar and Bose, 1964). A single clonal isolate was then maintained in drug free liquid suspension culture.

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Media

For liquid suspension culture the medium used was standard Eagle's basal medium supplemented with 150 ml. of normal horse serum, 10 units of penicillin G, and 0·5 g. of streptomycin base per litre.

For solid culture Eagle's basal medium containing 1 % washed agar was used.

Isolation of DNA

For isolation the modified phenol deproteinisation method of Kirby (Saito and Miura, 1963) was adopted. Resistant cell population grown in 1 litre medium for 72 hours at 37° C. in shake culture was harvested by centrifugation at 1000–1200 r.p.m. (0° C.) and washed once with saline-EDTA and resuspended in 6 ml. of saline-EDTA to which 50 ml. of Tris-sodium dodecyl sulphate solution was added. It was then frozen and thawed 4 times. Deproteinisation was done by equal volume of water saturated phenol (pH 9), and finally DNA precipitated by double volume of 95 % ethanol at 4° C. and dissolved in saline citrate solution (NaCl 0·15 M + Na_3-citrate 0·015 M). It was then dialysed overnight at 4° C. against the same saline citrate solution and stored at the same temperature. DNA was estimated by diphenyl amine method of Burton (1956).

Labelling of DNA

For labelling, cells were grown in bulk medium to which [$^{32}P_i$] KH_2PO_4 was added. And subsequently labelled DNA was isolated following the method described above.

Technique of transformation

In a typical transformation experiment 16×10^6 competent cells (42–44 hours of age), were washed once with balance salt solution (8·0 g. NaCl, 0·4 g. KCl, 0·35 g. $NaHCO_3$, 1·0 g. glucose per litre of water) and resuspended in phosphate buffered saline solution (7·0 g. NaCl, 0·4 g. KCl, 2·75 g. Na_2HPO_4, 0·25 g. NaH_2PO_4, 1·0 g. glucose per litre of water) containing 100 μg. spermine-HCl and 30 μg. donor DNA. The volume of the reaction mixture was adjusted to 2 ml. It was then incubated for 15 minutes at 37° C. Thereafter it was diluted hundred fold by non-selective basal medium and immediately plated in selective medium for 0 hour mutation. For phenotypic delay a definite aliquot of the diluted suspension was centrifuged, resuspended in the same volume of the non-selective medium overnight and plated as before.

RESULTS

Competency of the recipient population

The competency of the recipient population was measured in terms of labelled DNA uptake (Fig. 1). In all, 3 parallel sets of experiments were performed using different DNA preparations having different specific activities. It appears that the uptake curves in case of hydrolysed DNA varies from preparation to preparation. With one preparation there is a continuous rise (Fig. 1, curve I B), with a second more than one peak (Fig. 1, curve II B) and with a third a single peak in the curve (Fig. 1, curve III B). In the uptake of native DNA all the 3 different preparations behave basically in the same way, in the sense that there is a single

peak in the uptake, although a plateau (obviously due to insufficient data) has been observed in one case only (Fig. 1, curve I A). These peaks are again very sharp being at the forty second hour of cell growth. It may be taken to mean that the cells are competent only at the specific hour of growth and that competency persists only for a very short time in the generation cycle.

