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Comparison of SDS Gel Electrophoretic Patterns of Rat Liver Nuclear Proteins in Response to Starvation, Refeeding and Insulin Injection

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斷食, 再給食과 인슈린注射에 따른 쥐의 肝細胞核蛋白質에 대한 전기영동상의 분포양상 비교

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Summary

SDS gel electrophoresis has been employed to examine the changes in distribution of three major classes of nuclear proteins extracted from isolated liver nuclei in response to refeeding of starved rats with a fat-free high carbohydrate diet and following insulin injection into streptozotocin-diabetic rats. The relative quantity of electrophoretically separated proteins in the fraction showed marked changes with 0.14 M NaCl extracts, but not with histones and phenol soluble non-histone proteins. During 48h starvation at least five proteins ranging in molecular weight from 50,000 to 180,000 daltons decreased relative to normal controls while a protein with 36,000 daltons was increased. Refeeding the starved rats with a high carbohydrate diet reversed these changes over 24 h. Insulin injection into streptozotocin-diabetic rats increased levels of the set of five 0.14 M NaCl soluble proteins identified from refeeding experiment of starved rats. The 36,000 daltons protein was also diminished. These results indicate that changes in distribution of certain nuclear proteins in 0.14 M NaCl extracts are associated with the control of nuclear activity ralated to known insulin-signalled modulation and induction of cytosolic lipogenic enzymes.

Introduction

It has been established that starvation and insulin deficiency in animals are associated with impaired lipogenesis in liver and adipose tissue and that refeeding and insulin administration restore the ability of these tissues to from triglyceride and cholesterol from ingested carbohydrates and other acetyl precursors.[1-4] Gibson *et al.*[5] have shown that the metabolic adaptation of the liver tissue is closely linked to the regulation of the synthesis and degradation of the lipogenic set of enzymes and that insulin is the primary signal *in vivo* for initiating the induction of the key enzymes for lipogenesis.[5] Thus induction of enzymes in eucaryotic cells is ultimately con-

trolled by the selective transcription.

A number of evidences suggest that the selective transcription in higher eucaryotes is regulated by nuclear proteins, in particular, the tightly bound non-histone proteins.[6-12] Recent studies [13] have also pointed out the importance of the loosely bound non-histone proteins, which are dissociated from chromatin at low salt concentrations since loosely bound non-histone proteins also bind to DNA and stimulate the transcription of DNA. However, it has not been intensively investigated how insulin signal is correlated to the qualitative or quantitative changes of the major nuclear proteins such as nuclear sap proteins, histones and phenol soluble non-histone proteins.

The present investigation was undertaken to examine the unclear protein profiles on SDS gel electrophoresis in relation to the change of insulin environment in the cells caused by the variation of nutritional states of animals and insulin injection to diabetic animals.

The SDS gel electrophoresis analysis demonstrate that the marked concentration change of the nuclear proteins is associated with 0.14 M NaCl soluble proteins during the refeeding of starved rats with a high sucrose diet and following insulin injection into fed streptozo tocin-diabetic rats.

Materials and Methods

Materials. Electrophoresis reagents were purchased from Bio-Rad laboratories. The standard proteins for the estimation of molecular weights were obtained from Calbiochem. Sucrose and urea were supplied by Schwarz-Mann. Other biochemicals were obtained from Sigma.

Animals. Male Wistar rats, 200 to 250g in weight, were used. Rats from normal feeding group were given free access to water and Purina balanced stock diet. Rats for starvation experiments were fasted for 48 h. For refeeding experiments rats starved for 48 h were fed *ad libitum* with fat free high carbohydrate diet (General Biochemicals) for 24 h and 48 h. To induce diabetes, rats were fasted for 24 h and streptozotocin (Upjohn), 65mg/kg of body weight in 0.1 M citrate buffer, pH4.5 was injected intraperitoneally to rats anesthetized under ether. Animals were further starved for 24 h after injection of streptozotocin and thereafter fed *ad libitum* with balanced Purina diet. Glucose content of urine was determined with Diastix (Miles Laboratories) 48 h after injection of streptozotocin and periodically. Prior to injection of insulin diabetic rats were fasted for 24 h. The mixture of regular Iletin insulin (1 unit/100g body weigh) and protamine-zinc insulin (3 units/100g body weight) in saline solution was subcutaneously injected to diabetic rats anesthetized under ether every 12 h until animals were sacrificed. Immediately after insulin injection the animals were allowed to be fed with fat-free high carbohydrate diet *ad libilum*. For the control diabetic rats saline solution without insulin was injected instead. In all experiments the rats were sacrificed by decaptitation just before use.

