ON THE INACTIVATION OF TRANSFORMING DEOXYRIBONUCLEIC ACID BY HEAT

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Received February 19, 1962

Heating in aqueous solutions degrades DNA in various ways. Marmur, Doty and co-workers (1960a,b) showed that H-bonds break at or above the so-called melting temperature, this resulting in partial or complete separation of the strands. This melting out of DNA causes the nearly complete destruction of its transforming activity. Eigner and co-workers (1961) measured the hydrolysis rate of single-stranded DNA phosphate-diester bonds at several temperatures. They calculated a "half-life" of three hours for the DNA at 75 °C but they could not demonstrate any hydrolysis over a period of 20 hours at 57 °C in double-stranded DNA. We know from studies of degradation of transforming DNA by DNase that single-stranded breaks can cause the disappearance of its activity (Zamenhof and co-workers, 1953). Zamenhof and Greer (1958) reported that the heating of whole cells of Escherichia coli gave rise to large amounts of mutants among the survivors. They believed that the underlying mechanism was the splitting off of purines from the bacterial DNA (Greer and Zamenhof, 1959).

These reports led us to study whether the biologically observable destruction of three linked transforming markers by heat at relatively low temperatures was indicative of any of the above degradation mech-

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anisms. We especially wanted to find out whether sub-melting temperatures had any mutagenic effect *in vitro* on transforming DNA.

EXPERIMENTAL - Strains, media and transformation techniques have been fully described (Stuy, 1961a,b). The wild type sensitive strain Rd of *Haemophilus influenzae*, obtained from Grace Leidy (New York), served as the recipient test strain in most cases. Antibiotic-resistant (mutant) strains carrying the corresponding genetic markers are here designated Rd/SM, Rd/Ka_1 and so on, while their DNA extracts are termed DNA(SM), DNA(Ka_1) etc. DNA was heated in a thermostatted waterbath at 0.05 μg/ml in saline-citrate (0.15 M, 0.015 M, respectively; pH 7.0). Samples were quickly chilled after heating and stored at 4 °C. 0.1-ml amounts were mixed with 1.9 ml of transformable cells and gently shaken during 30 minutes at room temperature. The mixture contained about 10^6 marker equivalents per ml, so ensuring a linear transformation response to the numbers of active markers present.

RESULTS - (1) Inactivation. The streptomycin (SM), kanamycin (Ka_1) and cathomycin (Ca) resistance markers in the DNA studied were located on one particle (Goodgal, pers. comm.) (Fig. 1). For a detailed description of this marker system, see Stuy (1961a).

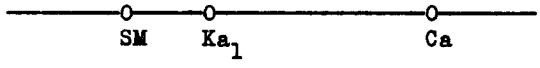

Fig. 1. The three-marker system

Inactivation at 75 °C proceeded almost exponentially (Fig. 2). Marker complexes were more sensitive than single markers. The sensitivity of the small complex SM-Ka_1 (slope of its inactivation curve) was much smaller than the sum of the sensitivities of the SM and Ka_1 markers. This great "overlapping" effect is believed to indicate that the inflicted damage extends its influence on subsequent transformation over DNA regions greater than the distance between the two mark-

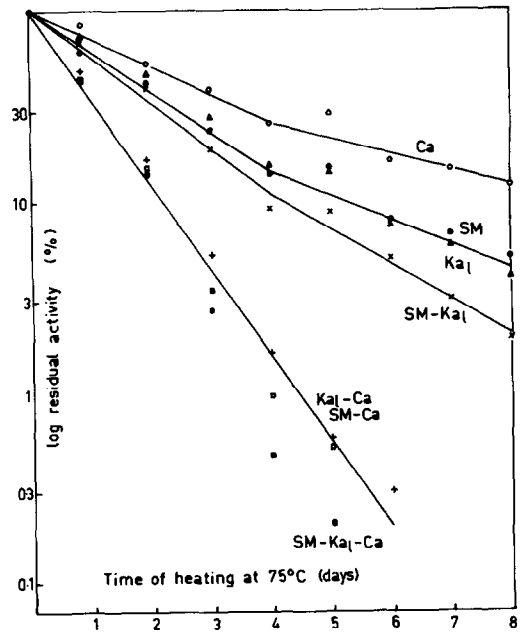

Fig. 2. Decrease in transforming activity of DNA(SM,Ka_1,Ca) during heating at 75 °C. Concentration: 0.05 μg/ml. Initial values in markers/ml x 10^{-6}; SM: 13, Ka_1: 11, Ca: 11, SM-Ka_1: 9.4, Ka_1-Ca: 5.4, SM-Ca: 5.0 and SM-Ka_1-Ca: 5.2.

