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Segregation of Histone Fractions from Purified Rat Pancreas Nuclei by Isoelectric Focusing (39220)

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(Introduced by M. Lipkin)

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The interaction of nucleoproteins with DNA has been suggested as a possible mechanism for the control of gene expression. Biochemical modifications of these proteins can be expected to exert a profound effect on such interaction (1-4). For example, modifications resulting from acylation and phosphorylation decrease the net positive charge of histones and are associated with the diffuse state of chromatin and increased transcription (1-4). On the other hand, reactions which increase the net positive charge of histones, such as methylation, or those leading to dimerization or aggregation, such as oxidation of thiol groups, are correlated with the condensed form of chromatin and decreased RNA synthesis. The variety, extent and rapidity of reactions that modify histones' structure have been studied extensively under various conditions and in several tissues (1-4).

The present report describes, for the first time, the use of isoelectric focusing in polyacrylamide gels for analysis of histones obtained from pure viable nuclei and the high degree of resolution achieved with regard to charge distinctions within the various histone fractions during pancreas development.

Materials and methods. Acrylamide (electrophoresis grade), *N,N,N',N'*-tetramethylenediamine (TEMED), and *N,N'*-methylenebisacrylamide (BIS) were obtained from Eastman Organic Chemicals, Eastman Kodak Co., Rochester, N. Y. BIS was further recrystallized from acetone as described by Loening (5). Ampholines 40% pH 3-10, pH 7-9, and 20% pH 9-11 (w/v), were purchased from LKB, Inc., Hicksville, N. Y. All other reagents were of the purified grade.

Animals and their treatment. All rats (CFN) were obtained from the Charles River Breeding Laboratories, Wilmington, Mass. Postnatal rats were obtained from pregnant rats received at midterm. The rats were fed a diet of Purina Laboratory Chow and water *ad libitum*.

Purification of nuclei. Rats were killed by decapitation and the pancreas removed. Portions, 0.1 to 1.0 g, were placed in a stainless steel hand-operated mincer with a screw-driven plunger and were forced through a perforated plate, 1.5 cm in diameter, containing 50 equidistant holes, 1 mm in diameter. This provided for the removal of connective tissue and the uniform disruption of cells. The minced tissue was homogenized in a glass homogenizer with a stainless steel hand-operated pestle (clearance 10/1000 in.) in a medium that consisted of 0.25 *M* sucrose, 5 m*M* Tris-HCl, pH 7.5, 0.2 m*M* $CaCl_2$, 2 m*M* $MgCl_2$, and 0.1 m*M* phenylmethyl sulfonyl fluoride (PMSF). The resulting homogenate was resuspended in a glass homogenizer with a stainless steel hand-operated pestle (clearance 2/1000 in.) and was filtered through three layers of nylon cloth to remove clumps of material consisting mainly of residual connective tissue, disrupted cells, and some amorphous masses of deformed nuclei as determined by light microscopy. The filtrate was centrifuged at 1100*g* × 10 min in a Sorvall RC2-B centrifuge and the sedimented material was suspended in a medium, twice its volume, that contained 0.25 *M* sucrose, 5 m*M* Tris-HCl, pH 7.5, 0.2 m*M* $CaCl_2$, 2 m*M* $MgCl_2$, 140 m*M* NaCl, and 0.1 m*M* PMSF, and was spun at 1100*g* × 10 min. The resulting pellet was layered over 1.7 *M* sucrose, 5 m*M* $MgCl_2$, 0.2 m*M* $CaCl_2$, 5 m*M* Tris-HCl, pH 7.5, 0.1 m*M* PMSF, and was spun at 82,500*g* × 60 min in a SW 27 rotor. The sediment was washed with 10 m*M* Tris-HCl, pH 7.0, 5

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m*M* $MgCl_2$, 0.1 m*M* PMSF, spun at 1100*g* × 10 min, followed by an additional wash with 1 m*M* HCl. The pure nuclei were collected by centrifugation at 1100*g* × 10 min, and were resuspended for future experiments. All purification procedures were carried out at 4°.

