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The Process of Proton Capture in the Alkali Denaturation of DNA

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SEZIONE II

(Fisica, chimica, geologia, paleontologia e mineralogia)

Biofisica. — *The Process of Proton Capture in the Alkali Denaturation of* DNA (*). Nota di ELISABETTA DORE (**) e CLARA FRONTALI (**), presentata (***) dal Corrisp. M. AGENO.

RIASSUNTO. — È stato messo in evidenza in precedenti lavori come il processo di denaturazione alcalina comporti la deprotonazione della molecola di DNA, e come questo meccanismo permetta di spiegare diverse caratteristiche della transizione alcalina.

Nel presente lavoro la deprotonazione viene messa direttamente in evidenza tramite l'abbassamento di pH che si verifica in una soluzione alcalina quando vi si immetta una quantità nota di DNA. Queste misure hanno richiesto la messa a punto di una tecnica molto accurata ed hanno consentito di ricavare una valutazione del numero di OH^- legati (ovvero di H^+ strappati) per ponte idrogeno presente nella molecola di DNA, in un intervallo di pH che copre la regione di transizione. I risultati mostrano un effetto cooperativo di deprotonazione, in corrispondenza della transizione alcalina. Vengono discusse possibili cause di errore ed eventuali effetti concomitanti.

In a series of papers [1–3] dealing with the mechanism of alkali denaturation of DNA, we have considered the capture of the protons linking complementary bases as a main feature of alkali denaturation, determining its very beginning, and limiting its rate (at least in the lower limit of the transition region). This process is expected to take place when a DNA molecule is introduced into a solution in which a large excess of OH^- ions is present.

We have also discussed [4] the basic difference between the alkaline and thermal melting processes, which is revealed through the study of the absorption spectra of melted DNA both at a high temperature and an elevated pH. In effect, the difference between those two spectra can be ascribed to the process of "deprotonation", which though effective in the case of alkali, is completely absent in the case of heat denaturation.

To further check our previous conclusions we then tried to demonstrate directly the deprotonation process, by measuring the drop in pH of an NaOH solution, due to addition of a given amount of DNA. The proton capture in effect is accompanied by the disappearence of an equivalent number of OH^- ions, with the formation of water molecules. (The excess negative charge then remains inside the double stranded structure, and causes its unwinding).

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In order to measure the drop in pH below the value of the corresponding blank, it is advisable to raise the concentration of DNA as much as possible; however the necessity of uniform distribution of the macromolecules, and of relatively large volumes of solution ($\sim$ 30 ml) to obtain good pH measurements in practice limit the concentration to about 100 μg/ml.

Under these conditions the difference in pH with respect to the corresponding blank turned out to be rather small, being of the order of a few hundredths of one pH unit in the transition region (between pH 11.7 and 12.0). Therefore a very sensitive instrument such as the Beckmann Research pH–Meter, accurate to 0.001 pH was needed. When using such an instrument,

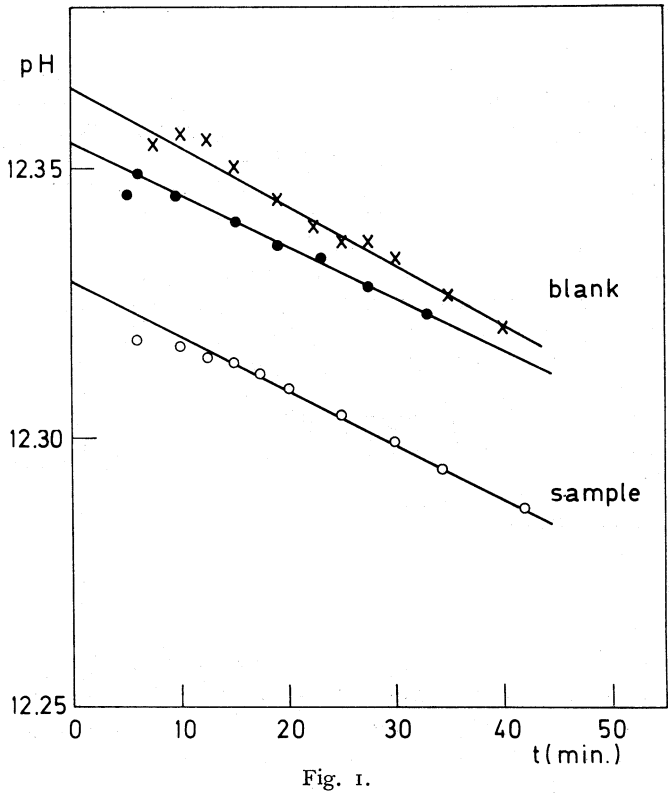

Fig. 1.

