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Transition to a New State of DNA in Solution, Possibly Related to Intraphage Condensed DNA

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Biofisica. — *Transition to a New State of* DNA *in Solution, Possibly Related to Intraphage Condensed* DNA. Nota di MARIO AGENO, ELISABETTA DORE (*) e CLARA FRONTALI (*), presentata (**) dal Corrisp. M. AGENO.

RIASSUNTO. — (Transizione del DNA in soluzione ad un nuovo stato) – Il fenomeno di aggregazione osservabile per DNA in soluzioni di elevata concentrazione salina a basso pH è riconducibile, quando si lavori a concentrazioni di DNA sufficientemente basse, ad una transizione del DNA ad un nuovo stato, in cui la molecola, pur mantenendo la struttura a doppia elica, mostra un effetto ipercromico del 20% circa. Viene suggerita l'ipotesi che questa transizione corrisponda alla condensazione del DNA, e che il nuovo stato abbia caratteristiche simili a quelle del DNA all'interno del fago.

INTRODUCTION.

The hypothesis of the existence of a " condensed " form of DNA under certain " in vivo " conditions, is not new. DNA is often found in a compact form when associated with protein, as is the case in chromosomes and in viruses, in particular in bacteriophages.

Very little is known about the mechanism of this structural modification. In electron microscope pictures of intracellular phage development [1] nuclei of " condensed " DNA are visible in the infected cell before the protein coat is assembled. This suggests that association with protein is not a requisite for condensation, but rather its consequence. E. Kellemberger [1] postulated the existence of a condensing enzyme to explain intracellular transition to the condensed state, but no idea as to its mode of action has ever been proposed.

Strong evidence that DNA within the head of a bacteriophage is in a different state from the one normally found in solution appears in the studies of Tikhonenko and co-workers [2–5], Inners and Bendet [6] and Maestre and Tinoco [7]. According to the first group of authors, intraphage DNA conformation is characterized by reduced hypochromism [3], higher reactivity with hydrophobic substances [2], a limited number of bases able to react with formaldehyde [4, 5] and loss of co-operativeness in thermal denaturation [5]. On the other hand, Maestre and Tinoco [7] have clearly shown that ORD spectra of intact bacteriphage are different from the ones given by the same phage suspension after osmotic shock. In the thorough discussion

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they present [7], the difference observed is interpred as a single Cotton effect, probably not arising from the interaction of DNA with proteins or polyamines, but rather from the dehydration of DNA. They also stress the fact that the observed structural alteration must involve the greater part of the molecule, and not only localized regions.

From the works of both Tikhonenko and co-workers [2–5] and Maestre and Tinoco [7], it appears that when released from phage, DNA readily turns into the well known extended form, normally found in solution. However, the inverse transition, namely, the transition from extended to condensed form, has not been obtained " in vitro " as yet.

Our search for " in vitro " conditions bringing about such a transition was oriented by the consideration of the role possibly played by proton donors or acceptors in the cellular mechanisms involving DNA [8]. The present paper reports the results of an investigation directed at ascertaining whether the addition of protons to DNA in solution could induce condensation. DNA is known to be " protonated " when introduced into an acid solution, and it is also well known that a low *p*H causes at least two important effects on DNA, both dependent on ionic strength, namely, denaturation and precipitation. So the first question to answer is: are there in a *p*H, NaCl molarity diagram, separate regions for the native, denatured, precipited, and possibly condensed states of DNA or does precipitation coincide with condensation? The experiments reported in this paper provide some evidence in favour of the latter hypothesis.

Materials and Methods.

T2 DNA was extracted from purified phage suspensions with the technique of Mandell and Hershey [9]. Final dialysis was against an 0.1 M NaCl solution.

In preparing DNA samples at increasing *p*H values or NaCl concentrations, we always avoided making subsequent additions of acid or salt to the same DNA sample, in order to reduce systematic errors as much as possible, and to eliminate temporary exposures of DNA to a non-homogeneous concentration of hydrogen ions or NaCl. On the contrary, DNA was added to independently prepared solutions, already adjusted to the desired *p*H and NaCl molarity. In some instances a small concentration (0.01 M) of citrate or formiate buffer was added to ensure that the *p*H did not vary during measurements. Accurate *p*H readings were taken with a Beckmann Research *p*H–meter equipped with E2 electrodes, only slightly sensitive to Na^+ ions. Optical density was measured in a Cary mod. 15 recording spectrophotometer, an expanded scale (0 ÷ 0.1 O.D.) being used in connection with a series of grey filters (prepared in our laboratory) to displace the O.D. expanded reading interval. Sedimentation velocity experiments were performed in a Spinco mod E. analytical ultracentrifuge at 30000 *rpm*.

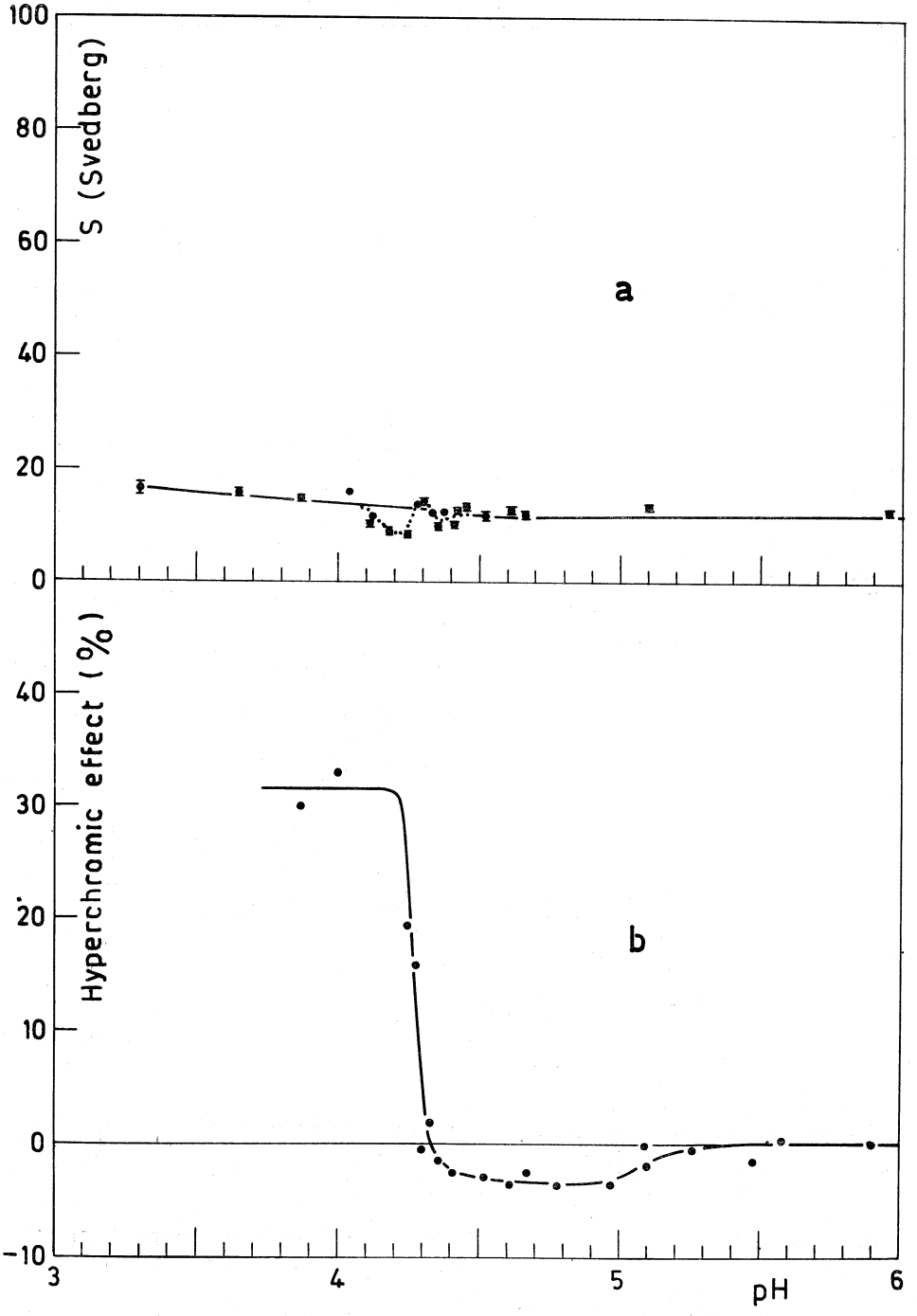

Fig. 1. – *a*) Sedimentation coefficient and *b*) hyperchromic effect as a function of *p*H for T2 DNA (20 μ g/ml) in 0.002 M NaCl. Maximum HCl concentration was $2 \cdot 10^{-4}$ M.