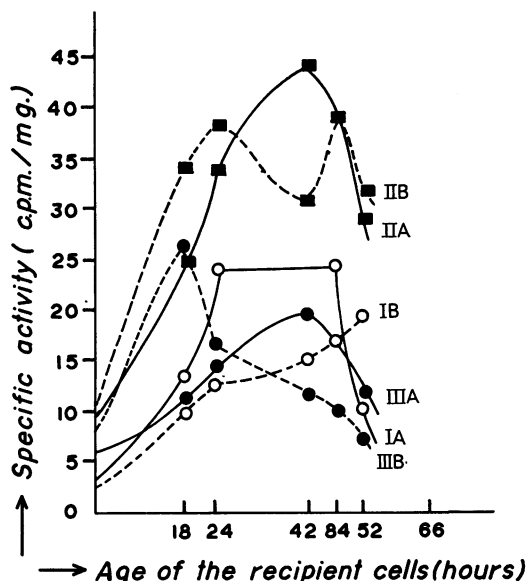

FIG. 1.—Competency of the sensitive recipient cells. Recipient cells (16×10^6) harvested at different points of their growth phase (0–52 hours), were exposed to 15 μg./ml. of native and hydrolysed (by 1·5 μg./ml. of DNase in presence of Mg^{++}ions at 37° C. for 2 hours) ^{32}P—DNA, at 37° C. for 15 minutes at pH 7·2. Cells after exposure were immediately centrifuged and washed repeatedly (usually for 4 times) with phosphate-buffered saline solution and then the pellet was plated in aluminium planchets and radioactive count taken in windowless gas flow counter (Tracer Lab. Inc., Sc. 16).

○————○, ○------○, represent uptake curves of intact and hydrolysed DNA (specific activity 565·3), by sensitive recipient cells respectively. ■————■, ■------■ and ●————●, ●------●, represent the same results of two separate experiments with specific activity of donor ^{32}P—DNA being 825·7 and 300·0 respectively.

Transformation reaction

Transformation reaction in the given system was performed with respect to 6-azathymine resistance marker, the donor DNA being obtained from the resistant cell line isolated at a concentration of 40·0 mM/ml. of the drug. Ten independent experiments were carried out using six different DNA preparations. The results are given in the Table I, and their statistical analysis in the Table II A and II B. It appears that DNA has no growth accelerating effect on the given cell line both at zero hour plating (Table II A, Column A_1 and B_1), and at 24 hours plating (Table II A, Column A_2 and B_2). The significantly higher colony count scored in selective medium in DNA treated system (Table I, Column A_3, A_4) over the control (Table I, Column B_3, B_4) may be taken as a positive evidence in favour of transformation (Table II A, Column A_3 and B_3, A_4 and B_4). The effect of

TABLE I.—*DNA- Mediated Genetic Transformation*

No. of Experiments	System	Cells plated in absence of the drug (inoculum: 5×10^2 cells/plate) 0 hour plating	Cells plated in absence of the drug 24 hours plating	Cells plated in presence of 40·0 mM/ml. of 6-azathymine (inoculum: 1×10^3 cells/plate) 0 hour plating	Cells plated in presence of 40·0 mM/ml. of 6-azathymine 24 hours plating
	Native DNA	A_1	A_2	A_3	A_4
(i)	. .	215	236	112	148
(ii)	. .	269	200	32	90
(iii)	. .	291	168	130	110
(iv)	. .	163	131	82	30
(v)	. .	324	195	67	20
(vi)	. .	223	237	19	90
(vii)	. .	236	240	41	30
(viii)	. .	246	236	17	19
(ix)	. .	200	281	45	10
(x)	. .	314	149	80	24
(xi)	. .	275	200	57	32
	Control	B_1	B_2	B_3	B_4
(i)	. .	172	292	21	75
(ii)	. .	184	235	16	108
(iii)	. .	240	113	92	25
(iv)	. .	245	132	80	27
(v)	. .	351	150	24	48
(vi)	. .	239	237	19	0
(vii)	. .	226	238	30	4
(viii)	. .	152	202	0	0
(ix)	. .	198	238	0	0
(x)	. .	267	133	3	18
(xi)	. .	260	100	3	19

16×10^6 recipient cells were exposed at the forty-second hour of growth to 15 μg./ml. of resistant donor DNA for 15 minutes at 37° C. pH 7·2, diluted hundredfold and plated in absence of the drug and also in presence of 40·0 mM/ml. of 6-azathymine both at zero hour and at 24 hours. (Preincubation was done in non-selective medium.)

Results are given in terms of colony counts/plate.

TABLE II A.—*Statistical Analysis of Table* I

Column	No. of experiments	t value	Conclusion
Comparison between figures with and without DNA			
A_1 and B_1	11	0·731	insignificant*
A_2 and B_2	11	0·641	insignificant*
A_3 and B_3	11	4·31	significant at 1% level
A_4 and B_4	11	3·180	significant at 1% level
Comparison between figures at 0 hour and 24 hours plating			
B_3 and B_4	11	0·578	insignificant*
A_3 and A_4	11	0·757	insignificant*

* Insignificant at 5% level.