the precision limit is no longer determined by the reading error; but rather by the difficulty of exactly repeating the manual procedures of dilution and mixing, both for the blank and the sample. In order to minimize these errors we used Marburg micropipettes in the dilutions, and carefully checked the mixing time which turned out to be a rather critical factor. Mixing was performed in a closed vessel, by means of a magnetic stirrer, at a constant low velocity; the presence of a small amount of air not free from CO_2, was however difficult to avoid without resorting to over-complicated systems. In practice we were satisfied when manual operations allowed the pH value of the blank to be reproduced to within 0.01 pH units.

We also found it necessary to standardize the pH–meter, with the Beckmann precision buffers, before every reading. The electrodes used were a standard calomel reference electrode, and a special E2 glass electrode suited for measurements in the high pH region.

The experimental procedure was as follows. In order to work at constant Na^+ concentration as in the previous experiments [2, 3], we prepared the denaturing solution, for every pH value, by mixing—at the time of the experiments—stock solutions of NaCl 0.1 M and NaOH 0.1 M in suitable proportions to give the chosen pH. To 26 ml of this solution, 4 ml of either NaCl 0.1 M (for the blank), or of a concentrated ($\sim$ 500 μg/ml) DNA solution (for the sample) were added and mixed for 60 sec in a water bath at 20° C. We made sure that the NaCl solution added had exactly the same pH (7.00) as the DNA solution. Since the pH of distilled water is usually low, a small addition of NaOH to the 0.1 N NaCl solution was necessary. The pH readings began some minutes after mixing, to allow thermal equilibrium at 20° C to be attained (and deprotonation to be definitely complete); readings were taken at intervals of 3–5 min over a period of about 40 minutes; during this time the solution, not being buffered, showed a drift in pH. It was therefore necessary to perform an extrapolation to zero time, in order to obtain a value not affected by the amount of CO_2 present in the air. In fig. 1 a typical set of measurements (two blanks and one sample) is given. The difference in pH between blank and sample can be easily converted into an evaluation of the number of OH^- ions which have disappeared during the process of deprotonation of DNA.

On the other hand, the same solutions were used to read the optical density at 2600 Å of the sample against the blank. This measurement yielded the final concentration of DNA, or the number of nucleotide pairs per unit volume. Taking into account the base composition of the DNA we used (DNA from bacteriophage T 2) the average number, n, of hydrogen bridges per nucleotide pair can be calculated ($n = 2.35$, based on the assumption that the GC pair possesses three H–bridges, and the AT pair only two). The subsequent step is the calculation of the number of OH^- ions which have disappeared per hydrogen bridge. The value so obtained, ΔOH^-/H–bridge, is given in fig. 2 against the pH of the blank (i.e. the pH the DNA is initially exposed to). In practice, since the measurements can be reproduced only to within one hundredth of a pH unit and the effect to be measured amounts to only a few hundredths, we were forced to re-measure both the blank and the sample several times, and to average out the resulting values of the parameter ΔOH^-/H–bridge.

The errors given in the graph of fig. 2 are standard errors, calculated for every pH value over a group of 4–6 measurements performed with DNA from different preparations. It should be remembered that different preparations may contain different amounts of impurities contributing to the effect of OH^- trapping. Protein impurities, e.g., can give such an effect. In the procedure we followed the measured effect was ascribed only to the addition of a given

amount of DNA, and we may therefore obtain values of the parameter ΔOH^-/H–bridge which are too high.

The curve in fig. 2 is composed of two distinct parts. First, between pH 8.4 and 11.6, there is a slow, almost linear increase of the number of bound OH^- ions. This increase can be thought of as due to the titration of the phosphate groups.