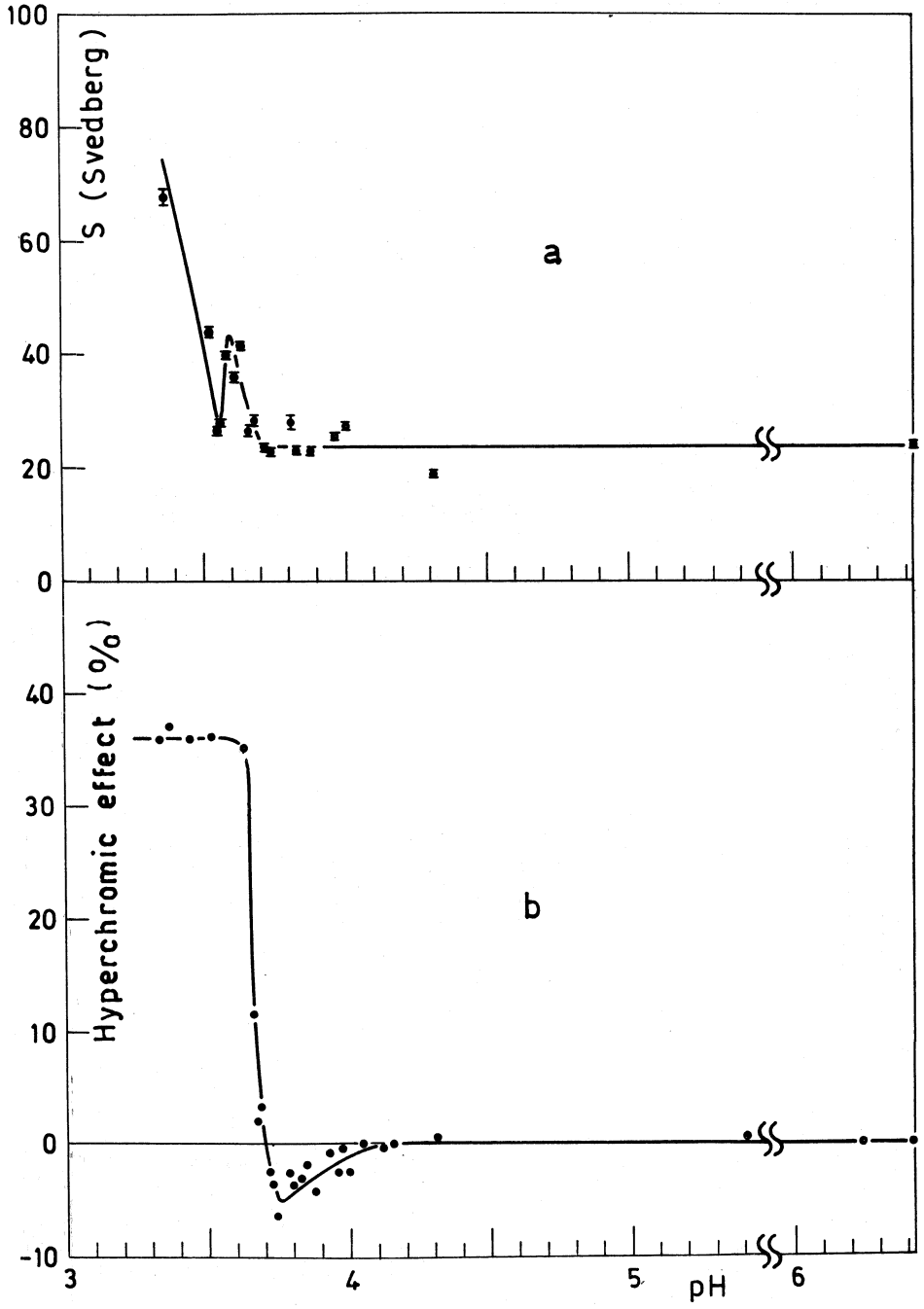

Fig. 2. – *a*) Sedimentation coefficient and *b*) hyperchromic effect as a function of *p*H for T2 DNA (20 μ g/ml) in 0.02 M citrate buffer.

DENATURATION AND PRECIPITATION.

In order to construct the phase diagram for DNA we systematically varied either the *p*H or NaCl molarity (at 20° C) of DNA solutions and followed the modifications of DNA conformation by optical density and sedimentation measurements.

A first set of curves was obtained by varying the *p*H at fixed $(Na^+ + H^+)Cl^-$ concentrations, over a range of 0.002 M to 0.65 M Cl^- (or Cl^- + formiate$^-$ when buffered solutions were used). The results did not depend on the presence of buffer. Furthermore, since the HCl concentration in the region investigated is negligible in comparison with the concentration of the salt, we usually refer simply to this latter.

Separate samples were prepared for each *p*H value by adding 0.4 ml of a concentrated (500 μg/ml) DNA solution to 10 ml solvent so that a final DNA concentration was 20 μg/ml.

Figs. 1–6 show typical curves obtained at the various NaCl molarities. The most striking feature in these graphs is the sedimentation peak, clearly resolved in 0.1 M NaCl (or 0.09 M NaCl, 0.01 M formiate buffer), less evident at both and lower NaCl concentrations. (In some instances alternative interpolations are presented by dotted lines). This same behaviour was first observed by Studier [10].

The decrease in optical density before denaturation corresponds to the observations of Bunville, Geiduschek, Rawitscher and Sturtevant [11] and of Zimmer [12, 13] and co-workers. According to these authors, this effect is mainly due to protonation of cytosine taking place before the onset of denaturation. The points corresponding in figs. 1–5 to midpoint (50 %) of hyperchromic transition, to the hypochromic minimum, and to the sedimentation peak, follow parallel straight lines in a *p*H, NaCl molarity diagram (fig. 7). In particular, the sedimentation peak closely follows the hyperchromic transition. These two effects could therefore reflect different aspects of the same phenomenon, namely, denaturation. The straight line they define divides the plane into the two regions corresponding to native and denatured DNA.

