## The Binding of Extra Histone and Protamine to Deoxyribonucleoprotein

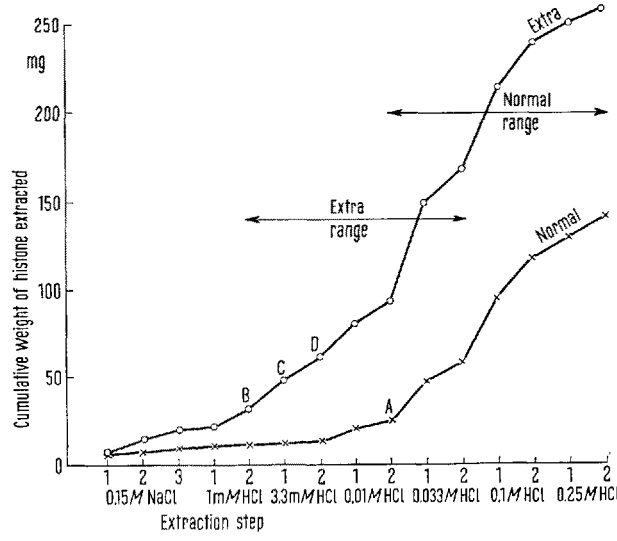
The nature of the binding of histones to DNA is of great interest in understanding the structure of chromatin and the expression of gene action. There is no doubt that positive groups in the histones are bound by salt linkages to phosphate groups on DNA, and the structure of the nucleoprotein is heterogeneous, quite apart from the continuous variation of nucleic acid bases along the length of the complex. In considering this structure, the question arose as to whether the nucleoprotein, as normally prepared, was saturated with histones or whether more binding sites were still available.

To study this, nucleoprotein from calf thymus was prepared by repeated cold saline washing. Such nucleoprotein has an N/P weight ratio of 4–4.4:1, and about 90% of the protein present is histone (DAVISON and BUTLER[1]). Only traces of these histones are removed by washing with 0.1–0.15 *M* NaCl. When this nucleoprotein was mixed with a solution of whole histone in 0.15 *M* NaCl, the extra histone was immediately removed from solution, and a series of experiments showed that an amount of histone at least equal to that already naturally present could be taken up. The extra histone, like the original, was not removed by further saline washing. If a great excess was added to the nucleoprotein, then that not taken up was enriched in the very lysine-rich type, F1. This behaviour is similar to that observed by JOHNS and BUTLER[2] on adding free DNA to an excess of whole histone.

An indication of the nature of the binding of this extra protein was shown by stripping off the histone again with acid. The saline-washed nucleoprotein-histone complex was extracted by stirring in the cold 3 times with 1 m*M* HCl, and then successively in the same way twice with 3.3 m*M*, 0.01 *M*, 0.033 *M*, 0.1 *M* and finally 0.25 *M* HCl. A similar extraction was done with a sample which had not received extra histone. The proteins were recovered from each supernatant, weighed, and the components identified from their patterns on starch and acrylamide gel[3] electrophoreses, and in some cases by amino acid analyses. The results are shown in the Figure. In the normal case, no histone was removed from the system until the 0.01 *M* HCl extraction began, the sample A being almost entirely histone fraction F1. With 0.033 *M* HCl, fraction F2b was also removed and so on. Where extra histone had been added however, the F1 fraction (sample B) appeared with the third extraction with 1 m*M* HCl, and the following samples C and D extracted by 3.3 m*M* HCl had fractions F2a and F2b in them.

The extra histone bound by the nucleoprotein could thus be removed by acid of about $^1/_{10}$ the concentration required to remove the same fractions from normal nucleoprotein. This corresponds to binding to an acid with a pK about 1 unit higher than usual, which suggests that most of it is bound to carboxyl groups rather than to phosphate groups. In view of the quantity of extra histone bound, the carboxyl groups chiefly involved must be those of the histone already present. After allowing for amide groups, since there are on average about half as many free carboxyl groups on the histones as there are positive charges, the saturation value for this type of extra binding would be an amount of histone equal to that already present, which is approximately the value found. It was also found that protamine (salmine) was readily bound to nucleoprotein from saline solution, and as in the histone experiment, most of it was easily removed by HCl in the range $10^{-3}$ to $10^{-2}$ *M*. However, about 10% of the added protamine was strongly bound and was only removed by 0.25 *N* HCl. The protamine could be easily distinguished from the histones on acrylamide gel electrophoresis and no histone was displaced from the nucleoprotein by this protamine binding. Conversely, the nucleoprotein was found to bind only a small amount of extra DNA under the same conditions. The limit was about 5% of the DNA already present.