ers (or, rather, mutated sites). This distance is estimated as of the order of hundred nucleotide pairs (Stuy, 1961a). The sensitivities of the large complexes Ka_1-Ca and SM-Ca were significantly greater than the sums of the sensitivities of the component markers ("unlinking" effect). Since the same phenomenon had been found in a study of DNase inactivation of this marker system (Stuy, 1961a), it is believed that the unlinking effect found here also signifies single-strand breakage.

DNA(SM,Ka_1,Ca) was heated at 80 and 85 °C with identical results (Fig. 3). The complex SM-Ka_1 was now as sensitive as the Ka_1 marker. We believe that this means a much more extended influence along the DNA chain by the inflicted damage with respect to DNA incorporation in the transformation process.

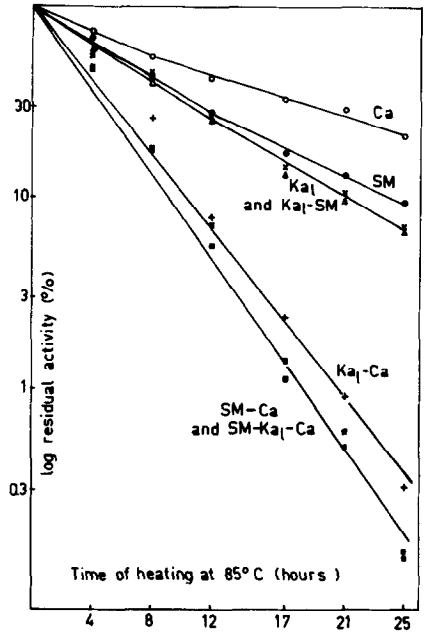

Fig. 3. Decrease in transforming activity of DNA(SM,Ka_1,Ca) during heating at 85 °C. Concentration: 0.05 μg/ml. Initial values in markers/ml x 10^{-6}; SM: 13, Ka_1: 14.5, Ca: 14.5, SM-Ka_1: 9.4, Ka_1-Ca: 8.0, SM-Ca: 7.0 and SM-Ka_1-Ca: 6.6.

(2) Mutagenic action. Heating at 75 °C of DNA(Ery6) (conferring resistance to 6 μg/ml of erythromycin) did not result in the appearance of marker activity with respect to resistance to streptomycin (200 μg/ml), kanamycin (9 μg/ml), cathomycin (2 μg/ml), viomycin (150 μg/ml), penicillin (0.5 μg/ml; C.A.v.S. isolated a spontaneous mutant resisting this concentration), aminopterin (10 μg/ml; Rd is sensitive to 3 μg) and oleandomycin (100 μg/ml). DNA(Ery6) was heated over periods of 0, 2, 4 and 6 days. The Ery6 marker dropped from 19 x 10^6 per ml to 11, 4.2 and 2.2 x 10^6, respectively. About 10^5 marker equivalents (sensitive loci) were plated and checked for resistance-transmitting activity. A few plates contained from 1 to 3 colonies while the others showed no growth at all. These colonies had certainly arisen from untransformed cells (spontaneous mutations) since they were also observed

in control experiments in which no DNA had been added to the recipient cells. The background on viomycin-containing plates is normally as high as 50 to 100. If there is any mutagenic action of heat on DNA, the induced frequency is smaller than 10^{-4}. No mutagenic action was observed at both other temperatures.