The optical density curve in fig. 6 is not achieved, because at sufficiently low *p*H and sufficiently high NaCl concentrations ($\gtrsim$ 0.5 M) precipitation takes place in the form of large aggregates sedimenting at a very high velocity. It is important to stress that in fig. 6, down to the *p*H where precipitation begins, DNA appear to be native if judged by O.D. and sedimentation. Experiments in the CsCl density gradient confirm that a single band is to be observed down to *p*H 2.72, while at a lower *p*H no band is present in he usual density range. More accurate experiments are under way for obtaining a definite band in low *p*H and high ionic strength. A further, striking property of the precipitate is made visible in fig. 8, where a full-size picture of a DNA solution 100 μg/ml in 1 M NaCl, *p*H 2.30, is shown. These rather

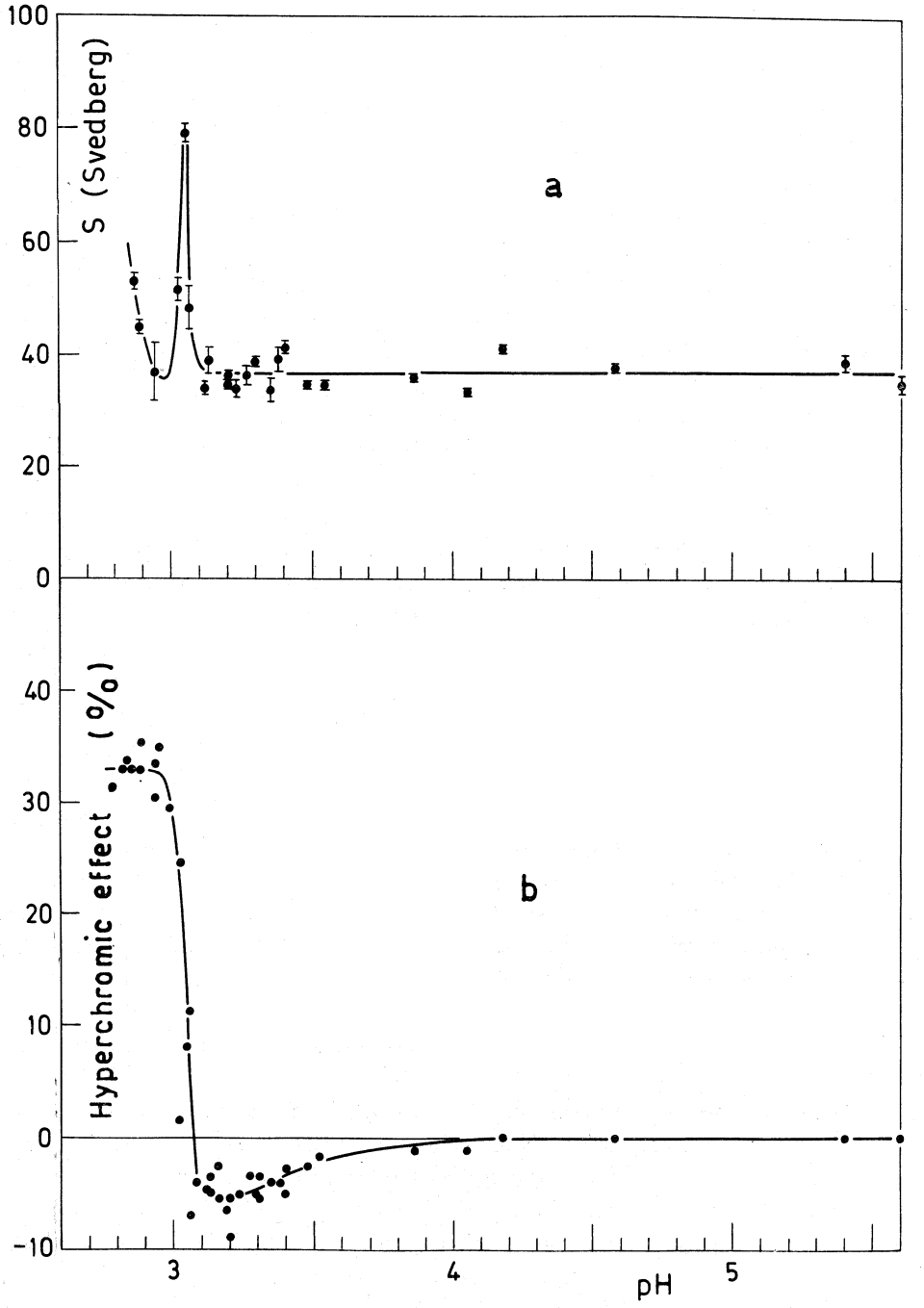

Fig. 3. – *a*) Sedimentation coefficient and *b*) hyperchromic effect as a function of *p*H for T2 DNA (20 μg/ml) in 0.09 M NaCl, 0.01 formiate buffer.

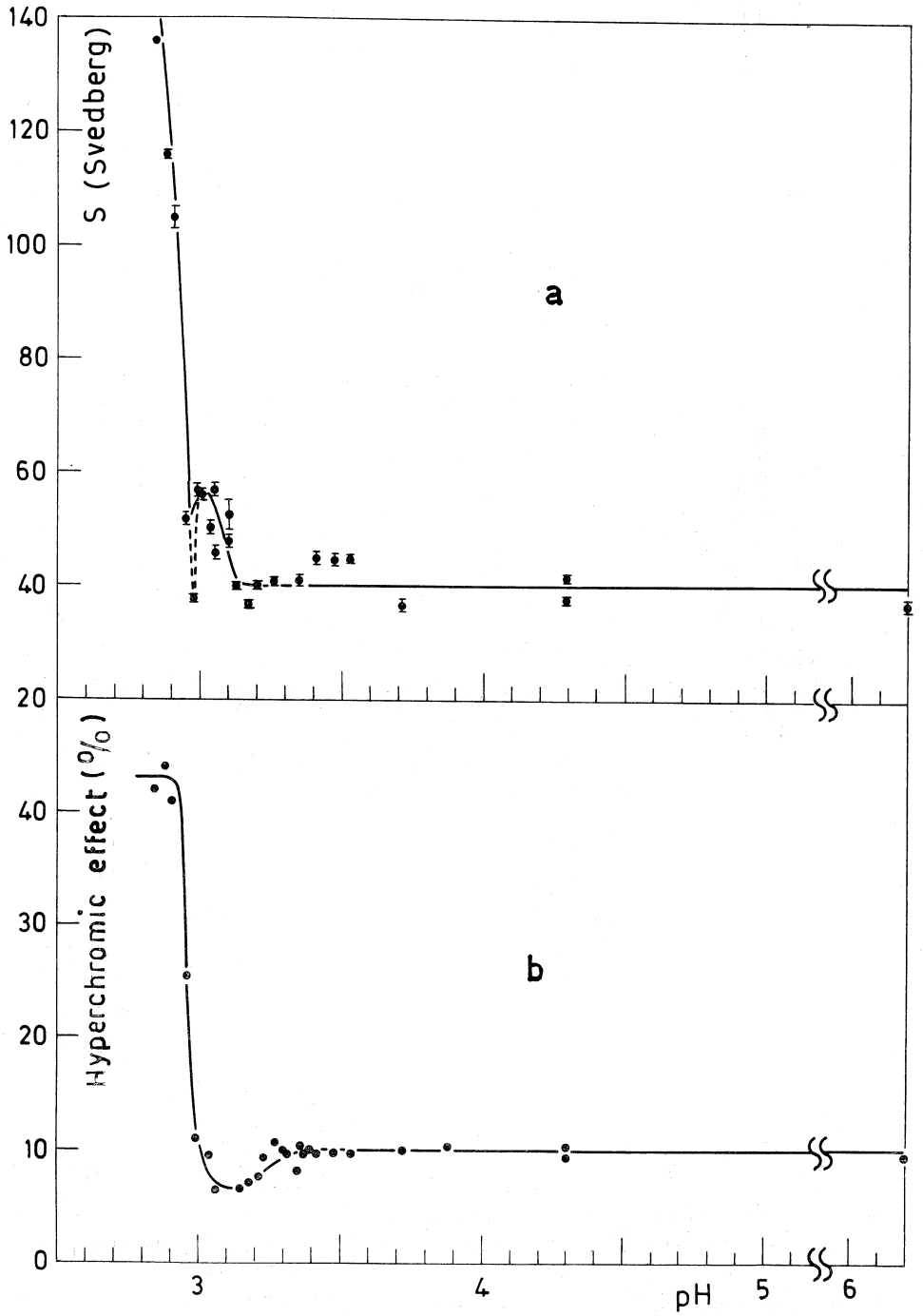

Fig. 4. – *a*) Sedimentation coefficient and *b*) hyperchromic effect as a function of *p*H for T2 DNA (20 μg/ml) in 0.16 M NaCl, 0.02 M citrate buffer.

