Phosphorylation of Dipteran Chromosomes and Rat Liver Nuclei

Abstract. *Side-chain modifications of the nucleoproteins are believed to be involved in the control of gene function. Rat liver nuclei and chromatin incubated in adenosine triphosphate labeled in the gamma position with phosphorus-32 demonstrated rapid phosphorylation in vitro of the nuclear proteins. Dipteran salivary glands incubated in either labeled adenosine triphosphate or orthophosphate labeled with phosphorus-32 showed that there is phosphorylation of chromosomal protein. The phosphorus is associated in protein from both liver and salivary gland nuclei predominantly with phosphoserine.*

It is believed that all somatic cells possess the total genetic information characteristic of the organism. The mechanisms involved in the selective expression of this information are unknown. Some of the proteins of the nucleus seem to be involved in this process (*1*). Histones, the basic proteins of the nucleus, may be involved in the control of gene expression, but for many cogent reasons it is thought unlikely that their primary structure alone possesses sufficient specificity for the complete control of the genetic apparatus (*2*).

The nonhistone nucleoproteins are also good candidates for participation in the control of gene expression. With the availability of more homogeneous nucleoprotein preparations, it has been demonstrated that there are acetyl, methyl, and phosphoryl side-chain alterations of both the histone and nonhistone nucleoproteins (*3*). Such enzymatic modifications of nucleoprotein may be involved in the control of gene expression. Also, side-chain modification may have biologic relevance, because one of the earliest responses of the lymphocyte to phytohemagglutinin is an increased phosphorylation of the nucleoproteins (*4*).

The nonhistone nuclear protein fraction is rich in phosphorus, is associated with DNA, has a higher turnover rate than the histones, and is thought to be associated with the puff regions of dipteran polytene chromosomes (*5*). To elucidate the biologic significance of nucleoprotein phosphorylation, we studied the phosphorylation in vitro of rat liver nuclei and the phosphorylation of chromosomal phosphoprotein by the surviving dipteran salivary gland.

Rat liver nuclei and chromatin were prepared with standard techniques (*6*). Nuclei were incubated at 30°C with adenosine triphosphate (ATP) labeled in the gamma position with ^{32}P (*7*) (0.1 mc/ml) in buffer containing 0.02*M* tris-(hydroxymethyl) aminomethane-hydrochloride, *p*H 8.5, 5 m*M* $MgCl_2$, 5 m*M* NaCl, and 0.5 m*M* ATP. In the incubations carried out at *p*H 9 and above, a 0.02*M* taurine buffer was used. ^{32}P-Orthophosphate (*7*) was not a suitable substrate, as ATP is presumably not generated by liver nuclei prepared in this manner. The nuclei were processed (*3*) to remove lipid and nucleic acids.

There is rapid phosphorylation in vitro of the proteins of liver nuclei. The incorporation was linear with time for the first 10 minutes, increasing from 0.27 to 0.85 mmole per milligram of protein at 2 and 10 minutes, respectively. The rate of phosphorylation was constant over a nuclei concentration ranging from 80 to 250 mg of DNA and was 2.2 mmole per milligram of DNA. Magnesium is an essential requirement for phosphoprotein phosphorylation in vitro. The rate of phosphorylation varied with the *p*H of the incubation medium, being 65 percent greater at *p*H 9.5 than at *p*H 6.5. Sharp decreases in rate occurred below *p*H 6.5 and above *p*H 9.5. The finding that the *p*H optimum is on the alkaline side is similar to that reported on the synthesis of RNA by isolated nuclei (*8*). The rate of phosphorylation of phosphoprotein with chromatin incubated with ^{32}P-labeled ATP is similar to that of the nuclear preparation. Acid hydrolysis of the labeled nuclear proteins, followed by high-voltage electrophoresis (*9*), demonstrated that phosphorus is associated predominantly with phosphoserine and to a lesser extent with phosphothreonine.