Preparation of nuclei and cytosol. Nuclei from rat livers were isolated by the procedure of Teng *et al.*.[9] The cytosol for the assay of malic enzyme was obtained by the centrifugation of crude homegenate of rat liver at 100,000×g for 1 h.

Extraction of nuclear proteins. Nuclear proteins were extracted from purified nuclei as described by Teng *et al.*.[9] The nuclear sap proteins were extracted with 6 to 10 ml of 0.14 M NaCl for purified nuclei pellets obtained from one rat liver. The procedure above was repeated once more for further extraction of nuclear sap proteins and the two extracts were combined. Histone and other basic proteins were extracted by two extractions of the nuclear residue with 6 to 10ml of 0.25 N HCl. Prior to extraction of phenol soluble proteins lipid was removed by two subsequent washes of the nuclear residue with 7ml of 1:1 (v/v) chloroform:methanol containing 0.2 N HCl and

7m*l* of 2 : 1 (v/v) chloroform:methanol containing 0.2 N HCl. The nuclei pellet was suspended in 5m*l* of cold ether for further lipid extraction. Phenol soluble non-histone proteins were extracted twice from the lipid removed nuclei pellet with the mixture of 5m*l* of 0.1M Tris-HCl, pH8.4 containing 10mM ethylenediamine tetraacetic acid (EDTA) and 0.14M 2-mercaptoethanol (buffer A) and 5m*l* of phenol saturated with buffer A. The subsequent procedure described by Teng *et al.*[9] was employed to restore phenol soluble proteins to aqueous phase.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis systems. Two different SDS-polyacrylamide gel electrophoresis systems were employed for this investigation. One of the electrophoresis systems was a modified method of Laemmli[19] for histone fractions. Resolving gels for histone fractions were composed of 15% acrylamide and 0.25% bisacrylamide instead. Electrophoresis buffer was 0.125 M Tris-0.192M glycine buffer, pH 8.9. Histone samples for electrophoresis were dialyzed against cold deionized water overnight at cold room. The second electrophoresis system used for all other protein fractions was a slightly modified method described by Weber and Osborn.[20] The gels were consisted of 10% acrylamide and 0.3% bisacrylamide in 0.1M sodium phosphate, pH 7.4 containing 0.1% SDS. Prior to application of protein samples for electrophoresis protein fractions; nuclear sap proteins, phenol soluble non-histone proteins and cytosolic proteins were dialyzed against 0.01M sodium phosphate, pH 7.4, 0.14 M 2-mercaptoethanol-0.2% soodium dodecyl sulfate at room temperature. Gels were stained with 0.2% Coomassie blue R250 in 50% methanol-7% acetic acid solution for 5h and destained in methanol-acetic acid-water (100: 140: 1760, v/v/v.) Destained gels were scanned at 600nm by Gilford Gel Scanner, Model 2500. The standard protein used for estimation of molecular weight were: γ-globulin (human) (160,000 daltons); bovine serum albumin (68,000 daltons); ovalbumin (43,000 daltons); chymotrypsinogen A (26,000 daltons); myoglobin (17,200 daltons) and cytochrome C (12,400 daltons).

Malate enzyme. (*E.C.* 1.1.1.38) **assay.** The enzyme activity of the cytosol fraction was measured spectrophotometrically according to the method of Hsu and Lardy[21]. The assay contained: 0.1 M Tris-HCl, pH 7.4, 5mM malic acid, 0.5mM $NADP^+$ and 1mM dithiothreitol.