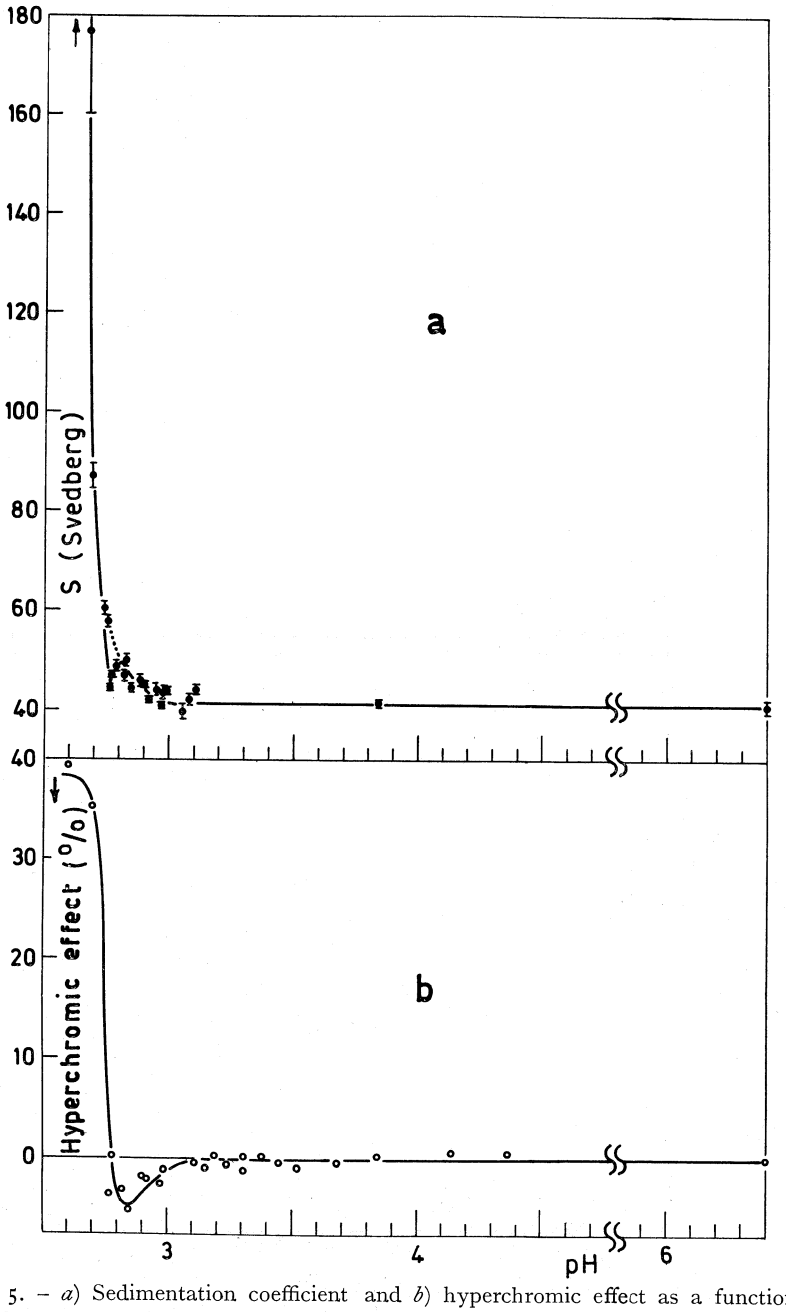

Fig. 5. – *a*) Sedimentation coefficient and *b*) hyperchromic effect as a function of *p*H for T2 DNA (20 μg/ml) in 0.40 M NaCl. Arrows indicate visible precipitation.

extreme conditions of DNA concentration and *p*H were chosen in order to show up the aggregation phenomenon. Evidently DNA molecules tend to form the maximum number of bonds either among themselves or with the beaker walls (or even with plastic material such as disposable pipette tips) and to present a minimum surface to the solvent.

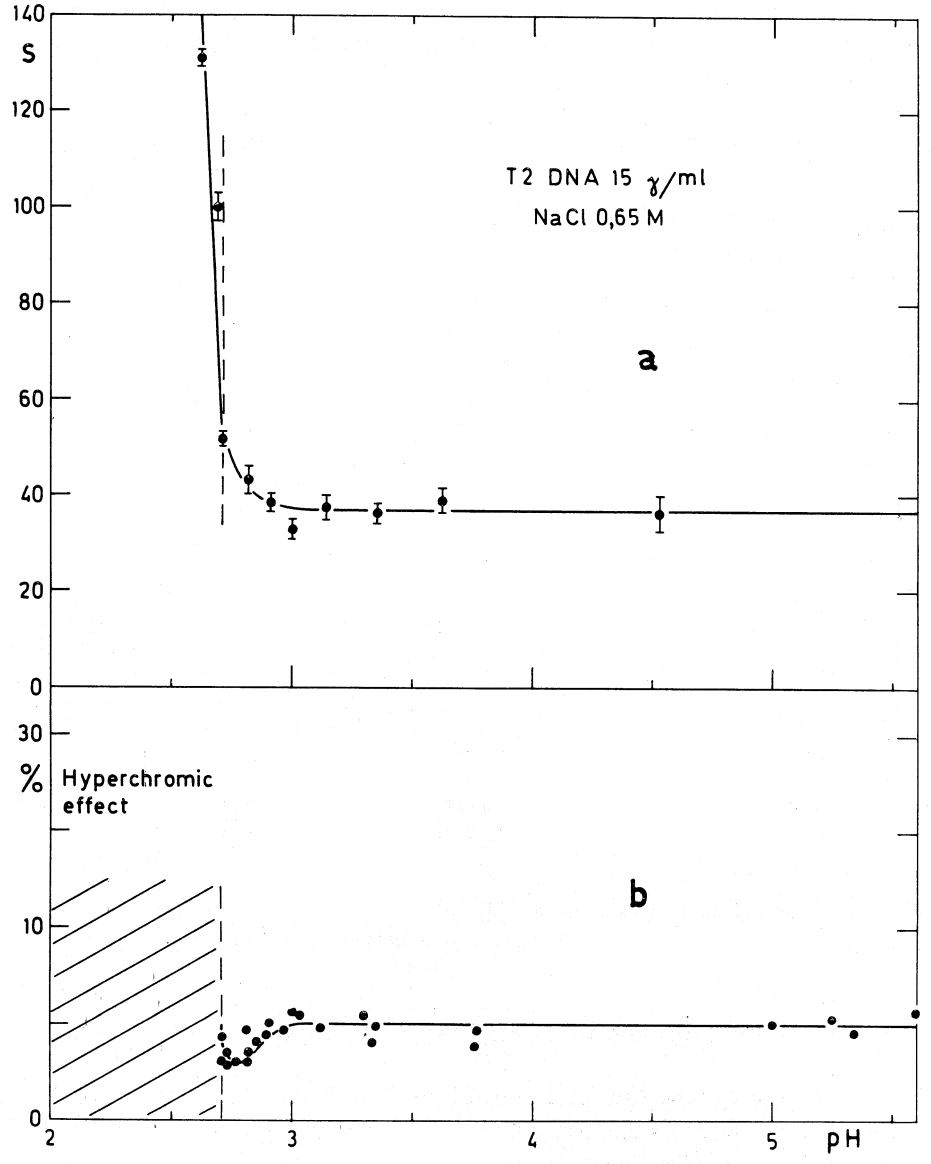

Fig. 6. – *a*) Sedimentation coefficient and *b*) hyperchromic effect as a function of $\not{p}$H for T2 DNA (15 μg/ml) in 0.65 M NaCl. Aggregation does not allow O. D. measurements to be made in the region corresponding to $\not{p}H < 2.7$.