TABLE II B.—*Statistical Analysis of Table* I

Comparison between figures with and without drug	t value	Conclusion
$\frac{A3}{A1} \longleftrightarrow \frac{B3}{B1}$	12·7429	Significant at 1% level
$\frac{A4}{A2} \longleftrightarrow \frac{B4}{B2}$	8·9180	Significant at 1% level

phenotypic delay in transformation was also studied. In another set of parallel experiments the cells after DNA exposure were preincubated for 24 hours in non-selective medium and plated as before. The statistical analysis of the columns B_3 and B_4 in Table I indicates the absence of any increase in the value of spontaneous mutation (Table II A, B_3 and B_4). So the columns A_3 and A_4 of the Table I were directly analysed which shows that there is no phenotypic delay in the system (Table II A, A_3 and A_4).

It seems desirable on account of the large spread in plating efficiency of drug sensitive and resistant cells to compare statistically the percentage of cells plated in absence and in presence of the drug with and without DNA. The independent tests so carried out (Table II B) indicate the data to be significant at 1% level which supports the claim already made in favour of DNA mediated genetic transformation (Table II A).

Effect of deoxyribonuclease and ribonuclease on the transforming activity of the preparation

The effect of deoxyribonuclease and ribonuclease on the transforming activity of the preparation was studied (Table III) and the data statistically analysed (Table IV). It appears that native DNA, deoxyribonuclease treated or ribonuclease treated DNA has no growth accelerating effect on the cell line both at zero hour plating (Table IV, Columns A and B, C and B, D and B) and at 24 hours (Table IV, Columns A_1 and B_1, C_1 and B_1, D_1 and B_1). The colony counts recorded in column A_2 and C_2 are significantly higher thant those in B_2 in Table IV, which again supports transformation. No significant difference in the colony counts has been observed between A_2 and D_2 and B_2 and C_2, which indicates that the transforming activity of the preparation is ribonuclease insensitive but deoxyribonuclease sensitive.

On statistical analysis of the data of the columns B_2 and B_3 in Table IV, no significant difference in the count has been observed, which shows that there is no increase in the number of spontaneous mutants between zero hour and 24 hours plating. Consequently the data of the columns (A_2 and A_3, C_2 and C_3, D_2 and D_3) were directly compared statistically. Absence of any significant difference may be taken to mean that there is no phenotypic delay in genetic expression, as observed before.

Effect of isologous and also unrelated DNA on the transforming activity of the preparation

In this experiment standard L-strain, and sensitive parent strain were used as the source of heterologous and isologous DNA respectively. The effect was

studied in 2 different concentrations (Table V). Isologous DNA does not affect, or does so very slightly, the colony count in non-selective medium. But in selective medium it adversely affects the count. Heterologous DNA on the other hand definitely decreases the colony count both in the non-selective and also in the selective medium. This may be taken to mean that the DNA preparations whether isologous or heterologous do not at any rate antagonise the action of the drug.

TABLE III.—*Effect of Deoxyribonuclease and Ribonuclease on Transforming Activity of the Preparation*

System	Cells plated in absence of the drug (Inoculumn: 5 × 10^2 cells/plate)				Cells plated in presence of 40·0 mM/ml. of 6-azathymne (Inoculum: 1 × 10^3 cells/plate)			
		0 hr plating		24 hr plating		0 hr plating		24 hr plating
		215		236		112		148
		269		200		32		90
		291		168		130		110
Native DNA	A	163	A_1	131	A_2	82	A_3	30
		314		149		80		24
		275		200		57		32
		329		195		67		30
		172		292		21		75
		184		235		16		108
		240		113		62		25
Control	B	245	B_1	132	B_2	40	B_3	27
		267		133		3		18
		260		100		3		19
		351		150		24		27
		200		184		6		19
		236		187		4		5
		245		100		93		45
Deoxyribonuclease treated DNA*	C	241	C_1	103	C_2	62	C_3	60
		274		271		10		9
		220		285		12		14
		317		145		30		4
		200		276		58		100
		163		200		60		90
		225		100		102		44
Ribonuclease treated DNA†	D	231	D_1	82	D_2	50	D_3	80
		271		100		65		30
		285		110		20		25
		268		130		70		10