Although there are presumably some gaps in the array of histones around DNA in nucleoprotein, such that RNA-polymerase or DNA-ase can approach the DNA closely, these results suggest that there are few sites available for binding cationic proteins in the same manner as the original histones, which have molecular weights of 10,000–25,000. The protamine experiment showed definite strong binding, probably to phosphate groups. In this case the protein has a molecular weight of only 4000 (see e.g. PHILLIPS[4]) and presumably could reach more restricted sites on the DNA. This does not conflict with the reports of considerable cationic dye binding to the nucleoprotein (DAVISON and BUTLER[1]; MIURA and OHBA[5]) since the dye is much smaller. The methods employed here however, would not detect firmly bound extra histone if it was less than about 20% of that added. The parallelism of the 2 curves in the Figure in the last 4 or 5 extraction




Removal of added histone from deoxyribonucleoprotein (DNP) (o——o), compared with removal from normal DNP (x——x) by successive extractions with HCl. In this example, the DNP obtained from about 5 g of calf thymus (see the text) was used in each case and 150 mg of calf thymus whole histone added to one sample. Histone first appeared in the samples marked A and B (very lysine-rich histone, type F1). The normal complement of histones and the extra histones were completely removed within the ranges shown by horizontal arrows (←——→). There were small losses in the recoveries of the 15 precipitates in each case.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steps, suggests that the proportion of strongly bound extra histones is in fact far less than this[6].

*Résumé.* La déoxyribonucléoprotéine (DNP) du thymus de veau peut lier de l'histone supplémentaire en solution physiologique. La plupart de cette protéine est très faiblement liée et s'est degagée par HCl de 3,3 m*M* ou moins. On suggère ainsi que l'histone supplémentaire soit combinée aux groupes carboxyl des histones originales du DNP. Néanmoins, environ 10% de la protamine, ajouté pareillement au DNP, fut liée fortement ce qui suggère qu'elle peut atteindre les groupes phosphates libres sur l'ADN. La protamine apparemment n'a pas deplacée les histones lorsqu'elle fut liée au DNP.

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## The $H_2O_2$-Production by Polymorphonuclear Leucocytes During Phagocytosis

In guinea-pig[1–6] as well as in human[7] polymorphonuclear (PMN) leucocytes, an increased rate of oxidation of $NADPH_2$ by a granule bound KCN-insensitive oxidase has been demonstrated during phagocytosis.

A $H_2O_2$-production associated with the stimulation of respiration in phagocytosing PMN leucocytes has been argued on the basis of an increased oxidation of formate[8–12], which is known to be oxidized by peroxidase or catalase in the presence of a $H_2O_2$-producing system[13]. In the present study, direct evidences for $H_2O_2$-accumulation during phagocytosis have been provided. Guinea-pig leucocytes (95% PMN) were obtained from peritoneal exudates and human blood leucocytes (70% PMN) were separated from red cells by dextran sedimentation[14]. When needed the leucocyte suspensions were purified from residual erythrocytes by means of a rapid hypotonic haemolysis[15]. The results presented here refer to the experiments with human PMN leucocytes. Quite similar results have been obtained by using guinea-pig leucocytes. Phagocytosis was performed by adding bacteria (*Bacillus subtilis*, filamentous forms, killed by heating 30 min at 120 °C, opsonized with fresh homologous serum 30 min at 37 °C and washed in saline) to a leucocyte suspension in calcium-free Krebs-Ringer phosphate solution incubated at 38 °C in a closed plastic chamber equipped with a Clark oxygen electrode (Yellow Spring Instr. Co., Ohio, USA). Experiments of phagocytosis were also performed in shaken Warburg vessels at 38 °C for 15 min and thereafter the leucocyte-bacteria mixtures were transferred into the plastic chamber. The $H_2O_2$ has been measured as oxygen liberated after adding catalase (Sigma).

The membrane coating the platinum surface of the electrode was shown to be impermeable to $H_2O_2$ according to the procedure described by Rorth and Jensen[16]. It has been preliminarily shown that small amounts of $H_2O_2$ were quantitatively recovered as $O_2$ liberated by catalase added in excess (Figure 1) and that endogenous catalase of intact leucocytes, measured as $O_2$-production from added $H_2O_2$, is almost completely inhibited by 2 m*M* KCN (Figure 1).

The addition of catalase to PMN leucocytes during phagocytosis in the presence of 2 m*M* KCN causes a liberation of oxygen and a modification of the rate of the oxygen uptake, indicating that an appreciable amount of $H_2O_2$ was accumulated (Figure 2 a, b). When catalase is added to PMN leucocytes during phagocytosis in the absence of KCN, the liberation of oxygen is hardly detectable (Figure 2 dotted traces).

In other experiments leucocyte-bacteria mixtures were incubated 15 min at 38 °C with or without KCN and then transferred to the plastic chamber for the measurements of $H_2O_2$ as indicated above. Figure 3 shows that after 15 min of phagocytosis an accumulation of $H_2O_2$ takes place only when KCN is present. These findings directly indicate that a $H_2O_2$-forming respiratory system is involved in the stimulated respiration of phagocytosing PMN leucocytes.

An approximate calculation of the ratio oxygen consumed/oxygen liberated after catalase, shows that in the early stage after addition of KCN nearly all the oxygen is consumed with stoichiometric accumulation of $H_2O_2$. The values from the experiment of Figure 2b are: 0.2 $\mu$ atoms of oxygen consumed and 0.08 $\mu$moles of $H_2O_2$ decomposed by catalase. In the long term experiments, when the measure of $H_2O_2$ is performed several min after addition of 2 m*M* KCN (Figure 2a) or after 15 min of preliminary incubation of phagocytosing PMN leucocytes at 38 °C (Figure 3), the amount of oxygen liberated by

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