Nuclei viability was determined by [^{3}H]ATP incorporation into the acid-insoluble precipitate as a function of time and nuclei concentration at 30°. The assay system consisted of 0.15 *M* KCl, 25 m*M* Tris-HCl, pH 7.8, 5 m*M* $Mg(Ac)_2$, 1 m*M* $MnCl_2$, 2.5 m*M* dithiothreitol, 300 μM ribonucleoside triphosphates, and 150 μM of [^{3}H]ATP in a total volume of 0.25 ml. The reaction was terminated, and the activity was determined as described by Marzluff *et al.* (6).

Isolation of the histone fractions. The purified nuclei were extracted successively for 1, 3, and 12 hr in 0.25 *N* HCl with constant stirring. These were centrifuged at 6000g × 15 min, and the supernatant containing the proteins soluble in 0.25 *N* HCl (PSH) was dialyzed twice, 3 hr each, against 100 vol of 0.4 *M* NaCl, 0.01 *M* Tris-HCl, pH 7.0. Biorex-70 (Bio-Rad Labs, Calif.) was preswollen overnight in 0.4 *M* NaCl, 0.01 *M* Tris-HCl, pH 7.0, and was packed into a column (0.5 × 1.5 cm) which was then preequilibrated with 100 vol of the same buffer. The PSH extract was applied to the column (1 mg PSH/100 mg Biorex-70), and 1-ml fractions were collected until the absorbance at 280 nm reached baseline levels. These fractions were pooled and considered collectively as nonhistone-containing fractions (NHF). The column was then eluted with 2.0 *M* NaCl, 0.01 *M* Tris-HCl, pH 7.0, and the fractions were pooled (Fig. 1).

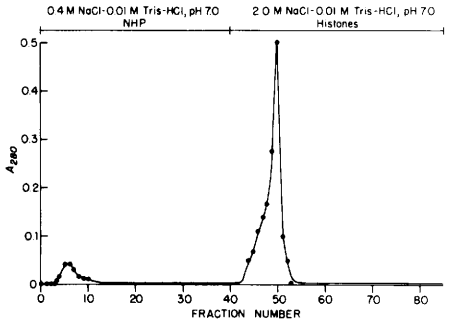

FIG. 1. Separation of histone and nonhistone fractions soluble in 0.25 *N* HCl by Biorex-70.

These were filtered through a column of Sephadex G-75 (Pharmacia) and were regarded collectively as the pure histone fractions. The Hl histone fraction was selectively purified essentially as described by Johns and Butler (7), and the H1 and H1° by removal from acrylamide gels according to Panyim and Chalkley (8). Both the NHF and the histone fractions were dialyzed extensively against 0.9 *N* acetic acid, concentrated by lyophilization and analyzed by means of isoelectric focusing on acrylamide gels and continuous acrylamide gel electrophoresis. Protein was determined according to Lowry *et al.* (9).

Preparation of acrylamide gels for electrofocusing. Pyrex gel tubes (length, 15.0 cm; o.d., 0.8 cm; i.d., 0.6 cm) were presoaked in sulfuric acid–dichromate cleaning solution, rinsed with distilled water, and coated with column coat (Canalco, Rockville, Md.). Each tube was tightly fitted with a rubber cap over one end and placed in a test tube rack in an upright position. A polymer solution, yielding gels whose compositions were 5% acrylmide, 0.16% BIS, 1.1% ampholytes in 6.25 *M* urea each, was prepared by mixing the following proportions of ingredients in order of addition: 4.0 ml of acrylamide–BIS monomer (25% and 0.8%, respectively, w/v), 0.08 ml of TEMED (stock), 10.0 ml of urea (12.5 *M*), 0.1 ml of ampholine (pH 3–10), 0.9 ml of ampholine (pH 9–11), and 4.59 ml of water. This mixture was degassed for 2 min under vacuum, and 0.33 ml of 2% ammonium persulfate was added with mixing. The solution was introduced immediately into the stoppered gel tubes, and the gel solution in each tube was overlaid with a layer of water to give a flat gel surface and provide for uniform polymerization (10). The gels were allowed to polymerize for about 30 min at room temperature and were "cured" overnight at 4°. The above volume of polymer solution was sufficient for casting four gels, each 14.5 cm long (4 ml/gel).