* 15 μg./ml. of donor DNA was treated with 1·5 μg./ml. of deoxyribonuclease (pancreas, B. grade, purchased from Calbiochem, U.S.A.) and Mg^{++}ion for 2 hours at 37° C.

† 15 μg./ml. of donor DNA was treated with 50 μg./ml. of ribonuclease (5 × crystallised, bovine pancreas, purchased from Sigma Chemical Company, U.S.A.) for 2 hours at 37° C.

16 × 10^6 recipient cells were exposed at the forty-second hour of growth to native as well as enzyme treated DNA as detailed above for 15 minutes at 37° C., then plated in absence of the drug and also in presence of 40·0 mM/ml. of 6-azathymine, both at 0 hour and after 24 hours. (Pre-incubation was done in non-selective medium.)

Results are given in terms of colony counts/plate.

TABLE IV.—*Statistical Analysis of Table III*

Column	No. of observations	t value	Conclusion
(1) B and A	7	0·344	Insignificant*
(2) B_1 and A_1	7	0·411	,,
(3) B and C	7	0·036	,,
(4) B_1 and C_1	7	0·387	,,
(5) B and D	7	0·648	,,
(6) B_1 and D_1	7	0·653	,,
(7) B_2 and B_3	7	1·15	,,
(8) A_2 and A_3	7	1·11	,,
(9) C_2 and C_3	7	0·955	,,
(10) D_2 and D_3	7	0·408	,,
(11) B_2 and C_2	7	0·726	,,
(12) B_3 and C_3	7	2·11	Significant at 5% level
(13) B_2 and D_2	7	3·34	Significant at 1% level
(14) B_3 and D_3	7	0·926	Insignificant*
(15) C_2 and D_2	7	2·56	Significant at 5% level
(16) C_3 and D_3	7	3·01	Significant at 1% level
(17) A_2 and D_2	7	1·17	Insignificant*
(18) A_3 and D_3	7	0·618	,,
(19) A_2 and C_2	7	3·39	Significant at 1% level
(20) A_3 and C_3	7	3·21	Significant at 1% level
(21) A_2 and B_2	7	4·01	Significant at 1% level
(22) A_3 and B_3	7	1·48	Insignificant*

* Insignificant at 5% level.

TABLE V.—*Effect of Isologous and Heterologous DNA on Transformation*

System	Non-selective medium*		Selective medium†	
	Colony	Plating efficiency (%)	Colony	Yield of trans-formants (%)
0 µgm./ml. resistant DNA	186	41	54	6·0
	230		68	
15 µg./ml resistant DNA	238	40	82	8·2
	168		83	
	200	32	66	
15 µg./ml. sensitive DNA	128		42	5·5
15 µg./ml. resistant DNA +	127	37	65	
15 µg./ml. sensitive DNA	150		45	5·4
50 µg./ml. sensitive DNA	236	38	50	5·6
	193		63	
15 µg./ml. resistant DNA +	150	36	18	2·3
50 µg./ml. sensitive DNA	156		28	
15 µg./ml. L-strain DNA	184	28	16	1·5
	114		20	
15 µg./ml. resistant DNA +	117	28	20	2·0
15 µg./ml. L-strain DNA	120		29	
50 µg./ml. L-strain DNA	150	30	38	2·4
	200		10	
15 µg./ml. resistant DNA +	159	31	35	3·6
50 µg./ml. L-strain DNA	176		38	

* No 6-azathymine and 5×10^2 cells/plate.
† 40·0 mM/ml. of 6-azathymine and 1×10^3 cells/plate.
Recipient cells exposed to varying DNA concentrations as detailed above at 37° C. for 15 minutes at pH 7·2.

