BIOORGANIC CHEMISTRY 13, 24–33 (1985)

The Effect of Cr(III) upon the Thermal Denaturation of DNA

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Received July 2, 1984

Thermal denaturation studies of Cr-DNA solutions at pH 6.0 were carried out by monitoring the uv absorbance at 260 nm. The melting curves of solutions of calf thymus and *Escherichia coli* DNA with added $Cr(ClO_4)_3$ were broadened and shifted to higher temperatures. As the ratio of Cr : DNA increased, the melting temperature increased until it reached a maximum at Cr : DNA ratios of 0.7 (*E. coli*) and 0.9 (calf thymus). At higher concentrations of Cr^{3+} the melting temperature decreased and then leveled off, but it never fell as low as that of the pure DNA.

INTRODUCTION

Chromium is a carcinogen in several valence states (*1, 2*). Epidemiologists have associated occupational exposure (mining, production, electrolytic plating) to hexavalent chromium compounds with lung and nasal cancer (*3, 4*). The cancer rates for chromium industry workers have been estimated to be from 3 to 38 times greater than in the general population (*1*). Hexavalent chromium is absorbed in the gastrointestinal and respiratory tracts and is then reduced to the trivalent form, which is probably the "ultimate carcinogen" (*1, 5*).

The mechanism of carcinogenesis for chromium probably involves damage to the DNA (*6*). Dichromate salts are mutagenic in *Escherichia coli* (*7*), *Bacillus subtilis* (*8*), *Salmonella* (*6*), and several hamster cell lines (*9, 10*). Cr(VI) (as dichromate or chromate) is the metal with the highest mutagenic activity in the standard Ames *Salmonella*/mammalian microsome assay. Cr(III) salts are several orders of magnitude less active in this test (*6, 7*). Chromosomal damage in human leukocytes and mutagenicity in *E. coli* is significantly higher for Cr(VI) than for Cr(III) (*11*). However, in several *in vitro* systems where effects due to cellular uptake are removed, Cr(III) is more potent than chromate in inducing misincorporation of bases during DNA synthesis (*12–14*). Recently, Okada *et al.* (*15*)

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0045-2068/85 $3.00

showed that Cr is accumulated in mouse liver cells after intraperitoneal injection of $CrCl_3$. In this study both Cr(III) and Cr(VI) were taken up; Cr(III) enhanced RNA synthesis while Cr(VI) inhibited it.

Cr(III), a d^3 ion, forms extremely inert complexes. It has great difficulty in passing through the cell membrane (*16*). In contrast, the chromate anion can penetrate the membrane by diffusion or by facilitated transport (*17*). Inside the cell, only the reduced form, Cr(III), is found (*1*). Several investigators (*16, 17*) have presented evidence that Cr(VI) is reduced to Cr(III) inside the cell by microsomal enzymes, specifically cytochrome *P*-450, although EPR studies (*18*) have found evidence for a long-lived Cr(V) intermediate which may also be involved in carcinogenesis. The final reduction product, Cr(III), then binds tightly to various cell components, especially DNA (*1, 17*).

In order to obtain information about Cr(III)–DNA interactions in solution, thermal denaturation studies of DNA in the presence of Cr(III) have been conducted. These studies are similar to those of Eichhorn and co-workers, who have observed DNA melting patterns with various metal ions in order to deduce the nature of the interaction between metal ion and double helix (*19–22*).

Two previous investigations of Cr-DNA thermal denaturation found a decrease in T_m[2] as the ratio of Cr : DNA(P) increased. Chin and Rodley (*23*) conducted their studies of calf thymus DNA at pH 4.2–4.3, in order to compare the behavior of Cr(III) with Cr(II). Under these acidic conditions, they had to add Mg^{2+} in order to stabilize the duplex form of DNA. Tamino *et al.* (*24*) observed the melting behavior of BHK hamster fibroblast and calf thymus DNA in unbuffered solutions. They observed that Cr(III) decreased the pH of the DNA solutions, but did not report the pH of the solutions used for thermal denaturation. Since the thermal stability of the DNA double helix decreases with pH (*25*), it is not clear whether the decrease in T_m was due to added Cr(III), to lowered pH, or to a combination of the two factors. In our experience (see Experimental Procedures), DNA melting experiments in unbuffered solutions gave poor results.