Biochemical analysis. The protein content was determined by either biuret method[22] or the method of Lowry *et al.*[23]. The protein samples dialyzed against 0.01M sodium phosphate, pH 7.4, 0.14M 2-mercaptoethanol-0.2%sodium dodecyl sulfate were precipitated in 10% trichloroacetic acid containing 2mM silicotungstic acid[24] and the precipitate was solubilized in 1N NaOH. The amount of DNA was estimated by a modified method of Burton [25].

Table 1. Content of protein and DNA in purified nuclei isolated from liver of rats maintained under different experimental conditions.

Fraction	Experimental conditions[a]	NF	48hS	24hRF	48hRF	DIAB	DI&INS
Crude homogenate	Vol. (m*l*)	55	32	118	102	93	90
	Protein (mg)	746	480	1180	1163	1153	1386
	DNA (mg)	11.4	9.6	19.2	19.2	16	16.9
	$\frac{\text{Protein}}{\text{DNA}}$	65.4	50	61.5	60.6	72	82

Vol (*l*)	4	4	10	10	5	10
Protein (*mg*)	27.2	17	37	34	34	35
DNA (mg)	7.09	4.7	10.2	10.8	9.72	8.88
$\frac{\text{Protein}}{\text{DNA}}$	3.84	3.62	3.63	3.15	3.5	3.94
Yield (%)[b]	62	49	53	56	60	53

a: NF indicates normal feeding rat group, 48hS; 48h starved rat, 24hRF; refed 48h starved rats with a fat-free diet for 24h, 48hRF; refed 48h starved rats with a fat-free diet for 48h, DIAB; diabetic rats maintained on normal regular diet for 4 days since diabetes was induced by streptozotocin, DI&INS; diabetic rats with insulin treatment for 24h.

b: yield of nuclei=100×ratio of DNA content of crude homogenate to DNA content of purified nuclei.

Results and Discussion

Table 1 presents a representative data on DNA and protein contents of crude homogenate and purified nuclei fractions isolated from liver of rats maintained under different experimental conditions. A significant result was observed as the ratios of protein to DNA were compared. Starvation led the ratio of protein to DNA for crude homogenate to marked decrease and refeeding of starved rats reversed the change in the ratio to the comparable level to normal feeding controls over 24h. However, any consistent trend were not observed with the change in the ratio of protein to DNA for purified nuclei fractions during starvation and refeeding of starved rats. Similarly, insulin injection into streptozotocin-diabetic rats enhanced significantly the ratio of protein to DNA for crude homogenate fraction, but there was no observable change with the ratios of protein to DNA for purified nuclei fractions. In the case of diabetic rats the ratio of protein to DNA for crude homogenate was usually higher as compared with that obtained from normal feeding rats. In the present investigation diabetic rats were sacrificed 72h after injection of streptozotocin to rats. The timing of sacrificing animals is coincident to the period that the highest level of lipogenic enzyme concentrations is maintained in liver cells in response to refeeding of starved rats[6]. Thus the increased protein concentration in diabetic rats may be ascribed to a temporary shoot up of protein biosynthesis caused by refeeding of starved rats after streptozotocin injection which was manipulated for inducing diabetes from normal rats.