If, in the phase diagram of fig. 7, we put a black dot whenever the $\not{p}$H and NaCl molarity are such as to give visible precipitates, we obtain the diagram shown in fig. 9. The continuous line is the one defined by the mid-point of hyperchromic transition and the sedimentation peak (see fig. 7) and divides the plane into the two regions of native and denatured DNA. The dashed line divides the native region into two zones, the upper one cor-

responding to DNA suspended in solution, the other to precipited DNA. The intersection of this line with the one dividing native from denatured DNA is at *p*H 2.60, NaCl 0.65 M.

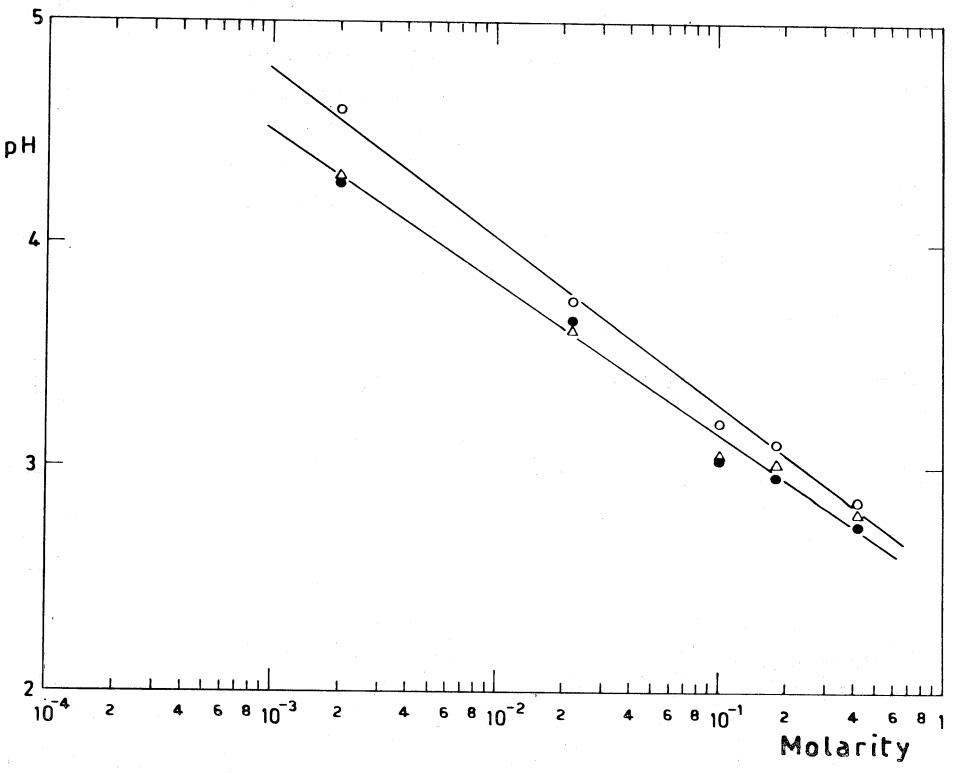

Fig. 7. – ○ Hypochromic minimum; ● midpoint (50%) of hyperchromic transition (pH_m); △ sedimentation peak derived from figs. 1–5, reported in the *p*H, molarity diagram.

Reversibility of aggregation and " in vitro " condensation.

Visibly aggregated DNA (20 μg/ml in 1 M NaCl, brought to *p*H 2.50) resuspended in 0.1 M NaCl, shows the CsCl density and the melting profile characteristic of native (figs. 10–11). It seems, therefore, that there is at least a certain region of the phase diagram that corresponds to DNA precipited in the native state. The transition seems to be reversible, but it must be said that resuspension under the conditions given above is never complete, probably because of entanglement of different molecules.

The problem is now that of understanding what has been altered in the native structure so that it becomes capable of forming many inter-(and probably intra-) molecular bonds. A possible interpretation is that the protonated sites in the DNA molecule become involved in the formation of many new bonds both both inter- and intra-molecularly (as well as with different substances) provided that the ion concentration is sufficiently high as to

shield electrostatic repulsions. Alternatively, one might think that the role of the high NaCl concentration is that of lowering water activity in the solution and bringing DNA to a dehydrated condition [7].

In any case, it is possible to put forward the following working hypothesis: whatever the mechanism leading to precipitation of visible aggregates, we suppose that when DNA concentration is sufficiently low as to avoid interaction between different molecules, the same mechanism leads to monomolecular condensation.

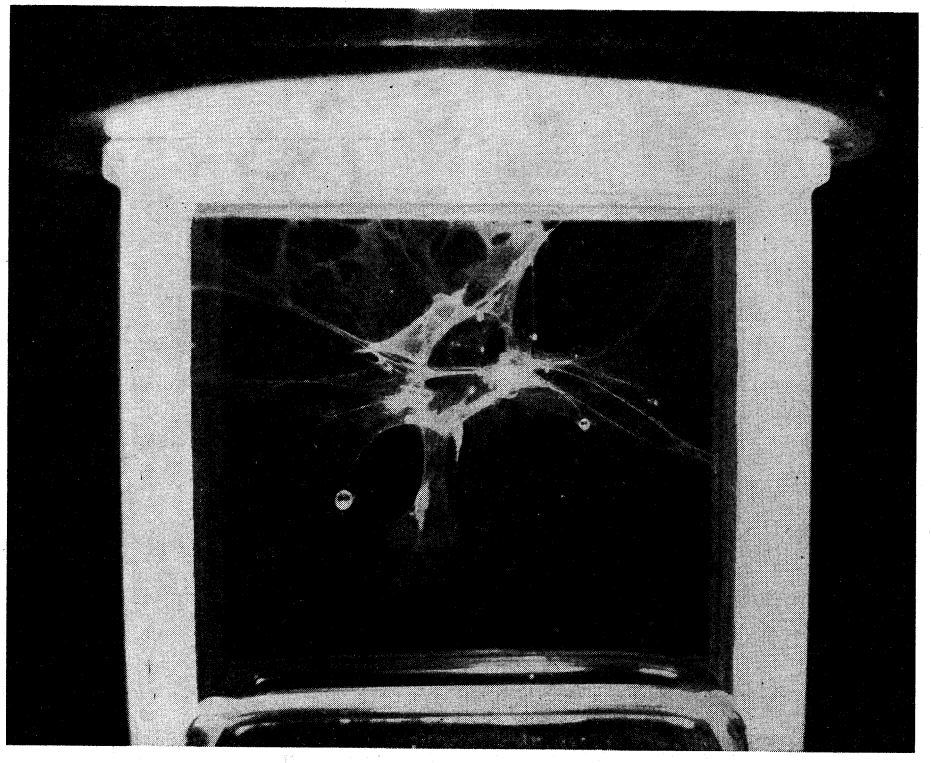

Fig. 8. – DNA precipitate, obtained by adding DNA to a final concentration of 100 μg/ml to a 1 M NaCl solution, at *p*H 2.30.

In order to obtain O.D. measurements not affected by intermolecular aggregation, (i.e. not dependent on concentration) we found it necessary to work at DNA concentrations lower than 4 μg/ml. Fig. 12 shows the O.D. curve obtained at fixed NaCl molarity (1 M) and at a DNA concentration 0.4 μg/ml in cuvettes of a 10.0 cm optical path. In these, as in the following measurements, DNA to be added to solutions having different *p*H and the same NaCl concentration was first equilibrated at the same molarity.

The two transitions observed in fig. 12 can tentatively be ascribed to condensation followed by denaturation. Both effects, according to this ten-tentative hypothesis, are accompanied by loss of hypochromism, so that the

the final plateau value corresponds to an increase over the native value of 60–70 %.