In this paper we report thermal denaturation studies of calf thymus and *E. Coli* DNAs in buffered solutions at pH 6.0 with Cr : DNA(P) ratios of 0 to 3. While the optimum pH is physiological, 6.0 was the highest pH which avoided precipitation of hydroxychromium species. Moreover, in contrast to the earlier investigations, the Cr-DNA solutions were equilibrated at ambient temperature for at least 6 days prior to the melting point determination, due to the slow rate of H_2O exchange for $Cr(H_2O)_6^{3+}$: 2×10^{-5} sec^{-1} ($t_{1/2} \sim 10$ hr) at 25°C (*26*).

EXPERIMENTAL PROCEDURES

Chemicals. All glassware, utensils, and distilled–deionized H_2O for solutions were autoclaved, and gloves were worn to avoid cleavage of the DNA. A buffer solution was prepared by adjusting the pH of a 5.0×10^{-3} M solution of Mes (Sigma) to 6.0 by adding 0.1 M NaOH. Following the method of Karlik *et al.* (*22*),

[2] Abbreviations used: Mes, 4-morpholineethanesulfonic acid; T_m, melting temperature.

either calf thymus DNA or *E. coli* DNA (Worthington) was dissolved by slow rotation for 48 hr in the Mes buffer to make solutions 10^{-4} M(P) DNA. Exact concentrations were calculated from absorbance measurements at 260 nm using ε_{260} of 6000 M^{-1} cm^{-1}. Some electrolyte is necessary to maintain the DNA double helix; the relatively low salt concentration used here is sufficient for this purpose, but not so great as to obliterate the effect of the metal ion (*19, 23*).

A stock solution of $Cr(ClO_4)_3 \cdot 6H_2O$ (G. Frederick Smith) was prepared by diluting 0.1 M $Cr(ClO_4)_3$ with Mes buffer. The ClO_4^- salt of Cr(III) was chosen since this anion would form the weakest bond with the metal ion (*27*). Cr-DNA solutions were prepared in various ratios of Cr : DNA(P), all with ionic strengths of 5.0×10^{-3} M.

These experiments were complicated by the fact that $[Cr(H_2O)_6]^{3+}$ solutions are quite acidic due to the formation of $[Cr(H_2O)_5OH]^{2+}$ ($pK = 4.1$) and $[Cr(H_2O)_4(OH)_2]^{+}$ ($pK = 5.6$) (*28*). The pH of 10^{-4} M(P) calf thymus DNA in 5.0×10^{-3} M $NaClO_4$ was 6.2. As the Cr^{3+} concentration increased to 5×10^{-5} M in unbuffered Cr-DNA mixtures (5.0×10^{-3} M $NaClO_4$) the pH dropped to 4.6. It was also found that the pH of Cr-DNA solutions became more acid over time. The pH of Cr-DNA solutions (5.0×10^{-3} M $NaClO_4$), initially adjusted to pH 6.0 with 0.10 M NaOH, decreased to as low as 5.2 during equilibration. At pH 5.5 and below the normal sigmoidal melting curve was not observed. Instead, there was a gradual rise in absorbance as the temperature increased from 55 to 95°C. The hyperchromicity ($A_{95°C}/A_{25°C}$) was 1.21, whereas the hyperchromicity of pure DNA solutions at pH 5.5 was 1.35.

It was apparent that meaningful results would be obtained only in a buffered system. The Mes buffer was chosen since it was presumed neither to intercalate between the bases of DNA nor to complex Cr^{3+}. At pH 6.0, good melting curves were obtained. The formation of various hydroxychromium species prevented use of higher pH.

Thermal denaturation. Absorbance readings (compensated for cuvette differences and corrected by a $NaClO_4$ solution blank) were taken at 260 nm on a Gilford 250 spectrophotometer equipped with an automatic cuvette selector and a Thermoset temperature controller. Quartz microcuvettes (0.30 ml capacity), stoppered to avoid evaporation of solutions during melting, were placed in a temperature-controlled cuvette block equipped with a thermistor for accurate temperature readout. The temperature was increased in 1°C increments near the melting point, allowing at least 2 min for the solutions to come to equilibrium at each temperature.