Standardisation of conditions for DNA mediated genetic transformation.

The conditions so far worked out to secure maximum transformation in the present system relate to pH, temperature, DNA concentration, and duration of DNA exposure. The results are graphically represented in Fig. 2, 3, 4 and 5. Transformation occurs best at pH 7·2 (Fig. 2) and at 37° C. (Fig. 3). Under these

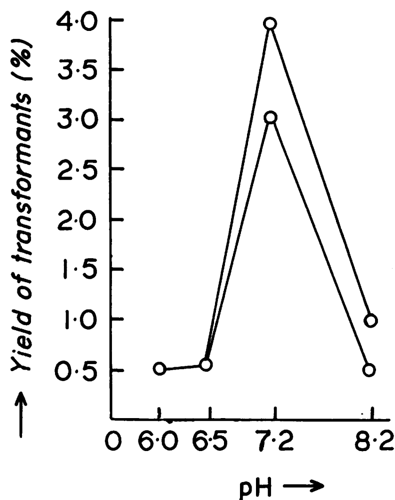

FIG. 2.—Effect of pH on transformation. Recipient cells (16 × 10^6), at the forty-second hour of growth phase were exposed to 15 μg./ml. of donor DNA at 37° C. for 15 minutes at different pH as given above and then plated in presence and also in absence of 40·0 mM/ml. of 6-azathymine.

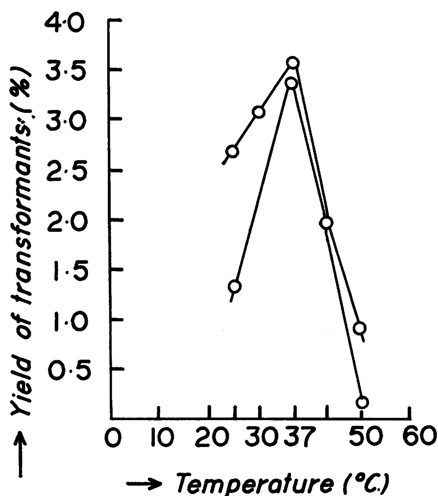

FIG. 3.—Effect of temperature on transformation. Recipient cells (16 × 10^6) at the forty-second hour of growth phase were exposed to 15 μg./ml. of donor DNA for 15 minutes, pH 7·2 at different temperatures as given above and then plated in presence and also in absence of 40·0 mM/ml. of 6-azathymine.

conditions the saturating DNA concentration appears to be 15 μg./ml. (Fig. 4) and the minimum time of exposure for maximum transformation at the saturating concentration is about 15 minutes (Fig. 5).

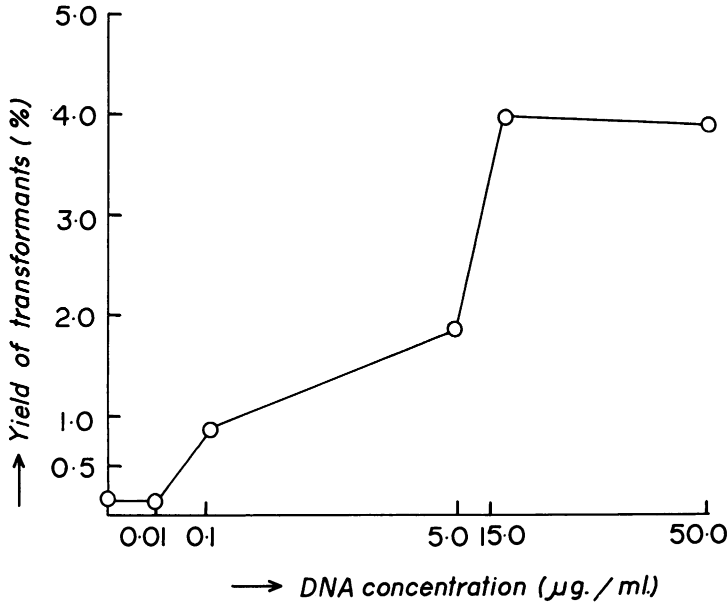

FIG. 4.—Effect of varying concentration of DNA on the yield of transformants. Recipient cells (16×10^6) at the forty-second hour of growth phase were exposed to different concentrations of donor DNA as detailed above for 15 minutes at 37° C., pH 7·2 and then plated in presence and also in absence of 40·0 mM/ml. of 6-azathymine.