There is one phosphate group per nucleotide, and therefore $1/(n/2) = 1/1{,}175 = 0.75$ phosphate groups per H–bridge. The initial slow increase could therefore be expected to cease on reaching 0.85 OH^-/H–bridge. This is in contrast with the finding that the linear part of the curve reaches a higher value (about 1 OH^-/H–bridge). The small difference between the expected and obtained value may well be due to impurities.

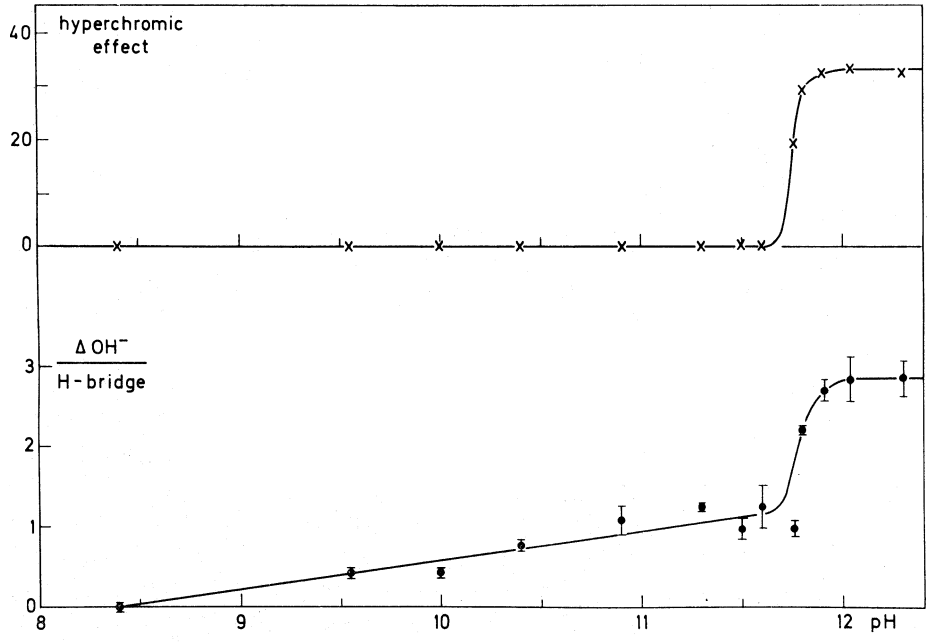

Fig. 2.

The second part of the curve corresponds to an abrupt increase in the number of trapped OH^- ions. Note that this happens just in the region of the alkaline transition, as is evident from the comparison with the hyperchromic effect, shown in the upper part of fig. 2. There is little doubt that this increase corresponds to the capture of the protons linking the complementary strands. The curve shows that the deprotonation effect is co-operative; the same suggestion is to be found in the well known titration curves of Gulland, Jordan, Taylor [5].

As to the size of the jump, it would be expected to be not less than 0.5 and not more than 1 OH^-/H–bridge. The value which is found ($\sim$ 1.6

OH^-/H–bridge) appears to be too large. However ist must be remembered that the higher the pH, the smaller the effect we measure, so that in the final plateau region the statistical errors are quite large. Furthermore, as a result of opening the double-standed structure, other hydrogen atoms in the molecule which could be captured by the same mechanism become suddenly accessible. Examples are the second hydrogen atom attached to the NH_2 group of Adenine, Guanine and 5–Hydroxymethylcytosine and the hydrogen atom belonging to the hydroxyl group of the non glucosylated hydroxymethylcytosines. If all of these points were deprotonated simultaneously to denaturation, a further contribution of about 1 OH^-/nucleotide, (i.e. ~ 0.8 OH^-/H–bridge) would be present, and the size of the jump would be in agreement with the expected increase.

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REFERENCES.

[1] M. AGENO, E. DORE e C. FRONTALI, « Ann. Ist. Super. Sanità », *2*, 629 (1966).
[2] M. AGENO, E. DORE e C. FRONTALI, « Atti Accad. Naz. Lincei, Rend. Classe sci. fis. mat. nat. », *42*, 580 (1967).
[3] M. AGENO, E. DORE, C. FRONTALI e F. PODO, ibid. in press (1968).
[4] M. AGENO, E. DORE e C. FRONTALI, ibid. in press (1968).
[5] J. M. GULLAND, D. O. JORDAN e H. F. W. TAYLOR, « J. Chem. Soc. », 1131 (1847).