But, if condensation and denaturation are two independent processes which can take place one after the other, their effect being additive, one should expect a condensation transition for denatured DNA too. This expectation is fulfilled, as shown in fig. 13, where the hyperchromic effect of a solution of acid-melted DNA at pH 1.9 is shown as a function of NaCl concentration, for two different DNA concentrations.

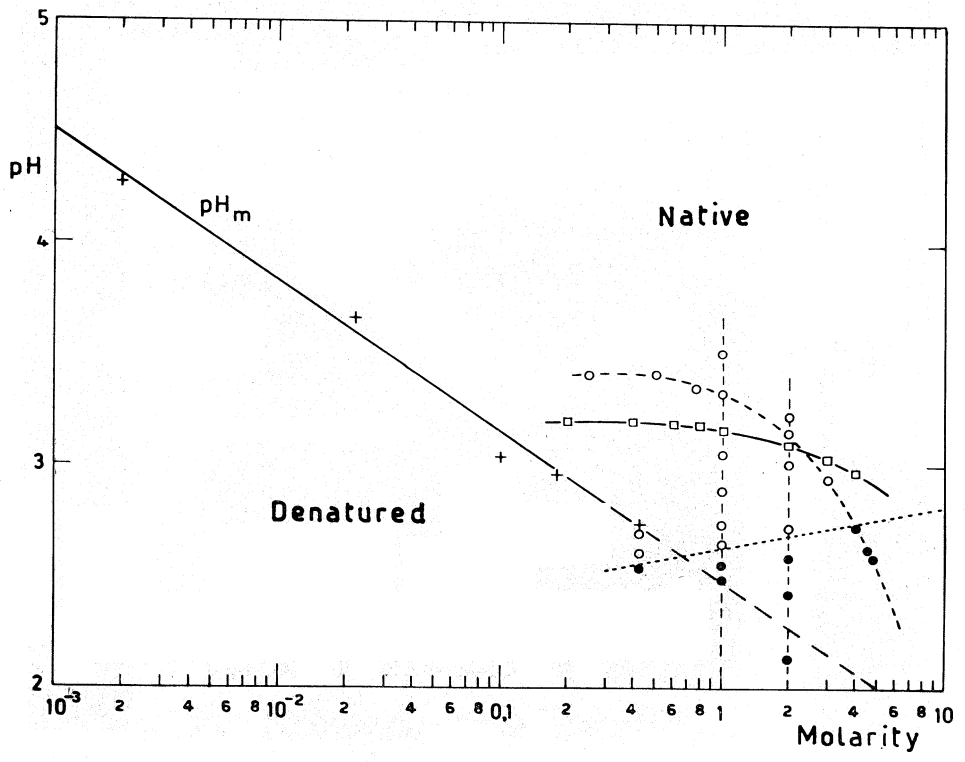

Fig. 9. – + $p\mathrm{H}_m$ derived from Figs. 1–5; black dots indicate pH and NaCl molarity conditions in which at DNA concentrations of 20 μg/ml aggregation is visible.

The curve was obtained by measuring the optical density of separate samples containing the same amount of HCl and different concentrations of NaCl. DNA, previously denatured by alkali and rapidly neutralized, was added to each sample to give a final concentration of 0.8 and 2.1 μg/ml respectively. Cuvettes of a 10.0 and 2.0 cm optical path were used for O.D. readings.

It must be said that although the HCl concentration was the same in every sample, the pH values measured with E2 glass and standard calomel electrodes in the Beckman Research pH-meter were not constant, but varied

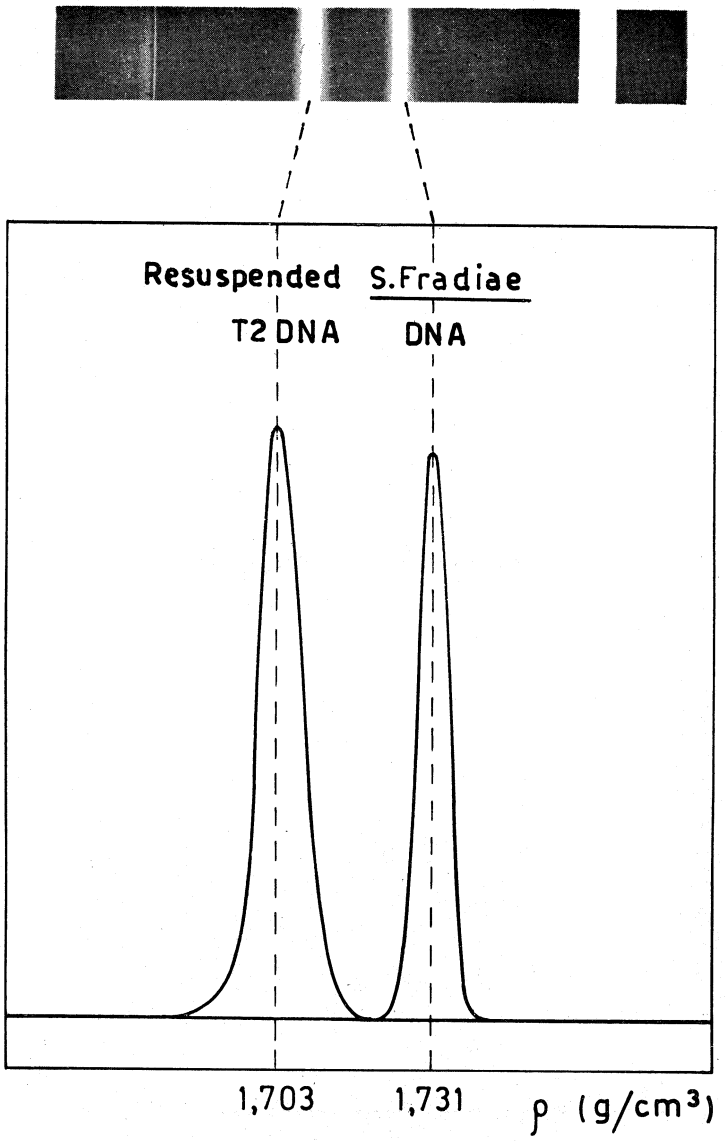

Fig. 10. – DNA precipited in 1 M NaCl, $\not p$H 2.50, resuspended in 0.1 M NaCl $\not p$H 7, bands in CsCl density gradient at density (1.703 g/cm³) almost identical to that of native T2 DNA (1.702 g/cm³). Reference density is provided by DNA from *Streptomyces fradiae*.

in the manner indicated in fig. 13. This is probably an effect due to permeability of the glass membrane to Na^+ ions. No correction was attempted, however, in view of the arbitrary nature of such corrections. The curve in fig. 13 shows that while acid-melted DNA in low ionic strength exhibits a 40 % hyperchromic effect, the increase in NaCl molarity brings about a transition to a final value of about 58 % hyperchromic effect.