Renaturation. Cooling curves were measured at slow rates of temperature decrease, typically 2 hr from 95 to 20°C, to allow renaturation to the fullest extent possible. No significant decrease in absorbance was noted after allowing the cooled DNA solutions to remain at 20°C for 12 hr. In other experiments, the Cr-DNA solutions were cooled rapidly from 95 to 20°C and the absorbance was recorded.

Data analysis. The T_m of the DNA samples was determined graphically in two different ways. Figure 1 shows two plots of relative absorbance versus temperature; T_m is the temperature at the midpoint of the relative absorbance increase

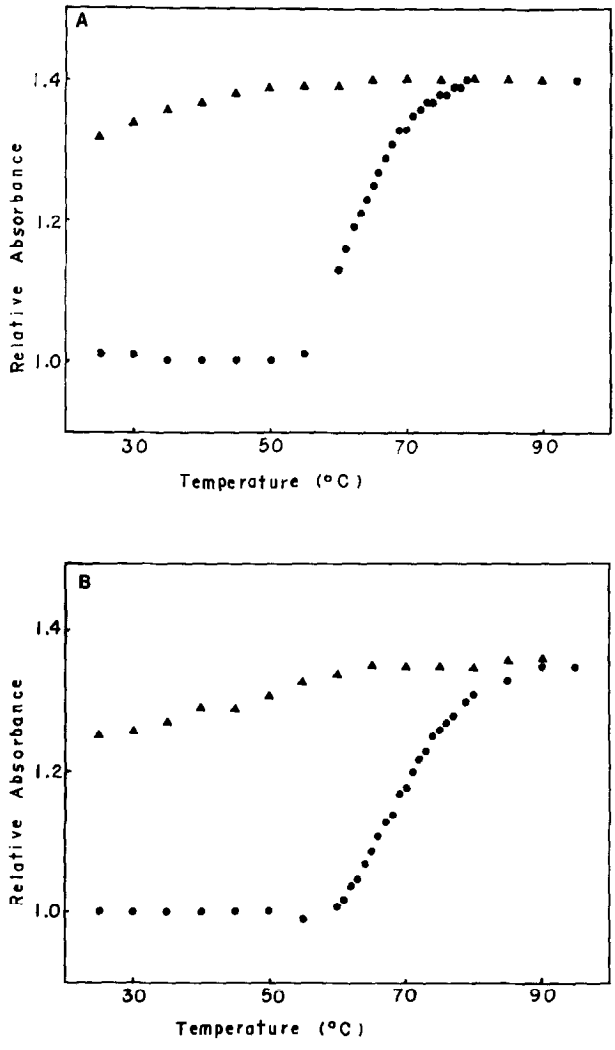

FIG. 1. Thermal denaturation (○) and renaturation (×) curves for (A) 1.0×10^{-4} M calf thymus DNA and (B) 1.0×10^{-4} M calf thymus DNA with 2.0×10^{-5} M $Cr(ClO_4)_3$; pH 6.0 (Mes buffer); ionic strength, 5.0×10^{-3} M. T_m (determined at 50% of absorbance rise): (A) 62.5°C, (B) 70°C.

(*29*). Or, normal probability paper (Keuffel & Esser) can be used to transform the gaussian shape of the derivative absorbance curve to a straight line, as in Fig. 2 (*30*). T_m is the point of maximum slope for a broad transition only when the absorbance data are plotted versus T^{-1} (*31*). Therefore, plots of percentage absorbance increase versus T^{-1} were drawn on normal probability paper; T_m is the temperature where the straight line crosses the 50 percentile point. The two methods gave similar values of T_m; the reported values were obtained by the derivative method, and in all cases but one are the means of either two or three determinations.