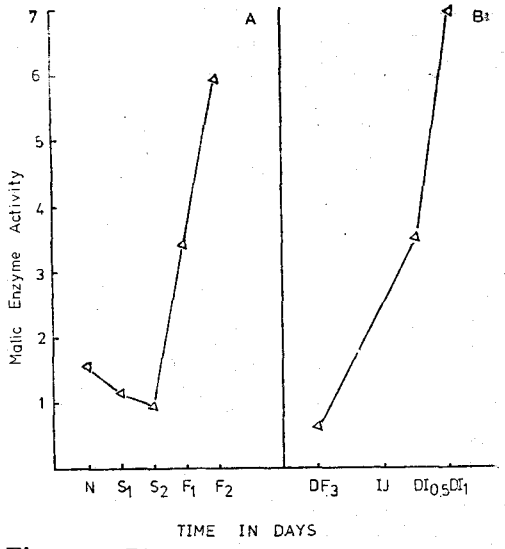

Figure 1. Effect of the change in nutritional states (Figure A) and insulin injection (Figure B) on the activity of malic enzyme from rat liver cytosol. The details of the enzyme preparation and assay system are given in the text. The ordinate is expressed in terms of ⊿OD at 340nm×100/min/mg of protein. Rats previously maintained on a balanced diet (N)were starved for 2 days(S_1 and S_2) and then refed a fat-free high carbohydrate diet for 2 days (F_1 and F_2)(Figure A). Diabetic rats induced with streptozotocin were maintained on a balanced diet for 3 days. (DF 3). Insulin was injected to diabetic rats starved for 1 day (IJ) and then fed with a fat-free high carbohydrate diet for 1 day ($DI_{0.5}$ and DI_1) (FigureB). Each point is the average value of the enzyme activity in three or more rat livers.

The ratios of protein to DNA for purified nuclei fractions varied from 2.7 to 4.4, although Teng *et al.*[9] observed that the ratio of total nuclear protein to DNA is 4.2 in highly purified rat liver nuclei. Their experimental value is based on the result obtained from a typical nuclei preparation. Yields of nuclei shown in Table 1 are close to the value, 52% as reported by Teng *et al.*[9]. These results suggest that our nuclei preparations were comparable to those obtained by Teng *et al.*[9].

To make sure that animals used for the experiment were in the proper adaptive response, malic enzyme activity was measured for the centrifugal supernatant. (100,000× g for 1 h) obtained from livers of rats under the different experimental conditions. The results shown in Fig. 1 are in good agreement with the earlier report[5] that malic enzyme activity of cytosol rises or falls in response to insulin signal.

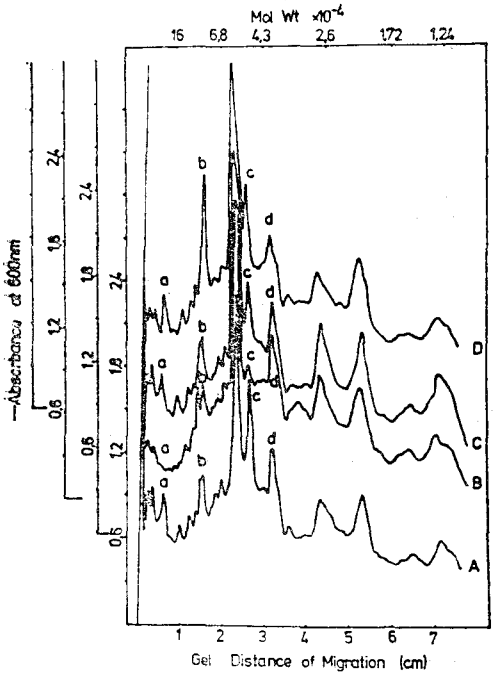

Figure 2. Electrophoretic patterns of 0.14M NaCl soluble proteins extracted from liver nuclei of rats with different nutritional states. The details of the experiments are given in the text. An aliquot of 35μg protein was electrophorized on a tube. The capital letters of alphabet indicate the nutritional states of rat: A-normal feeding, B-48 h starvation, C-24 h after refeeding of 48 h starved rat, and D-48 h after refeeding of 48 h starved rat. The small letters of alphabet indicate the molecular weights of the major protein peaks: a-180 kilodaltons, b-100 kilodaltons, c-50 kilodaltons, and d-36 kilodaltons.

Figure 2 shows the SDS gel electrophoresis pattern of 0.14M NaCl soluble proteins extracted from purified liver nuclei of rats with different nutritional states. The salient feature is that the relative concentrations of 0.14M NaCl soluble proteins change significantly during starvation and refeeding of rats. During 48 h starvation at least five protein bands with molecular weights of 50, 100, 105, 160, and 180 kilodaltons decreased in 0.14M NaCl extract fraction relative to normal controls, while a single peak at 36 kilodaltons was increased. Refeeding the starved rats with a fat-free high sucrose diet reversed these changes over 24h. Further refeeding of starved rats for 48h intensified the change in the quantities of the proteins sensitive to the nutritional states.