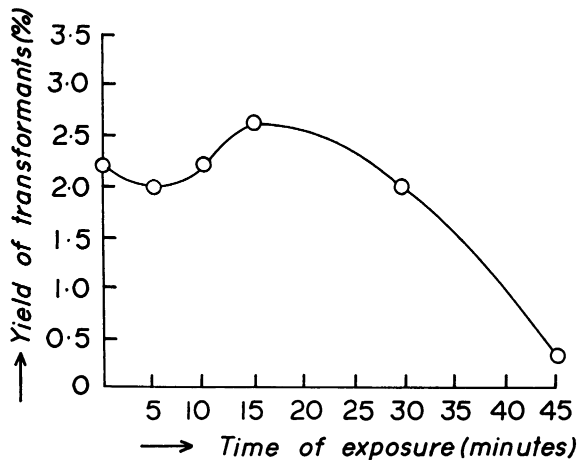

FIG. 5.—Effect of different duration of DNA exposure on the yield of transformants. Recipient cells (16×10^6) at the forty-second hour of growth phase were exposed to 15 μg./ml. of DNA at 37° C. pH 7·2 for different lengths of time and then plated in absence and in presence of 40·0 mM/ml. of 6-azathymine.

DISCUSSION

The present work aims at genetic transformation in mammalian cells. As the recipient, human uvular carcinoma cell was used and the marker selected was 6-azathymine resistance, the resistant strain being an isolate in a single step at a level of 40·0 mM/ml. of the drug. For transformation, competency was first determined and found to be at a growth period of 24–44 hours. The competent cells were then exposed to the action of resistant DNA and plated in selective and non-selective medium at zero hour and at 24 hours. In this system the rate of spontaneous mutation to 40·0 mM/ml. drug resistance is very high which tends to mask the result of genetic transformation. It has, however, not been possible for the elimination of spontaneous mutation to use donor cells of higher drug resistance because of their genetic instability. Hence the data was subjected to statistical analysis. It appears that DNA preparations have got no nongenetical effect like growth acceleration on the given cell line (Table I). Isologous sensitive DNA which is very similar in structure to resistant DNA has also got no drug antagonising action (Table V). In view of these considerations the significantly higher colony count in the native DNA treated system over the control may be considered as an evidence in favour of genetic transformation (Table I). The transforming activity of the preparation is sensitive to deoxyribonuclease and insensitive to ribonuclease (Table III) which shows that it is a case of DNA mediated genetic transformation.

In these transformation experiments no phenotypic delay has been observed. This may be taken to interpret that the duplication time in mammalian cells is sufficiently long to allow for genetic expression.

The condition for transformation reaction has been standardized. The pH and temperature dependence of transformation may be an indication that it might be an energy linked enzymic process as observed in microbial systems (Stuy and Stern, 1964).

Both isologous and heterologous DNA inhibits transformation. The former may inhibit by competition at the site of synapsis and the latter by non-specific association or in some unknown way.

Transformation in diploid cells raises an interesting query. Simultaneous replacement of the allelic region by incoming chromosome pair is rather unlikely (Podgejetskaja *et al.*, 1964). Partial replacement can, however, account for the development of resistance provided the mutant character is a dominant one.

SUMMARY

1. An extracellular preparation of DNA brings about genetic transformation in a strain of sensitive human uvular carcinoma, with reference to 6-azathymine resistance marker.

2. No phenotypic delay has been observed in this system.

3. Deoxyribonuclease inactivates the biological activity of the preparation whereas ribonuclease has got no action on it.

4. Isologous DNA preparation inhibits transformation but not viability, whereas heterologous DNA preparation inhibits both.

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malignant tissue material saccording to the specific requirements after the operations.

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