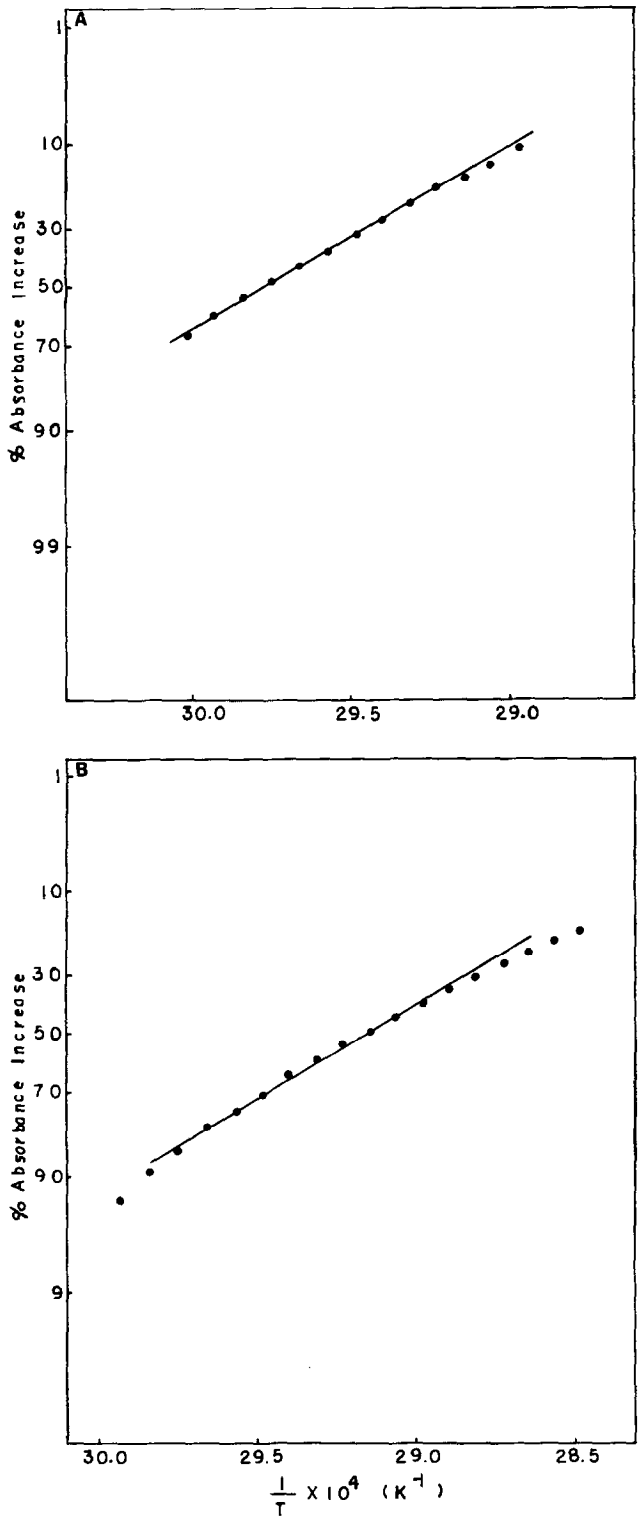

FIG. 2. Normal probability plots for thermal denaturation experiments shown in Fig. 1: (A) 1.0×10^{-4} M calf thymus DNA and (B) 1.0×10^{-4} M calf thymus DNA with 2.0×10^{-5} M $Cr(ClO_4)_3$; pH 6.0 (Mes buffer); ionic strength, 5.0×10^{-3} M. % absorbance increase = $(A_{max} - A_T)/(A_{max} - A_{25°C})$. T_m (determined at 50 percentile point): (A) 62.6°C, (B) 69.9°C.

RESULTS

Melting behavior for calf thymus and *E. coli* DNA in solutions containing various concentrations of $Cr(ClO_4)_3$ was monitored by measuring the uv absorbance at 260 nm. The absorbance of the solutions increased by as much as 38% as the solutions were heated. Thus, the denaturation of the DNA from the double-stranded structure to a less ordered state could be monitored. The increased molar absorptivity (hyperchromicity) results from the unstacking of the bases in the denatured DNA (*29, 31*).

The absorbance rise took place over a relatively broad temperature range (about 25°C), which is typical of DNA heating curves at low ionic strength. Both the shape of the denaturation curve (Fig. 1A) and the melting temperature for the sample of pure DNA (Table 1) are quite similar to that obtained by other workers (*21*). Likewise, the cooling curve of pure DNA (Fig. 1A) is similar to that observed previously (*21*). The slight decrease in absorption upon cooling the DNA solution to 20°C represents random restacking of the bases, without regeneration of the full duplex structure.