Such transition is reversible: this was easily demonstrated by diluting a sample at the beginning of the plateau region by a factor of 2.50 (exact dilution was made possible by the use if Marburg micropipettes), with an HCl

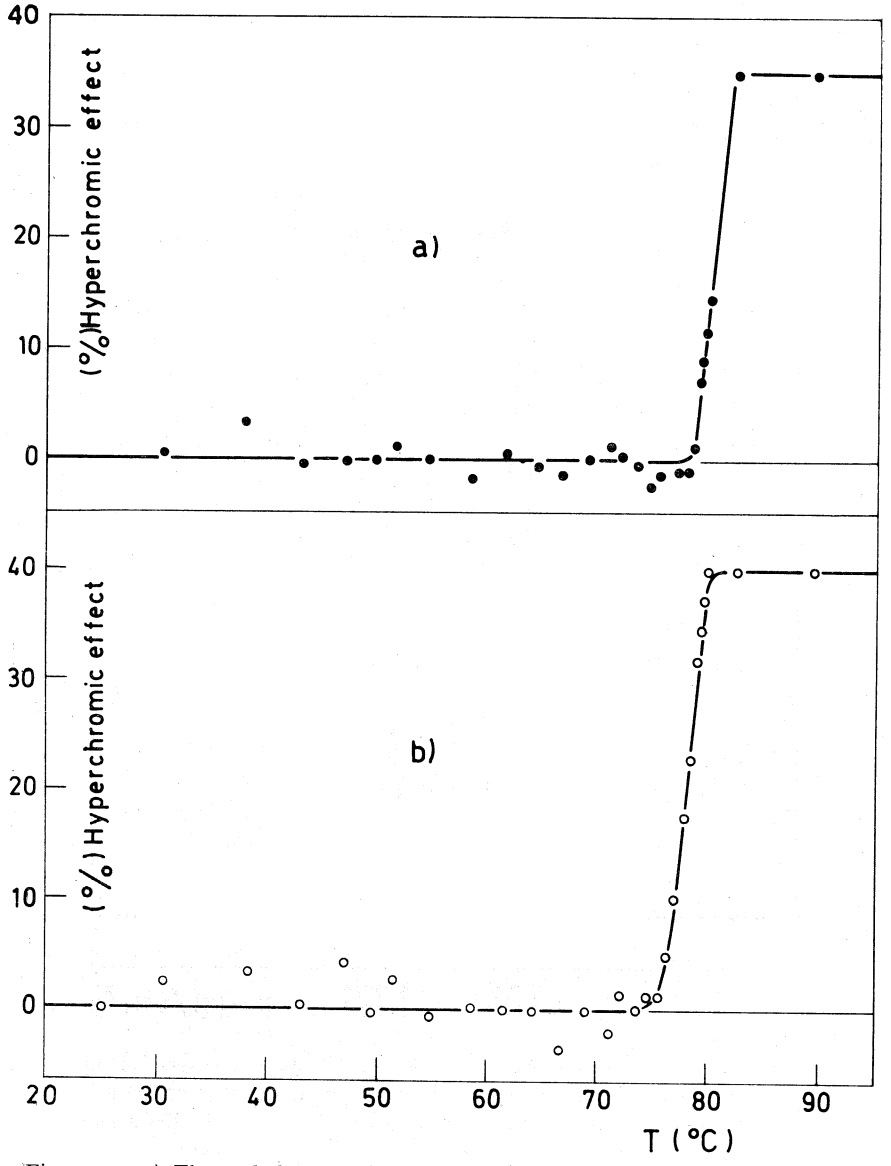

Fig. 11. – *a*) Thermal denaturation profile of native T2 DNA in 0.1 M NaCl. *b*) Thermal denaturation profile of T2 DNA precipited in 1 M NaCl *p*H 2.50, and resuspended in 0.1 M NaCl *p*H 7.

solution having the same concentration as the sample, and by repeating the O.D. measurements in a cylindrical cuvette having a 5.0 cm optical path instead of the 2.0 cm cuvettes previously used. The result is a complete recovery of the O.D. value corresponding to 40 % hyperchromic effect.

A whole set of curves of the hyperchromic effect either at fixed NaCl molarity (e.g. fig. 12) or at fixed HCl molarity (e.g. fig. 13) were easily and reproducibly obtained. The result of such a systematic scanning of the NaCl,

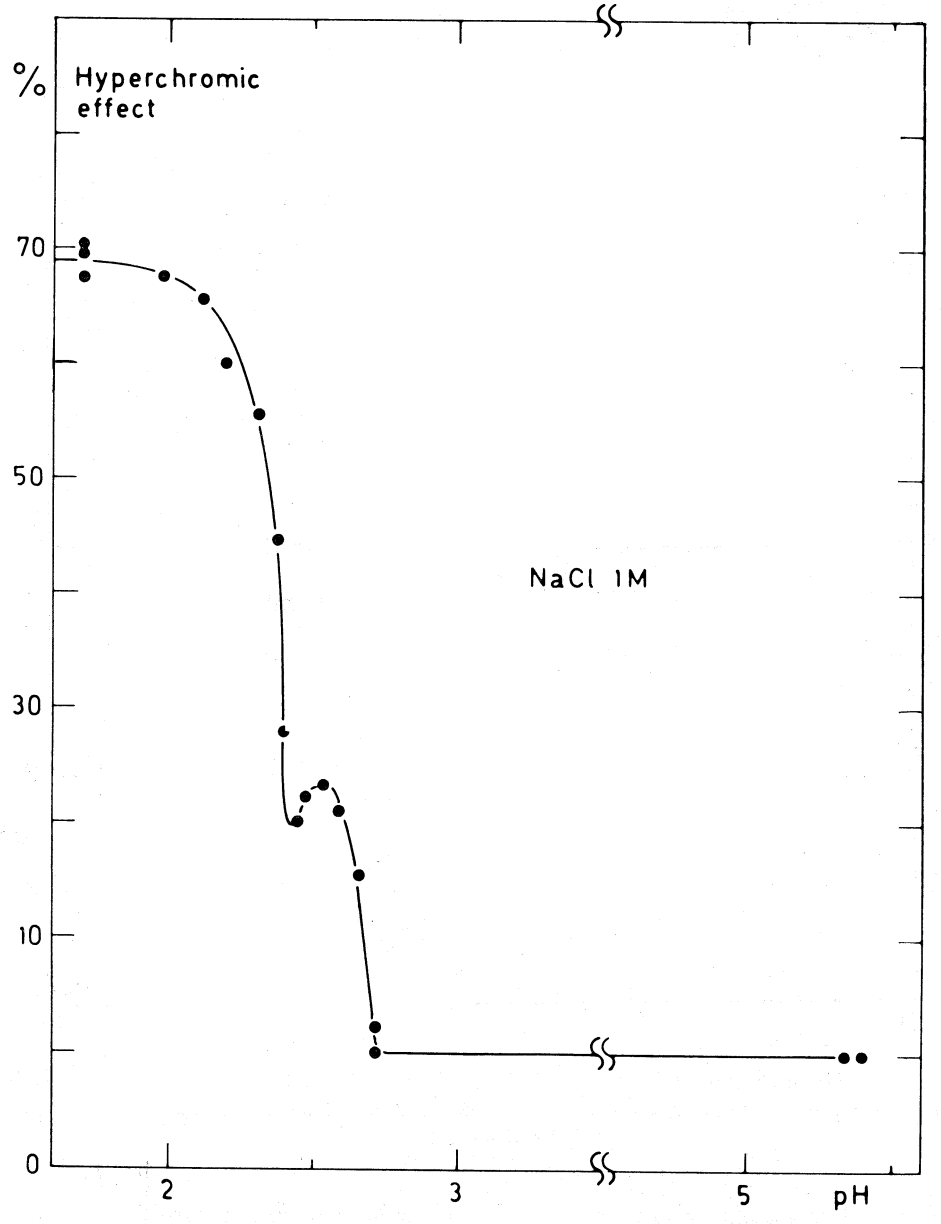

Fig. 12. – Hyperchromic effect as a function of pH for T2 DNA 0.4 μg/ml in 1 M NaCl.

pH phase diagram indicates the existence of three different transitions, which can consistently be interpreted as condensation of native DNA, denaturation of condensed DNA and condensation of denatured DNA. The midpoints