(3) Fate of damaged markers. Inactivated markers, regarded as induced back-mutations or suppressor-mutations, could be expected to be incorporated by recipient cells just as spontaneous mutations are. This was tested as follows. If every inactivated Ca marker replaces the chromosomal Ca marker in recipient Rd/Ca cells, the survival of the complex SM-Ca should be equally low when measured on the strains Rd and Rd/Ca. On the other hand, if every inactivated Ca marker is rejected by recipient cells, then the survival of the SM-Ca complex as measured on strain Rd/Ca should be equal to that of the SM marker alone. The latter case was clearly observed for the two markers studied (Table I). To narrow

Table I

Survival of DNA(SM,Ka_1,Ca) heated at 74 °C (6 days) and at 85 °C (25 hours) as measured on the strains Rd, Rd/SM and Rd/Ca of H. influenzae.

Marker(s)	Survival (%) at 74 °C on			Survival (%) at 85 °C on		
	Rd	Rd/SM	Rd/Ca	Rd	Rd/SM	Rd/Ca
SM	7.8	-	6.4	6.3	-	5.5
Ca	24	23	-	18	16	-
SM-Ca	0.21	23	6.2	0.13	17	5.8

a possible discrepancy down to less than one per cent, 76 Ca-transformed clones from the cross: heated (74 °C) DNA x Rd/SM, and 76 SM-transformed clones from the cross: heated DNA x Rd/Ca, were streaked on SM-agar and Ca-agar, respectively. All cultures grew on these media. This demonstrates that recipient cells do not exchange (active) chromosomal markers for inactivated ones in transforming DNA.

DISCUSSION - We are unable to conclude definitely from the results obtained whether the decreased transforming activity of heated DNA had been caused by depurination, strand breakage, breakage of H-bonds or by a combination of these effects. We believe, however, that the results indicate a dominating role by strand breakage because (a) the picture obtained at 75 °C closely resembles that of DNase degradation (Stuy, 1961a; overlapping and unlinking effect), (b) depurination alone would probably not cause unlinking of SM and Ca markers by analogy to UV (Stuy, 1962) and nitrous acid effects (Stuy, 1961c) and (c) at the higher temperatures of 80 and 85 °C the expected H-bond breakage, resulting in more or less collapsed areas around breaks, seems to show up as an enhanced overlapping effect.

Marker inactivation by heat is in effect a rejection by recipient cells of areas around a "hit" in the DNA. Markers may remain unaffected but they "disappear" from the reaction mixture when they become located on such areas. Accordingly, they are scored as inactivated.

We have failed to demonstrate <u>in vitro</u> mutagenesis by exposing purified transforming DNA to sub-melting heat. Some of the induced changes may have been mutational if inflicted in bacteriophage DNA or in DNA inside a living bacterium. But in the transformation process all damaged DNA areas block their own incorporation into chromosomal DNA. Spontaneous mutational changes are readily incorporated. We conclude, therefore, that sub-melting heat affects rather seriously the secondary DNA structure besides, or through, the observed chemical alterations. In this connection, we prefer to think of rather extensive H-bond breakage with perhaps a faulty reunion.

ACKNOWLEDGEMENTS - The able assistance of Miss Joke Bloemendal must be gratefully mentioned. The kanamycin used was a generous gift by Bristol Laboratories, Syracuse, New York.

REFERENCES

Doty, P., Marmur, J., Eigner, J. and Schildkraut, C., Proc. nat. Acad. Sci. 46 (1960b) 461.

Eigner, J., Boedtker, H. and Michaels, G., Biochim. biophys. Acta 51 (1961) 165.

Greer, S. and Zamenhof, S., Fed. Proc. 18 (1959) 238.

Marmur, J. and Lane, D., Proc. nat. Acad. Sci. 46 (1960a) 453.

Stuy, J.H., Doctoral thesis, Utrecht (1961a) (reprints available).

Stuy, J.H., Rad. Res. 15 (1961b) 45.

Stuy, J.H., Biochem. biophys. Res. Comm. 6 (1961c) 328.

Stuy, J.H., J. Photochem. Photobiol. 1 (1962) 40.

Zamenhof, S. and Greer, S., Nature 182 (1958) 611.

Zamenhof, S., Alexander, H.E. and Leidy, G., J. exp. Med. 98 (1953) 373.