Preparation of electrolyte solutions. Since the histones were applied in their cationic form, the anolyte, 500 ml of 25 m*M* phosphoric acid, was used as the upper electrolyte, and the catholyte, 500 ml of 50 m*M* sodium hydroxide, as the lower electrolyte. The anodal solution was placed in its com-

partment after gel tubes were properly oriented. Electrolyte solutions were kept at 4° prior to use.

Sample application and electrofocusing. Gels were prerun for 30 min at 1 mA/gel, and a sample of 150 to 200 μg of histones in a volume of 200 μl that contained 6.25 *M* urea was carefully layered through the anolyte solution onto the gel top. After sample application had been completed, current was readjusted to 1 mA/gel (four gels per each run). As the ampholytes and proteins approached their isoelectric point, conductivity in the gels decreased with a concomitant rise in voltage. When the voltage reached 400 V (27.6 V/cm), it was maintained at this level for 4 hr at which time the current had fallen to a low, but a constant level of 1.5 mA, and the proteins were focused at their isoelectric point. Total electrofocusing time was 7.5 to 8 hr at 4°.

Staining for proteins. Gels were removed by rimming with water and stained essentially as described by Spencer and King (11). In our hands, better visualization of the bands was obtained by increasing the Coomassie blue concentration to 0.05% (w/v). Gels were destained in acetic acid/H_2O/methanol (5:70:25, by vol) for a period of 48 hr. The intensity of the bands can be accentuated by repeating the staining procedure.

Determination of pH. The pH gradient of each gel was determined by the method of Finlayson and Chrambach (12). Gels were sectioned into 0.2 cm slices, each immersed in 0.5 ml of 0.01 *M* KCl for 1 hr and the pH determined.

Continuous acrylamide gel electrophoresis. Electrophoresis was conducted in 0.9 *N* acetic acid–6.25 *M* urea as described by Panyim and Chalkley (8). Prior to sample application, gel tubes (length, 20 cm; o.d., 0.8 cm; i.d., 0.6 cm) were pre-electrophoresed for 3 hr at 5 mA/gel. Following sample application, the current was readjusted to 0.67 mA/gel; total electrophoresis time was 18 hr. Staining was accomplished with 0.2% amido black–0.1% Coomassie blue (w/v) in acetic acid/ethanol/H_2O (1:10:10, by vol) for 4 hr, followed by a destaining period of 24 hr in 5% acetic acid.

Iodination of the histone H2B fraction. Iodination of purified H2B fraction was carried out in the presence of 0.025 m*M* ^{125}I-labeled sodium iodide (1.8 mCi/μmole) and 0.25 m*M* chloramine-T in 0.1 *M* citrate buffer, pH 6.0, for 10 min at 4°. The reaction was terminated with 0.125 m*M* $Na_2S_2O_5$, and the mixture was passed through an AG 11 A8 Ion Retardation Resin (Bio-Rad, Calif.) column (0.7 × 10 cm) and eluted with 0.01 *M* citrate buffer. The iodinated histone fraction was collected, dialyzed twice against 1000 ml of 0.25 *N* HCl for 3 hr, and kept lyophilized until ready for use.

Results and discussion. From the data presented herein, a number of points regarding the use of isoelectric focusing for the separation of histones became evident. Of particular importance was the inclusion of 6.25 *M* urea in the gel composition since histones may undergo extensive aggregation at the neutral and alkaline ranges of the pH. For similar reasons, sample application was best achieved in the presence of 6.25 *M* urea as a carrier for histones. The procedure of prerunning gels prior to sample application discharged excessive persulfate and provided for partial formation of the pH gradient (10). Removal of excess persulfate was essential as it could have oxidized the carrier ampholytes and so alter the pH gradient. In addition, a portion of the ampholytes occupied the space on top of the gel and acted as an initial buffer zone for the proteins during sample application.