When rat liver nuclei were incubated with ^{32}P-labeled ATP for 10 minutes and then incubated for up to 30 minutes in the presence of unlabeled ATP, the specific activity of the isolated phosphoprotein remained constant. Perhaps an explanation for these results, which are at variance with ^{32}P-incorporation experiments with thymus nuclei (*3*), is that the total phosphorus pool of the phosphoproteins is large in liver compared with the amount turned over. A decrease in radioactivity would, therefore, not be noted by the design of these experiments. Of course the nuclear isolation procedure or other factors may have interfered with the normal metabolism of the phosphoproteins in such a way that turnover was impaired. Therefore, isolated nuclei or chromatin possess proteins which may be phosphorylated in vitro, but the results do not provide evidence for implicating these proteins in the control of gene expression.

To determine whether nucleoprotein phosphorylation is involved in the control of gene expression, we studied the phosphorylation of the proteins of the giant chromosomes of the dipteran salivary gland, because in these interphase cells the polytene chromosomes contain regions which puff and are considered to be actively engaged in gene activity, that is, synthesis of RNA (*10*).

Salivary glands of late 4th instar *Sciara coprophila* larvae were incubated at 20°C in a modified Cannon buffer (*11*) with either ^{32}P-orthophosphate (1 mc/ml) or ^{32}P-labeled ATP (100 μc/ml). They were then rinsed, briefly fixed in 45 percent acetic acid, and squashed in 1 percent lactic acid–orcein; the slides were then processed and prepared for autoradiography by coating with Ilford L-4 emulsion, 2 to 5 dilution (*12*). Lipid was removed by ether-alcohol and chloroform-methanol extraction (*9*). Histone was removed by acid extraction before formalin fixation (*13*). Nucleic acids were extracted by extensive treatment with hot trichloroacetic acid (TCA) with agitation. Nucleic acid extraction was monitored by incubating the glands with H^3-uridine or H^3-thymidine (*14*) (10 μc/ml for 10 minutes) and by demonstrating that tritiated material was removed by the hot TCA treatment. In addition, the completeness of the extraction procedures was assessed by the alkaline fast-green stain for histones (*15*) and with a modified Feulgen reaction (*16*). All preparations treated with hot TCA were Feulgen negative, and the chromosomes could be visualized only with phase microscopy.

Autoradiograms of salivary glands incubated with radioactive phosphate and then processed (*17*) had numerous grains over the chromosomes both in puffed regions and regions without puffs (Fig. 1, a and b). Thus, in preparations in which histone, lipid, and nucleic acid had been removed, active phosphorylation of the nonhistone proteins of the chromosomes was demonstrated. Nuclei from many glands which had been incubated with ^{32}P-orthophosphate were prepared, and the phosphoprotein was extracted and hydrolyzed in acid; the product was separated by high-voltage electrophoresis (*9*). Radioactivity was associated with the phosphoserine

and phosphothreonine of the sample. Therefore, there is active phosphorylation of the chromosomal protein by the surviving tissue, and the phosphorus is predominantly associated with the phosphoserine. Quantitation of the extent of phosphorylation is difficult because there is an indeterminate loss of phosphorylated amino acid as a result of the acid hydrolysis (*9*).

Salivary glands were incubated with ^{32}P-labeled ATP and processed to remove histone, nucleic acids, and lipid. The chromosomes of glands incubated (30 minutes) with ^{32}P-labeled ATP were extensively labeled (Fig. 2). Because the substrate was labeled in the gamma position and was of relatively low specific activity and because the incubation medium contained phosphate ions, it is concluded that the phosphorus from ATP was transferred to the chromosomal phosphoprotein. Glands in which nucleic acid was not extracted were not noticeably more labeled than those from which nucleic acids had been removed with hot TCA.

To relate phosphorylation to gene activity, short-interval incorporation studies were undertaken. Salivary glands were incubated for 30 seconds and 1, 2, and 4 minutes and then processed. After such intervals, radioactivity was discretely localized to some segments of the chromosomes. With increasing incubation times the radioactivity was more diffusely spread over the surface of the chromosome. When glands were incubated with the labeled compound for 30 seconds (Fig. 3a) and then with the unlabeled compound for 9 minutes and 30 seconds, the radioactivity appeared to come off the chromosomes (Fig. 3b). Confirmatory studies with ^{32}P-labeled ATP as substrate in incubations ranging from 1 (Fig. 4) to 30 minutes (Fig. 2) showed that the pattern of labeling changed from a discrete to a diffuse one.