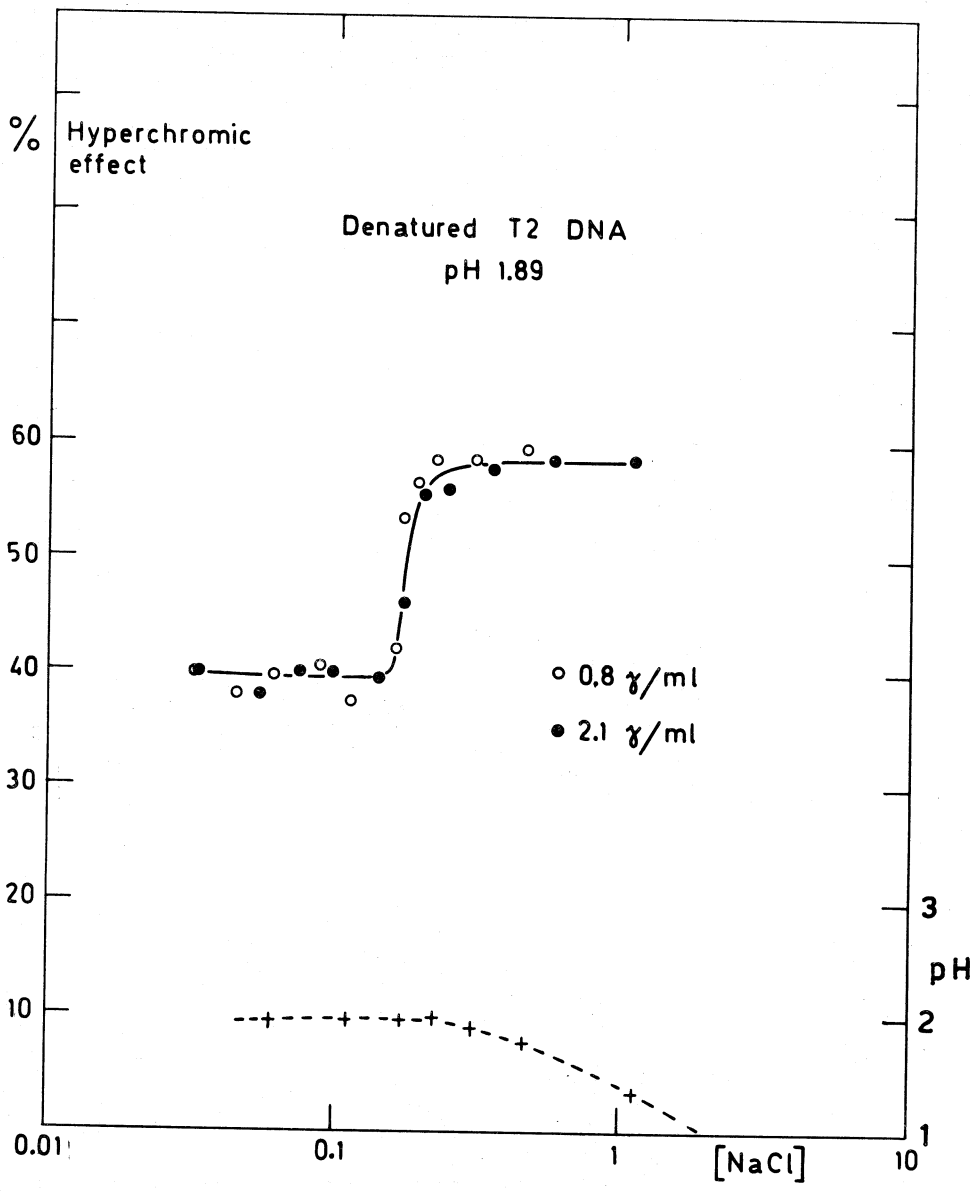

Fig. 13. – Hyperchromic effect as a function of NaCl molarity for alkalidenatured T2 DNA, ● at 2.1 μg/ml; ○ at 0.8 μg/ml. High NaCl concentration influences *p*H reading as indicated by dashed line.

of such transitions are reported in fig. 14, where different symbols are used to indicate points obtained in the different ways listed.

There are several features to be observed in such a graph, apart from the agreement between different sets of experiments.

a) The denaturation midpoints for condensed DNA fall onto the same straight line, already defined by the denaturation points of noncondensed DNA.

b) Although in the region of the square the different transitions are too near to be clearly distinguished one from the other, the interpolated, triple point (*p*H 2.58, NaCl 0.7 M) compares well with the one previously determined on the basis of the observation of visible precipitates.

c) Condensation of native and denatured DNA takes place in a well defined region of the plane, which coincides with the region in which, at higher DNA concentration, visible precipitates appear.

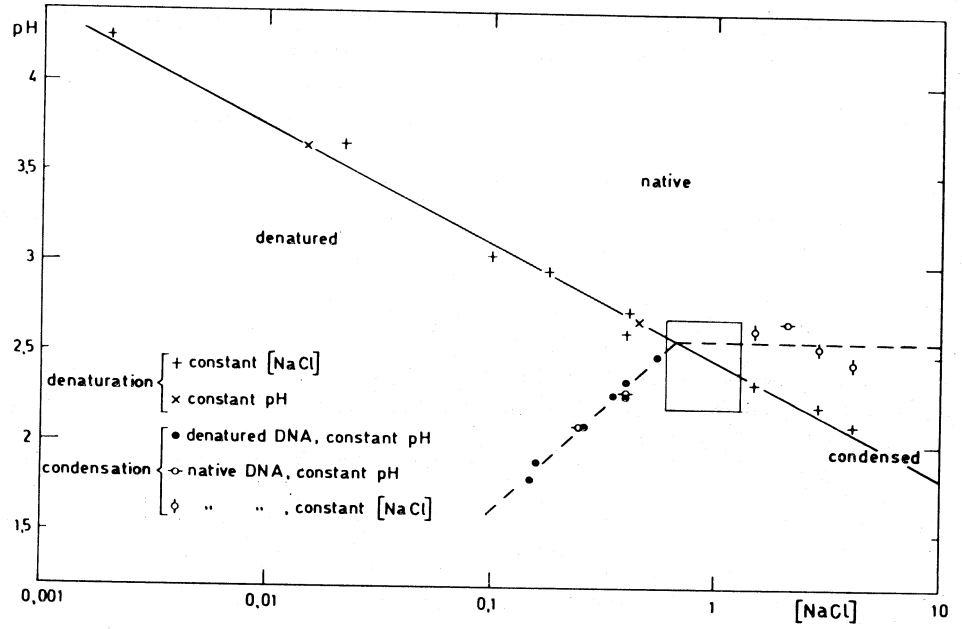

Fig. 14. – Phase diagram showing the regions corresponding to native, denatured and condensation transitions observed on both native and denatured DNA.

CONCLUSION.

It has been shown that, if sufficiently low DNA concentrations are used, the well known aggregation observed at low *p*H and high ionic strength is amenable to a highly reproducible and sharp transition to a new state, characterized by a certain degree of hyperchromism (about 20 %).

The transition can be observed for both native and denatured DNA, and seems to be reversible. The latter observation rules out the possibility (rather unlikely) that the observed level of hyperchromism is due to hydrolysis.

In the phase diagram (*p*H, NaCl molarity) in which we have drawn a straight line dividing the region of native from that of denatured DNA, the midpoints of the new transition observed for native and denatured DNA respectively define two straight lines. These two lines intersect the previously defined denaturation line in a triple point, located at *p*H 2.58, NaCl = 0.7 M. The denaturation line continues across the region thus identified with the

same slope. This is, however, probably a mere coincidence, given the uncertainties in the *p*H measurements in a high NaCl concentration.

We have tentatively interpreted this new state as similar to the condensed state observed in some " in vivo " situations. This interpretation must be confirmed by sedimentation measurements and ORD studies. The tentative hypothesis proposed is attractive, however, and immediately suggests a possible model of action for the condensing enzyme whose existence was postulated to explain " in vivo " condensation. This enzyme should be able to orderly protonate definite sites in the molecule, so as to create the possibility of new bonds with other points of the same molecules, or with the phage proteins. The coarse aggregation phenomena observed " in vitro " could, on the other hand, be due to the simultaneous and disordered protonation of every possible site in the molecule.

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