The pH gradient generated during the electrofocusing procedure linearly spanned the range of pH 7.5–10.5. This linear gradient was observed in our reference gels that contained 20 μg of cytochrome *c* as a marker and in gels that contained the histone fractions in amounts up to 200 μg. Reproducibility of pH gradients was optimal at a concentration of 1.1% ampholytes that consisted of a mixture of pH 3–10 and pH 9–11 (1:9, by vol). An ampholyte mixture of pH 7–9 and pH 9–11 (1:9, by vol) was found inferior to that described above. The electrolytes' concentration, current-voltage, and time specifications provided an equilibrium pattern of the proteins and the proper pH gradient. Any change in these parameters effected an ampholyte drift which re-

sulted in disfiguration of the pH gradient and band diffusion. The factors involved in maintaining an adequate pH gradient have been discussed by Miles *et al.* (13).

Nuclei isolation was carried out at relatively mild conditions of cell breakage, in the absence of detergent, which minimized rupture of nuclei and provided for the removal of cytoplasmic tags as seen by light and phasecontrast microscopy. The use of Triton X-100 removes the outer membrane of the nucleus (14) and thus may alter considerably structural and metabolic parameters of this organelle. The isolated nuclei used in the present report have retained the double membrane configuration (Fig. 2) and were, therefore, found suitable for studies of structure and function. [^{3}H]ATP incorporation into the acid-insoluble precipitate by the intact nuclei preparation was linear with nuclei concentration and time at 30° (Figs. 3A and 3B). This nuclei preparation would appear to be useful in studying the control of gene expression by nucleoproteins (6).

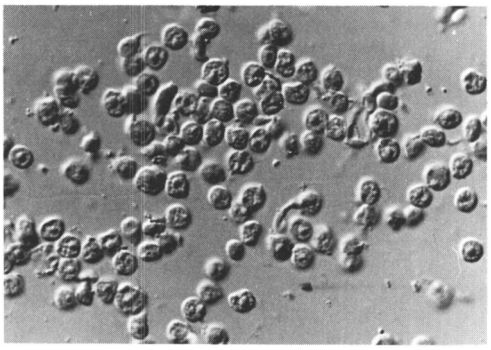

FIG. 2. Phase-contrast microscopy of purified rat pancreas nuclei.

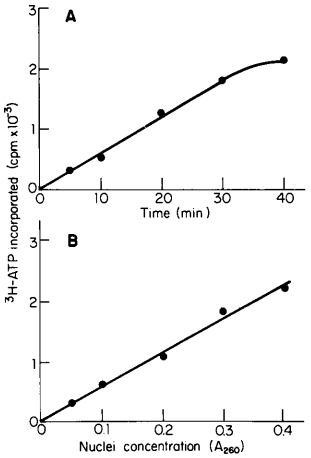

FIG. 3. [^{3}H]ATP incorporation into the acid-insoluble precipitate by intact nuclei preparation of rat pancreas. (A) As a function of time at a nuclei concentration of 0.3 A_{260}; (B) as a function of nuclei concentration for 30 min.

The quality of isolation of histones was determined by the use of a ^{125}I-labeled H2B fraction. The iodinated fraction was carried through the entire purification procedure of histones from pancreas of adult rats. The isolated histones that contained the ^{125}I-labeled H2B fraction were compared with an untreated ^{125}I-labeled H2B fraction by isoelectric focusing. Figure 4 shows that the peaks of radioactivity of the two samples were identical and coincided exactly with that of a stained, purified H2B fraction, indicating the lack of proteolytic activity under our experimental conditions. This is of particular importance in the analysis of segregated histone fractions by isoelectric focusing.