The electrophoretic patterns of histones and phenol soluble non-histone protein fractions were also examined during the refeeding of starved rats with a fat-free high sucrose diet. But it was found that the variation of nutritional states for rats did not bring about consistent and significant changes in the electrophoretic patterns qualitatively and quantitatively (not shown). Our observation is somewhat different from the finding that the striking changes of the electrophoretic profile occur in phenol soluble non-histone proteins in response to starvation with Physarum polycephalum and Hela cells growing exponentially in the culture medium.[26,27] The discrepancy may be ascribed to the different experimental subjects employed. In other words, it is conceivable that the composition ratio of proteins such as structural proteins, metabolic proteins and regulatory proteins in chromatin may be greatly variable among different cells. In case that

phenol soluble non-histone protein fractions contain negligibly small amount of regulatory proteins as compared with concentrations of structural proteins, in particular, the electrophoretic analysis method may not be able to pick up the qualitatively or quantitatively delicate changes of regulatory proteins most sensitive to cell adaptation unless regulatory proteins are selectively concentrated using purification techniques.

In the case of insulin injection to feed diabetic rats the electrophoretic profile of 0.14M NaCl soluble proteins shows the essentially identical pattern to that observed during refeeding of starved rats. Levels of the set of five nuclear

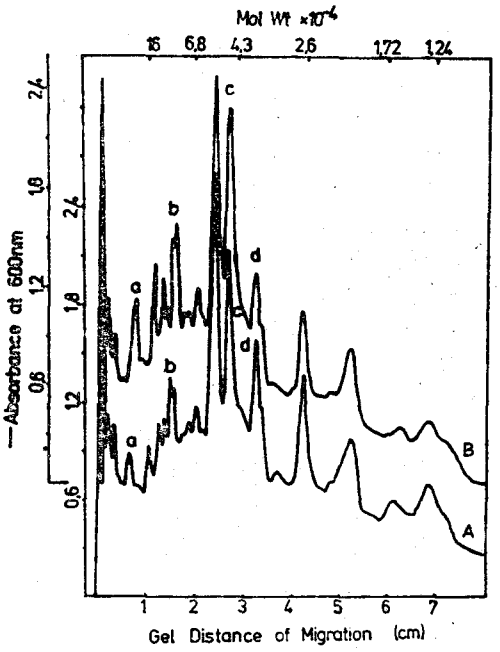

Figure 3. Effect of insulin injection on the change of the electrophoretic patterns of 0.14 M NaCl soluble proteins extracted from liver nuclei of diabetic rats. The details of the experiments are given in the text. An aliquot of 35μg protein was electrophorized on a tube. A and B indicate the electrophoretic pettern obtained from fed streptozotocin-diabetic rats without and with insulin injection, respectively. The small letters of alphabet indicate the molecular weights of the major protein peaks: a-180 kilodaltons, b-100 kilodaltons, c-50 kilodaltons, and d-36 kilodaltons.

proteins identified earlier increased significantly, while the 36 kilodalton protein was diminished (Fig.3). The histone and phenol soluble non-histone proteins appeared to be invariant as observed with the experiments using the variation of nutritional states (not shown).

The electrophoretic analysis of cytosolic proteins was also performed to find quantitative or qualitative changes during the refeeding of starved rats with a high sucrose diet and following insulin injection into fed streptozotocin-diabetic rats. Some quantitative changes of protein bands are anticipated from cytosol fractions since cytosol contains metabolic enzymes such as some of lipogenic enzymes sensitive to metabolic adaptation. However, cytosol fraction did not exhibit any cosistent trend of changes in proteins as judged by SDS gel electrophoresis.