Figure 1B is typical of the absorbance curves obtained from Cr-DNA solutions. Comparison of Figs. 1A and B shows a shift in the melting curve to higher temperature and a definite broadening of the absorbance transition. As the

TABLE 1

THERMAL DENATURATION OF CALF THYMUS DNA WITH Cr^{3+}

[DNA] ($\times 10^5$ M(P))	[Cr^{3+}] ($\times 10^5$ M)	T_m (°C)	Hyperchromicity[a]
10	0	61.4	1.37
10	1	69.2	1.38
10	2	70.5	1.35
10	3	71.5	1.36
10	4	72.3	1.34
10	5	74.9	1.31
10	6	72.7	1.32
10	7	73.3	1.29
10	8	77.0	1.27
10	9	73.4	1.28
10	10	74.8	1.25
5	11	71.4	1.28
5	12	65.4	1.31
5	13	66.7	1.29
5	14	66.8	1.25
5	15	66.4	1.26

Note. T_m and hyperchromicity (relative absorbance increase at 260 nm during melting) values are the means of either two or three experiments at pH 6.0 (Mes buffer), ionic strength 5.0×10^{-3} M. Average standard deviation in T_m was 0.9°C.

[a] $Abs_{95°C}/Abs_{25°C}$.

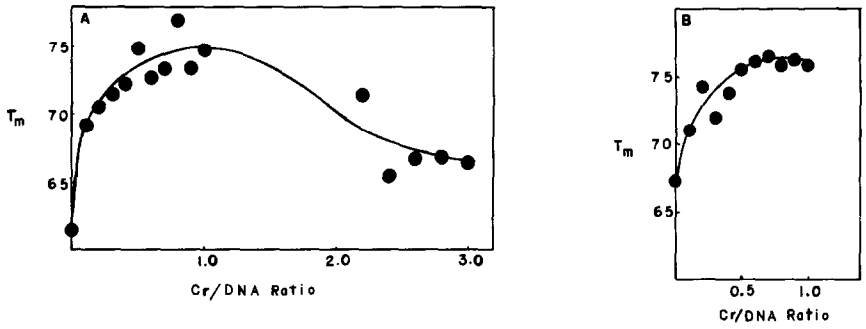

FIG. 3. T_m values for (A) calf thymus DNA and (B) *E. coli* DNA at various Cr/DNA(P) ratios; pH 6.0 (Mes buffer); ionic strength, 5.0×10^{-3} M.

Cr : DNA(P) ratio increased to 1.0, T_m steadily increased. The melting point of the pure calf thymus DNA solution was 61.4°C; at a Cr : DNA(P) ratio of 0.8, T_m was 77.0°C. At Cr : DNA(P) ratios above 1.0, T_m fell to 65°C, and then remained constant at 67°C as the Cr : DNA(P) ratio increased from 2.6 to 3.0. Although T_m decreased at high Cr^{3+} concentrations, it never fell as low as T_m for pure DNA.

Changes in T_m as a function of Cr : DNA(P) ratio at constant pH (6.0) and ionic strength (5.0×10^{-3}) are summarized in Table 1 and Fig. 3A. The temperature range of the melting transition increased as T_m rose, and then decreased as T_m fell at highest Cr : DNA(P) ratios. For solutions with Cr : DNA(P) ratios of 0.3 to 1.0 the absorbance was still increasing slightly at 95°C, the highest temperature at which absorbance measurements were made. There was some decrease in hyperchromicity at higher Cr^{3+} concentrations (see Table 1) but not as much as the 35% decrease which had been seen in DNA melting experiments with Al^{3+} (*22*). As expected, the melting point of the pure *E. coli* DNA (67.3°C) was higher than pure calf thymus DNA due to the higher G–C content of *E. coli* DNA. The absorbance transition of *E. coli* DNA was also broadened from less than 20°C for the pure DNA to 30°C or more when Cr^{3+} was present, even at the lowest concentrations. The T_m of *E. coli* DNA increased with increasing Cr^{3+} concentration to 76.5°C at a Cr : DNA(P) ratio of 0.7, and then fell slightly (Table 2, Fig. 3B).

Renaturation experiments were performed both by gradual cooling of the Cr-DNA solutions and by rapid cooling from 95 to 20°C. There was no consistent tendency for the added Cr^{3+} to facilitate renaturation.