There is active phosphorylation of the proteins of both dipteran chromosomes and rat liver nuclei and chromatin. Therefore, one of the prerequisites for implicating a class of proteins in the control of gene action has been fulfilled, that is, the nonhistone phosphoproteins undergo active metabolism at the sites of RNA synthesis. These proteins may be involved in the control of gene action.

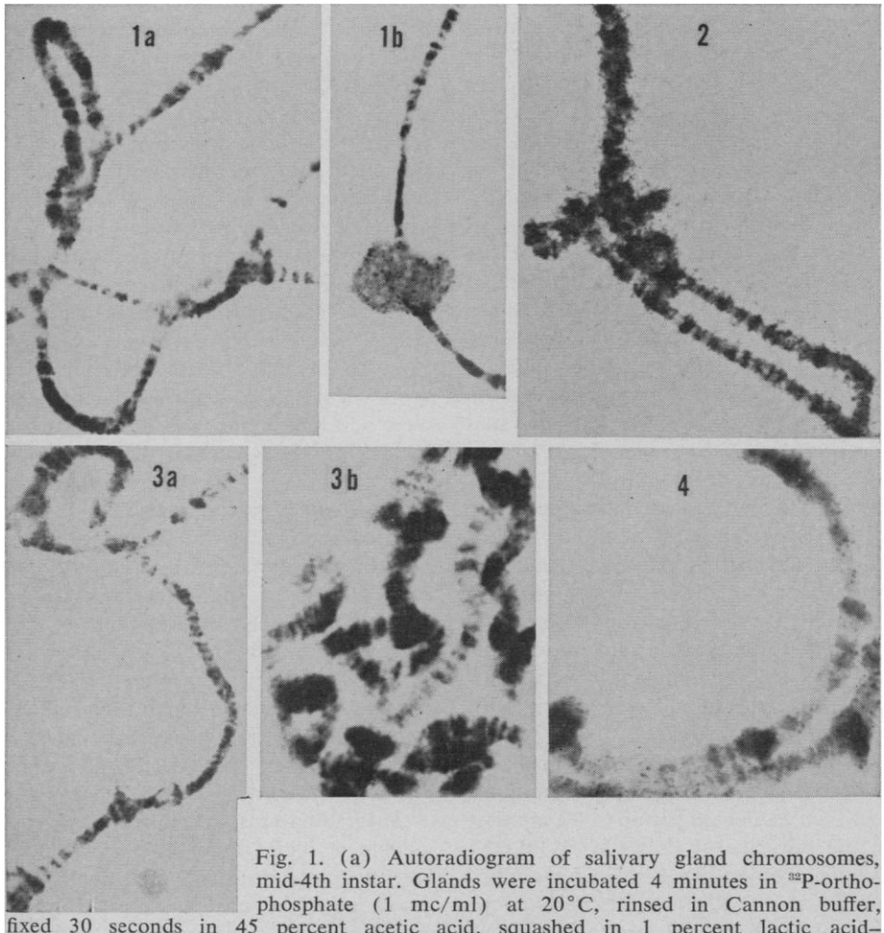

Fig. 1. (a) Autoradiogram of salivary gland chromosomes, mid-4th instar. Glands were incubated 4 minutes in ^{32}P-orthophosphate (1 mc/ml) at 20°C, rinsed in Cannon buffer, fixed 30 seconds in 45 percent acetic acid, squashed in 1 percent lactic acid–orcein, and processed for autoradiography (*17*). Note grains discretely localized over bands (× 1092). (b) Autoradiogram of salivary gland chromosome, late 4th instar, incubated 10 minutes in ^{32}P-orthophosphate (1 mc/ml) and processed as described in (a). Note grains in puffed region (× 1092). Fig. 2. Autoradiogram of salivary gland chromosomes after being incubated (30 minutes) in ^{32}P-ATP gamma and processed as described in Fig. 1a. Note extensive labeling along chromosomes (× 1092). Fig. 3. Salivary glands incubated in ^{32}P-orthophosphate. (a) Autoradiogram of chromosomes processed as described in Fig. 1a after incubation for 30 seconds (× 1092). (b) Autoradiogram of salivary gland chromosomes incubated 30 seconds in ^{32}P-orthophosphate. Immediately after incubation glands were transferred to Cannon medium containing 0.02*M* NaH_2PO_4 for 9 minutes and 30 seconds. The chromosomes are virtually unlabeled (× 1092). Fig. 4. Autoradiogram of salivary gland chromosomes incubated 1 minute in ^{32}P-ATP (100 μc/ml) and processed as described in Fig. 1a. Note the discrete labeling pattern as compared with the ^{32}P-ATP incubation (30 minutes) in Fig. 2 (× 1092).