The change in the concentration of nucleoplasmic proteins in response to nutritional states and insulin injection may result from the following possibilities; redistribution of nuclear proteins, particularly non-histone proteins through non-random movement of proteins between cytoplasm and nucleus, the change in the rate of synthesis or degradation of nuclear proteins in nucleus, and modification of affinity properties of nuclear proteins to chromatin. However, there is evidence that most of nuclear proteins including histones are not synthesized in the nucleus of most cells.[28] The change in the affinity of nucleoplasmic proteins to chromatin may be insignificant to effect a marked change of concentration of nuclear sap proteins since our data indicate that phenol soluble non-histone proteins appeared to be invariant with the variation of nutritional states. Considerable information has been accumulated to support that non-random movement of proteins between cytoplasm and nucleus accompanies a general change in nuclear activity.[29] In addition, there have been a number of studies on the transport of hormone receptor proteins into nucleus in response to,

hormone action.[30,31] In view of these evidences, it seems likely that the translocation of the shuttling proteins between cytoplasm and nucleus results in the observed quantitative changes in nucleoplasmic proteins in response to glucagon and insulin signals.

Recently, it has been shown that genome regulating proteins have an appreciable non-specific affinity for other DNA regions as well as for their specific target DNA sequences.[32-34] The non-specific binding property of genome regulating proteins implies the importance of dynamic equilibrium of nucleoplasmic proteins between chromatin and nucleoplasm in the nucleus. Consequently, the regulation of gene expression may be influenced by the relative concentration of nucleoplasmic non-histone proteins. Thus the present results indicate that changes in the relative concentration of individual nuclear sap proteins may be associated with the regulation of nuclear activity related to induction of cytosolic lipogenic enzymes.

Acknowledgments

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요 약

쥐를 48시간 굶긴 뒤에 탄수화물 농도가 높은 먹이를 주거나 streptozotocin을 이용하여 당뇨병을 가지게 만든 쥐에 인슈린 주사를 주었을 때 쥐 간세포의 핵단백질 중 가장 주요한 0.14 M NaCl에 녹는 핵단백질, histones 그리고 페놀에 녹는 핵단백질의 분포 변화를 조사하고자 SDS gel 전기영동법을 이용하였다. 각 핵단백질 분획을 전기영동법으로 분리시킨 단백질의 상대량을 비교하였을 때 0.14 M NaCl 추출물은 현저한 변화를 나타내었으나 histones과 페놀로 추출된 핵단백질 분획들은 분리된 단백질 상대량에 큰 변화가 없었다.

48시간 굶긴 쥐와 정상적으로 먹이를 준 쥐의 0.14 M NaCl 추출 핵단백질 분획을 비교하였을 때 분자량이 50,000과 180,000 daltons 사이에 있는 적어도 5개의 핵단백질의 농도가 다른 핵단백질에 비교하여 크게 감소되었다. 반면 분자량이 36,000 daltons 단백질의 농도는 48시간 굶주린 상태에 있었던 쥐에서 증가되었다. 48시간 굶긴 쥐에게 다시 탄수화물 농도가 높은 먹이를 주었을 때 24시간 동안에 정상적으로 먹이를 준 쥐에서 관찰한 핵단백질 분포 양상과 비슷한 결과를 얻었다.

당뇨병을 가진 쥐에게 인슈린 주사를 준 쥐와 인슈린주사를 주지 않은 당뇨병 쥐의 0.14 M NaCl 추출 핵단백질 분획을 비교 조사한 결과는 굶은 쥐에게 탄수화물 농도가 높은 먹이를 준 다음에 얻은 뒤의 결과와 정성적으로 유사하였다. 여기서 얻은 실험결과들은 0.14 M NaCl 추출 핵단백질 중에 있는 어떤 핵단백질의 분포 변화가 이미 알려진 인슈린 신호에 따라 조정되고 cytosol에 있는 지방합성에 관하는 효소(lipogenic enzymes)들의 유도에 관련된 세포핵 활성조절에 영향을 끼치고 있음을 시사해 준다.