DISCUSSION

In all DNA melting experiments reported here, the pH was held constant at 6.0 by the addition of Mes buffer. Thus, the changes in the T_m which were observed can be ascribed to the effect of added Cr^{3+}, rather than to changes in pH. Furthermore, sufficient time was allowed for the Cr^{3+} to bind to the DNA before the melting experiments were conducted.

From these experiments, it appears that there are several types of interaction

TABLE 2

THERMAL DENATURATION OF *E. coli* DNA WITH Cr^{3+}

[DNA] ($\times 10^5$ M(P))	[Cr^{3+}] ($\times 10^5$ M)	T_m (°C)	Hyperchromicity[a]
10	0	67.3	1.33
10	1	71.1	1.35
10	2	74.3	1.33
10	3	71.9[b]	1.32
10	4	73.8	1.32
10	5	75.6	1.32
10	6	76.1	1.29
10	7	76.5	1.29
10	8	75.8	1.28
10	9	76.2	1.28
10	10	75.8	1.26

Note. T_m and hyperchromicity (relative absorbance increase at 260 nm during melting) values are the means of two experiments at pH 6.0 (Mes buffer), ionic strength 5.0×10^{-3} M.

[a] $Abs_{95°C}/Abs_{25°C}$.

[b] One experiment.

between the trivalent chromium ion and DNA. At low Cr : P ratios, the primary effect seems to be a stabilization of the double helix, evidenced by the rise in T_m with increased Cr^{3+} concentration. Stabilization of the native conformation might result from either of two mechanisms: (1) Binding of the Cr^{3+} ion to bases on both DNA strands (crosslinking) to delay the transition from double helix to single coil (*22*); or (2) Nonspecific interaction of the positively charged metal ion with the negative phosphate oxygens to reduce the electrical repulsion between phosphate groups, stabilizing the DNA in duplex form (*20, 32*). Crosslinking by the metal ion would hold the bases "in register" so that the double helix would be reformed upon cooling. This facilitated denaturation has been observed with Al^{3+} (*22*), the other trivalent ion studied in this way, as well as with Cu^{2+} (*19*) and Zn^{2+} (*21*). The denaturation experiments reported here do not support crosslinking by Cr^{3+}. Chromium–phosphate binding (mechanism 2) is consistent with the results of NMR experiments (*33*), the synthesis of various Cr(III) nucleoside triphosphates (*34*), and the counterion stabilization model of Manning (*32*).

At Cr : P ratios greater than 1.0 the native conformation of the DNA is apparently disturbed; T_m decreases, then levels off to a value 5°C higher than that of pure DNA. In this respect the thermal denaturation behavior of Cr-DNA solutions is quite similar to that of Mn-DNA or Zn-DNA. For example, T_m of 5×10^{-5} M(P) calf thymus DNA solutions (pH 6.3 to 6.5, ionic strength, 5×10^{-3} M) increases from 63.0°C for the pure DNA solution to maxima of 72.0°C for a 1 : 1 mol ratio of Zn^{2+}: DNA(P) (*21*) and 75°C for a 1.5 : 1 mol ratio of Mn^{2+} : DNA(P) (*20*). At higher ratios, the T_m decreases slowly, but never falls as low as the T_m of the pure DNA.

The behavior of Cr-DNA solutions at high Cr concentrations could be due to several factors. Interruption of the hydrogen bonding between complementary base pairs would decrease T_m, as occurs with Cu^{2+} (*20*). Evidence for both DNA cleavage and crosslinking by chromium was found in chick embryo hepatocytes (*5*); cleavage would lower T_m, whereas crosslinking would raise T_m. Another possibility is cation-induced toroidal condensation of the DNA, which has been observed at high concentrations of other trivalent cations, including spermidine and Co^{3+} (*35*). The condensation is temperature- as well as concentration-dependent, being favored at higher temperatures (*36*).

Further experiments with this system at different pH and ionic strengths need to be carried out to help further elucidate the effect of Cr^{3+} upon the melting behavior of DNA. At present, we are trying to crystallize complexes of Cr(III) with various nucleosides and nucleotides in order to investigate Cr(III) binding to DNA in as great detail as possible.

ACKNOWLEDGMENTS

We thank Dr. Jenny P. Glusker, in whose laboratory this work was carried out, for her encouragement and helpful discussion. This work was supported by Grants IN-140 and BC-242 from the American Cancer Society, and CA09035-06 from the National Cancer Institute, DHEW.

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