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17. After the cover slip was removed with liquid nitrogen, slides were passed through absolute alcohol, ether-alcohol, and chloroform-methanol (for removal of lipids), and then hydrated to saline. Slides were then placed in 0.2*N* HCl (20 minutes, cold) for histone removal, in 10 percent TCA (90°C, 20 minutes), dipped in 70 percent alcohol and 5 percent TCA, and rinsed under running tap water. Slides were fixed for 5 minutes in 10 percent neutral formalin, rinsed in saline, and processed for autoradiography as follows: slides were dipped individually in 2 to 5 dilution of Ilford L-4 nuclear track emulsion, dried overnight, and stored in refrigerator. Slides were developed in D-19 (5 minutes), rinsed, and fixed in hypo. Photomicrographs were taken on the Leitz Ortho-Lux microscope with the Ortho-Mat camera, using Adox KB-14 film.
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Axonal Outgrowth and Cell Migration in vitro from Nervous System of Cockroach Embryos

Abstract. *Brain and ganglia of embryonic* Periplaneta americana *were grown for 2 to 3 weeks in a chemically defined medium. Nerve fibers sprouted vigorously from intact brains and ganglia, interconnecting nerve cultures, and adjacent cultures of the digestive tract. Glia, tracheal, and nerve cells were present in large numbers in the migratory zone surrounding the explants.*

We report the successful in vitro culture of the brain, the ganglia, and the gut of embryonic cockroaches. Success in this case hinged on the discovery of a suitable culture medium consisting of five parts of Schneider insect solution (Grand Island Biological Company) to which was added four parts of Eagle basal medium (Microbiological Associates). The two solutions were mixed just prior to use.

Roach embryos were obtained from oöthecae that were collected daily and incubated at 29°C to obtain embryos that were 16 to 18 days old. Brains, as well as subesophageal and thoracic ganglia and the entire digestive tract, were surgically removed under aseptic conditions and gently pressed onto a small cover slip. The latter was placed in a culture chamber consisting of glass vessels (internal diameter, 13 mm; height, 7 mm) containing 0.18 ml of the above-mentioned medium. Five to six culture chambers were placed in each petri dish and stored in a desiccator containing 95 percent air and 5 percent carbon dioxide. Incubation was at 29°C in a water-saturated atmosphere, attained by placing wet cotton in the desiccator and in the petri dish. The cultures were examined daily with an inverted microscope. After 1 or 2 weeks, they were studied and photographed by Zeiss-Nomarski optics and then stained with toluidin blue or by a modified Cajal technique (*1*).

Up to 95 percent of the explants showed substantial development, including the outgrowth of axons and the centrifugal migration of other cell types. The following description is based on a study of 500 cultures, each containing 5 to 6 explants.

By the end of the 2nd day of culture, the brains or ganglia showed axonal

Fig. 1. Photomicrograph of living culture taken with the interference differential (Zeiss-Nomarski) microscope, showing nerve cells in the migratory zone around brain explant from a 16-day embryo 12 days in vitro. Large dilatations are evident on the axon of two nerve fibers. Arrow points to junction of the axon of one nerve cell with another nerve fiber; *n*, nerve cells (× 300). Fig. 2. Silver-stained (Cajal-DeCastro) preparation of the migratory area around brain explant from an 18-day embryo 9 days in vitro. Nerve fibers are encrusted on the surface of a large glial cell. To the left, arrows point to a fragment of glial cytoplasm adhering to nerve fibers (× 640). Fig. 3. Photomicrograph of living culture taken with the interference differential (Zeiss-Normarski) microscope, showing migratory area around brain explant of a 16-day embryo 15 days in vitro; *n*, nerve cells (× 256).