References

(1) Stetten, D. and Boxer, G. E.: J.Biol. Chem., **156**, 271(1944)
(2) Chaikoff, I. L: Harvey Lectures, **47**, 99 (1951-1952)
(3) Tepperman, J. and Tepperman, H. M: Am. J. Physiol., **19**, 55(1958)
(4) Gibson, D. M: J. Chem. Educ., **42**, 236 (1965)
(5) Gibson, D.M., Lyons, R. T., Scott, D. F. and Muto, Y: Adv. Enz. Reg., **10**, 187 (1972)
(6) Paul, J. and Gilmour, R. S: J. Mol. Biol., **16**, 242(1966)
(7) Paul, J. and Gilmour, R. S: J. Mol. Biol., **34**, 305(1968)
(8) Spelsberg, T.C. and Hnilica, L. S: Biochim. Biophys. Acta, **195**, 63(1969)
(9) Teng, C. S., Teng, C. T. and Allfrey, V. G: J. Biol. Chem., **246**, 3597(1971)
(10) Spelsberg, T. C., Steggles, A. W. and O'Malley, B.W: J. Biol. Chem., **246**, 4188

(1971)
(11) Kostraba, N. C. and Wang, T. Y.: Biochim. Biophys. Acta, **262**, 169(1972)
(12) Stein, G. S., Spelsberg, T.C. and Kleinsmith, L. J.: Science, **183**, 817(1974)
(13) Comings, D. E. and Tack, L. O.: Exp. Cell Res., **82**, 175(1973)
(14) Fujitani, H. and Holoubek, V.: Biochem. Biophys. Res. Commun., **54**, 1300(1973)
(15) Kostrba, N. C., Montagna, R. A. and Wang, T. Y.: J. Biol. Chem., **250**, 1548 (1975)
(16) Comings, D. E. and Harris, D. C.: J. Cell Biol., **70**, 440(1976)
(17) Prestayko, A. W., Crane, P. M. and Busch, H.: Biochemistry, **15**, 414(1976)
(18) Umansky, S. R., Zotova, R. N. and Kovalev, Y. I.: Eur. J. Biochem., **65**, 503(1976)
(19) Laemmli, U. K.: Nature, **227**, 680(1970)
(20) Weber, K. and Osborn, M.: J. Biol. Chem., **244**, 4406(1969)
(21) Hsu, R. Y. and Lardy, H. A.: J. Biol. Chem., **242**, 520(1967)
(22) Gomall, A. G., Bardawill, C. J. and David, M. M.: J. Biol. Chem. **177**, 751(1949)
(23) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J.: J. Biol. Chem., **193**, 265(1951)
(24) Kleinsmith, L. J. and Kish, V. M. in O'Malley, B. W. and Hardman, J. G. eds., Methods in Enzymology, **11**, (14), Academic Press, New York, pp. 180(1975)
(25) Giles, K. W. and Myers, A.: Nature, **206**, 93(1965)
(26) Le Stourgeon, W. M., Wray, W. and Rusch, H. P.: Exptl. Cell Res., **79**, 487(1973)
(27) Le Stourgeon, W.M. and Rusch, H. P.: Arch. Biochem. Biophys., **155**, 144(1973)
(28) Goldstein, L.: Advan. Cell Biol., **1**, 187 (1970)
(29) Goldstein, L. in Busch, H. ed., The Cell Nucleus, Vol. I, Academic Press, New York, pp. 387(1974)
(30) Spelsberg, T. C.: in Cameron J. L. and Jeter, J. R. eds., Acidic Proteins of the Nucleus, Academic Press, New York, pp. 247(1974)
(31) Spelsberg, T. C., Webster, R. and Pikler, G. M.: in Stein, G. S. and Kleinsm ith, L. J., eds., Chromosomal Proteins and their Role in the regulation of Gene Expression, Academic Press, New York, pp. 153. (1975)
(32) Lin, S-Y. and Riggs, A. D.: Nature, **228**, 1184(1970)
(33) von Hippel, P. H., Revzin, A., Gross, C. A. and Wang, A. C.: Proc. Natl. Acad. Sci. USA, **71**, 4808(1974)
(34) Kao-Huang, Y., Revzin, A., Butler, A. P., O'Conner, P., Noble, D. W. and von Hippel, P. H.: Proc. Natl. Acad. Sci. USA, **74**, 4228(1977)