Table I shows the amount of proteins recovered from the Biorex-70 column following application of the PSH extract. Of the total protein recovered, the NHF constituted about 44 and 7.5% for newborns and adult pancreas, respectively. These were present in significant amounts in the embryonic pancreas as well (unpublished observations). Thus, a correlation appears to exist between pancreas differentiation and the proportion of nonhistones to histones that exist in the 0.25 *N* HCl extract. This sug-

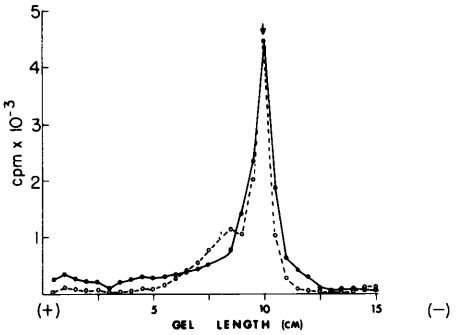

FIG. 4. Segregation of ^{125}I-labeled H2B fraction by isoelectric focusing. Solid line, treated fraction. Dashed line, untreated fraction. Arrow shows location of the stained purified fraction on gel.

TABLE I. SEPARATION OF PSF FROM NEWBORN AND ADULT PANCREATIC NUCLEI ON BIOREX-70[a]

State of development	NHP	Histone fractions
Newborn pancreas	155 μg (44.4%)	195 μg (55.6%)
Adult pancreas	172 μg (7.5%)	2140 μg (92.5%)

[a] Column recovery was 85% and 91% for the newborn and adult, respectively. Numbers in parentheses denote fraction as percentage of total protein added.

gests that proteins of the nonhistone-type soluble in 0.25 *N* HCl may play an important role in pancreas development and possibly in other organs as well.

The histone pattern obtained by electrophoretic separation and that obtained by electrofocusing are shown in Figs. 5 and 6. The number of bands by the latter method was considerably larger than that by the former procedure, indicating the high degree of microheterogeneity that exists within the histone fractions. In an applied sense, the technique of electrofocusing revealed substantial differences in the banding pattern of histones extracted from rat pancreas at Day 6 postpartum as compared with that at Day 3 postweaned (Fig. 6). No such differences were discernible by gel electrophoresis (Fig. 5). The banding pattern of histones that focused at the pH values of about 9.6 to 9.9 (Fig. 6) was found throughout pancreas development and constituted the major fraction of total histone's protein on the gels (unpublished observations). The significance of these results is currently under investigation. The increase in the number of bands and the changes in the focusing pattern that became noticeable during pancreas development may be due, in part, to the appearance of an H1° fraction (Fig. 5; refs. 15–17). We found that this fraction was missing during the earlier stages of pancreas development (embryonal and postnatal) and could be first discerned on about Day 9 postpartum (Fig. 5). It is of interest, however, that the number of bands focused was maximal in histone extracts isolated from pancreas of a 15-day embryo (unpublished observation). This was followed by a decline in the number of bands found at birth and a gradual increase thereafter. Selective removal of the H1 fraction from the histone extracts of a 6-day postpartum and of adult pancreas indicated that this fraction was responsible, in large measure, for the protein species at the acidic range of the isofocusing gels (Fig. 7A and 7B). In contrast, the H1° fraction isolated from adult pancreas segregated to a lesser degree than the H1 fraction and was largely associated with the basic portion of the pH profile (Fig. 7). The latter results may be consistent with reports on the possible role of the H1° fraction in organ development and neoplasia (15–18). Whether the H1 and the H1° histone fractions are transcribed sequentially off the same gene or are coded by two distinct genes remains to be established.

Heretofore, organ development apropos of chromosomal proteins has been described principally as a function of the acidic nucleoproteins (1, 2, 18). The technique of isofocusing provided a sensitive measure of histone's modification in relation to organ development. The present results indicate that pancreatic cell differentiation and secretory activity (19) are associated with changes in the microheterogeneous nature of histones and support the concept that histones too, the H1 fraction in particular (1–4), may be involved in the regulation of gene expression and of pancreatic cell differentiation. The variety, extent, and rapidity of reactions that modify histones in rela-

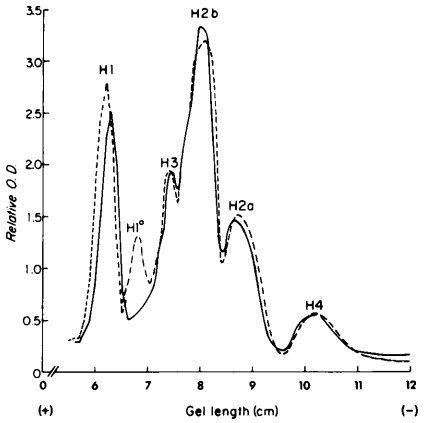

FIG. 5. Separation of histones by continuous gel electrophoresis. Solid line from rat pancreas at Day 6 postpartum. Dashed line from rat pancreas at Day 3 postweaned.

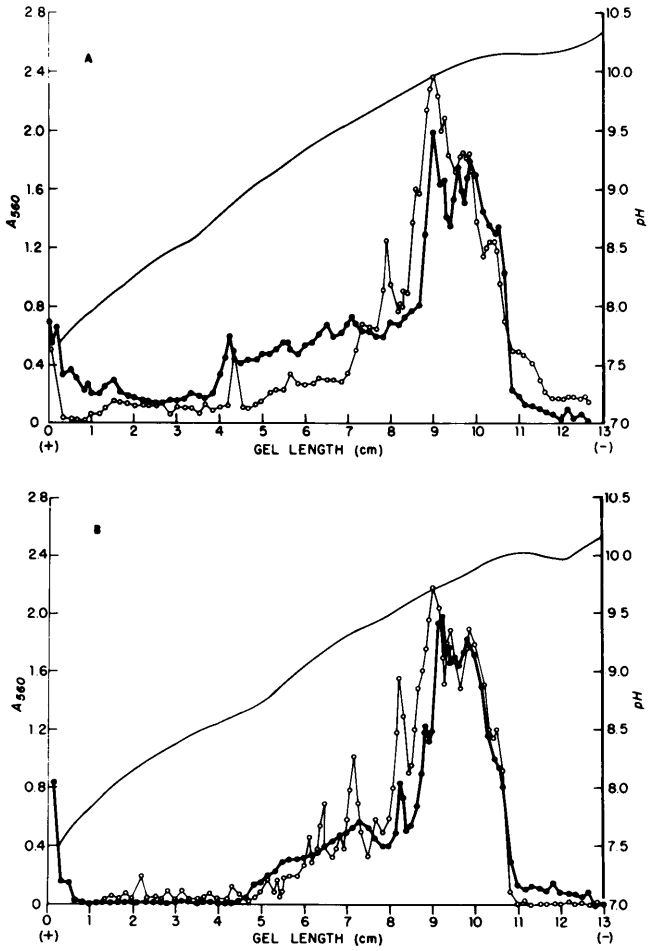

FIG. 6. Segregation of histones by isoelectric focusing. (A) From rat pancreas at Day 6 postpartum; (B) from rat pancreas at Day 3 postweaned. Closed circles represent the PSH extract and opened circles the histones separated by Biorex-70. pH Profile is represented by diagonal line.

tion to these processes remain to be elucidated.

The extension of the high resolution separation technique of isoelectric focusing in polyacrylamide gel matrices to histone fractions represents a sensitive method complementing procedures involving gel electrophoresis (1, 2, 15) or gel electrophoresis in anionic detergents (20, 21). While separation of histones by electrofocusing represents a more sensitive charge distinction within the various fractions, it does not necessarily demonstrate discrete species of protein. Combination of both electrophoretic and electrofocusing techniques with and without detergent (22, 23) would enable a demonstration of discrete species of proteins representing subfractions within the various histones. We have also been able to adequately resolve basic ribosomal proteins by the method described herein.

Summary. The present report describes the purification of nuclei from rat pancreas and the use of isoelectric focusing for the segregation of histone fractions, a heterogeneous mixture with respect to charge. This procedure employs gels of 5% polyacrylamide as support matrices, in 6.25 *M* urea, and spans a pH range of 7.5–10.8. It was, therefore, found adequate for study of the microheterogeneity of histones. The banding pattern of histone fractions in pancreas

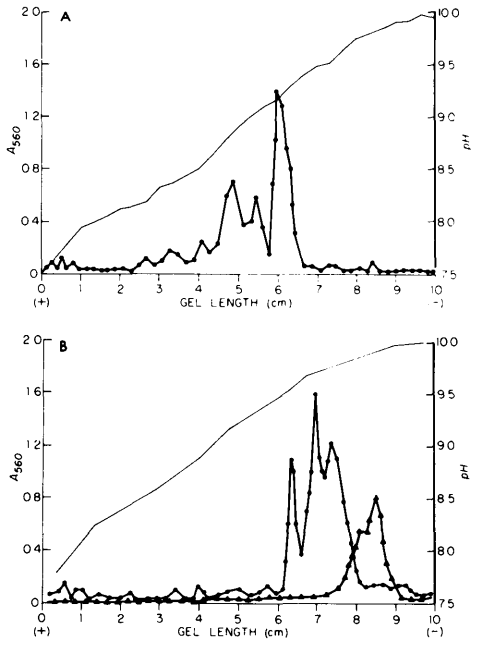

FIG. 7. Segregation of the H1 and H1° histone fractions by isoelectric focusing. (A) From rat pancreas at Day 6 postpartum; (B) from rat pancreas at Day 3 postweaned. Closed circles represent the H1 and closed triangles the H1° histone fractions.

development using electrofocusing is presented.

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1. Allfrey, V. G., *in* "Histones" (D. M. P. Phillips, ed.), pp. 241-248. Plenum Press, New York (1971).
2. Hnilica, L. S., *in* "The Structure and Biological Function of Histones," pp. 1-123. The Chemical Rubber Company, Cleveland (1972).
3. Kopelovich, L., and Wolfe, G., Fed. Proc. **34,** 530 (1975).
4. Kopelovich, L., and Wolfe, G., Proc. Amer. Assoc. Cancer Res. **16,** 118 (1975).
5. Loening, U. E., Biochem. J. **102,** 251-257 (1967).
6. Marzluff, W. F., Murphy, E. C., and Huang, R. C. C., Biochemistry **12,** 3440-3446 (1973).
7. Johns, E. W., and Butler, J. A. V., Biochem. J. **82,** 15-18 (1962).
8. Panyim, S., and Chalkley, R., Arch. Biochem. Biophys. **130,** 337-346 (1969).
9. Lowry, O. H., Rosenbrough, N. J., farr, A. L., and Randal, R., J. Biol. Chem. **193,** 256-261 (1951).
10. Righetti, P., and Drysdale, J. W., Biochem. Biophys. Acta **236,** 17-28 (1971).
11. Spencer, E. M., and King, T. P., J. Biol. Chem. **246,** 201-208 (1970).
12. Finlayson, G. R., and Chrambach, A., Anal. Biochem. **40,** 292-311 (1971).
13. Miles, L. E. M., Simmons, J. E., and Chrambach, A., Anal. Biochem. **49,** 109-117 (1972).
14. MacGillivray, A., Cameron, A., Krauze, R., Rickwood, D., and Paul, J., Biochem. Biophys. Acta **277,** 384-402 (1972).
15. Panyim, S., Bilek, D., and Chalkley, R., J. Biol. Chem. **246,** 4206-4215 (1971).
16. Varricchio, F., and Fitzgerald, P. J., Fed. Proc. **32,** 2276 (1973).
17. Marks, D. B., Kanefsky, T., Keller, B. J., and Marks, A. D., Cancer Res. **35,** 886-889 (1975).
18. MacGillivray, A. F., and Rickwood, D., *in* "Biochemistry of Cell Differentiation" (J. Paul, ed.), pp. 301-361. University Park Press, Baltimore (1974).
19. Pictet, R. L., and Rutter, W. J., *in* "The Handbook of Physiology" (D. F. Steiner and N. Freinkel, eds.), pp. 25-26. Williams and Wilkins Co., Baltimore (1972).
20. Elgin, S. C. R., and Bonner, J., Biochemistry **9,** 4440-4447 (1970).
21. Zweidler, A., and Cohen, L. H., Amer. Soc. Cell Biol. **59,** 378 (1973).
22. O'Farrell, P. H., J. Biol. Chem. **250,** 4007-4021 (1975).
23. Scheele, G. A., J. Biol. Chem. **250,** 5375-5385 (1975).

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