tioning as an energy source. The effect of glucose derivatives such as 3-O-methyl-D-glucose and 2-deoxy-D-glucose on the fertilization of mouse ova in vitro was therefore examined.

No effect was observed in the case of 3-O-methyl-D-glucose (table 2, exp. 1). On the other hand, 2-deoxy-D-glucose (1.2 mM) suppressed fertilization when the glucose concentration was reduced to 1.2 mM while no effect was observed in the presence of 6 mM glucose (table 2, Exp. 2). It was assumed that the latter worked as a glucose uptake inhibitor.

However, phloridzin, known as a glucose uptake inhibitor, showed no fertilization inhibitory effect, but stimulated fertilization in low glucose concentrations. Even in the presence of 0.15 mM glucose, in which no fertilization was observed in the control group, the addition of the reagent to the medium enabled the sperm to penetrate the ova. Since phloridzin is a glucoside of phloretine, the authors suspected the release of a glucose moiety from the reagent. However, phloridzin per se failed to induce sperm capacitation. Furthermore, the effect of phloretin on in vitro fertilization of mouse ova was also examined, and only a detrimental effect was observed. It was interesting that these two reagents had an opposite effect on sperm capacitation, because the only difference in the structures of phloridzin and phloretin is the glucose moiety. The fact that a small amount of glucose had to be contained in the medium in order for phloridzin to express its activity might be related to the observation of Fraser and Quinn[8]: 'Once the spermatozoa had been primed by glucose, the removal of exogenous glucose did not block fertilization.'

In the guinea pig, it was reported that glucose retarded both the sperm acrosome reaction[2] and sperm respiration[3]. In human sperm, glucose was more effective than fructose in enabling them to fuse with zona-free hamster ova[4]. Although the requirement of glucose in sperm capacitation might vary from species to species, the mechanisms of how the exogenously added energy source affect the physiological function of sperm are important aspects of the capacitation process. Phloridzin will serve as a useful reagent for the investigation to clarify the roler of glucose in mouse sperm capacitation.

Table 2. The effect of glucose concentration and glucose uptake inhibitors on mouse fertilization in vitro

| Glucose concentration (mM) | Ova penetrated (%) | |
|---|---|---|
| Exp. 1 | Control | 3-O-Methyl-D-glucose (1.2 mM) |
| 6.0 | 60 ± 6 | 63 ± 2 |
| 2.4 | 56 ± 3 | 57 ± 11 |
| 1.2 | 56 ± 8 | 46 ± 10 |
| 0.6 | 28 ± 9 | 21 ± 4 |
| Exp. 2 | Control | 2-Deoxy-D-glucose (1.2 mM) |
| 6.0 | 68 ± 10 | 59 ± 5 |
| 2.4 | 48 ± 6 | 23 ± 7* |
| 1.2 | 45 ± 6 | 1 ± 1** |
| 0.6 | 17 ± 3 | 1 ± 1** |
| Exp. 3 | Control | Phloretin-2'-β-D-glucose (500 μM) |
| 6.0 | 77 ± 10 | 79 ± 2 |
| 1.2 | 68 ± 7 | 86 ± 7 |
| 0.6 | 7 ± 4 | 69 ± 5** |
| 0.3 | 2 ± 1 | 63 ± 5** |
| 0.15 | 0 | 53 ± 10*** |
| 0 | 0 | 0 |
| Exp. 4 | Control | Phloretin |
| 6.0 | 60 ± 3 | (500 μM) 0*** |
| | | (100 μM) 5 ± 3** |
| | | (5 μM) 26 ± 2** |

Values are mean ± SE of 3–7 independent tests. *$p < 0.05$, **$p < 0.01$. ***Student's t-test was not applicable. Epididymal sperm prepared in fructose-KRB were added to the ova. The assessment of fertilization was done at 90 min after the sperm addition.

Acknowledgment. We are grateful to S. Cook for her assistance in preparing the manuscript.

1 To whom requests for reprints should be sent.
2 Rogers, B.J., and Yanagimachi R., Biol. Reprod. *13* (1975) 568.
3 Rogers, B.J., Chang, L., and Yanagimachi, R., J. exp. Zool. *207* (1979) 107.
4 Munemasa, K.H., Cash, M.K.M., and Rogers, B.J., Biol. Reprod. *28*, suppl. 1 (1983) 39a.
5 Niwa, K., and Iritani, A., J. Reprod. Fert. *53* (1978) 267.
6 Hoppe, P.C., Biol. Reprod. *15* (1976) 39.
7 Aonuma, S., Okabe, M., and Kawaguchi, M., J. Reprod. Fert. *53* (1978) 179.
8 Fraser, L.R., and Quinn, P.J., J. Reprod. Fert. *61* (1981) 25.

0014-4754/86/040398-02$1.50 + 0.20/0


## Gametes contain angiotensin converting enzyme (kininase II)[1]

J.R. Brentjens, S. Matsuo, G.A. Andres, P.R.B. Caldwell[2] and L. Zamboni[3]

*Departments of Pathology, Microbiology and Medicine, State University of New York at Buffalo, 204 Farber Hall, Buffalo (New York 14214, USA), 21 May 1985*

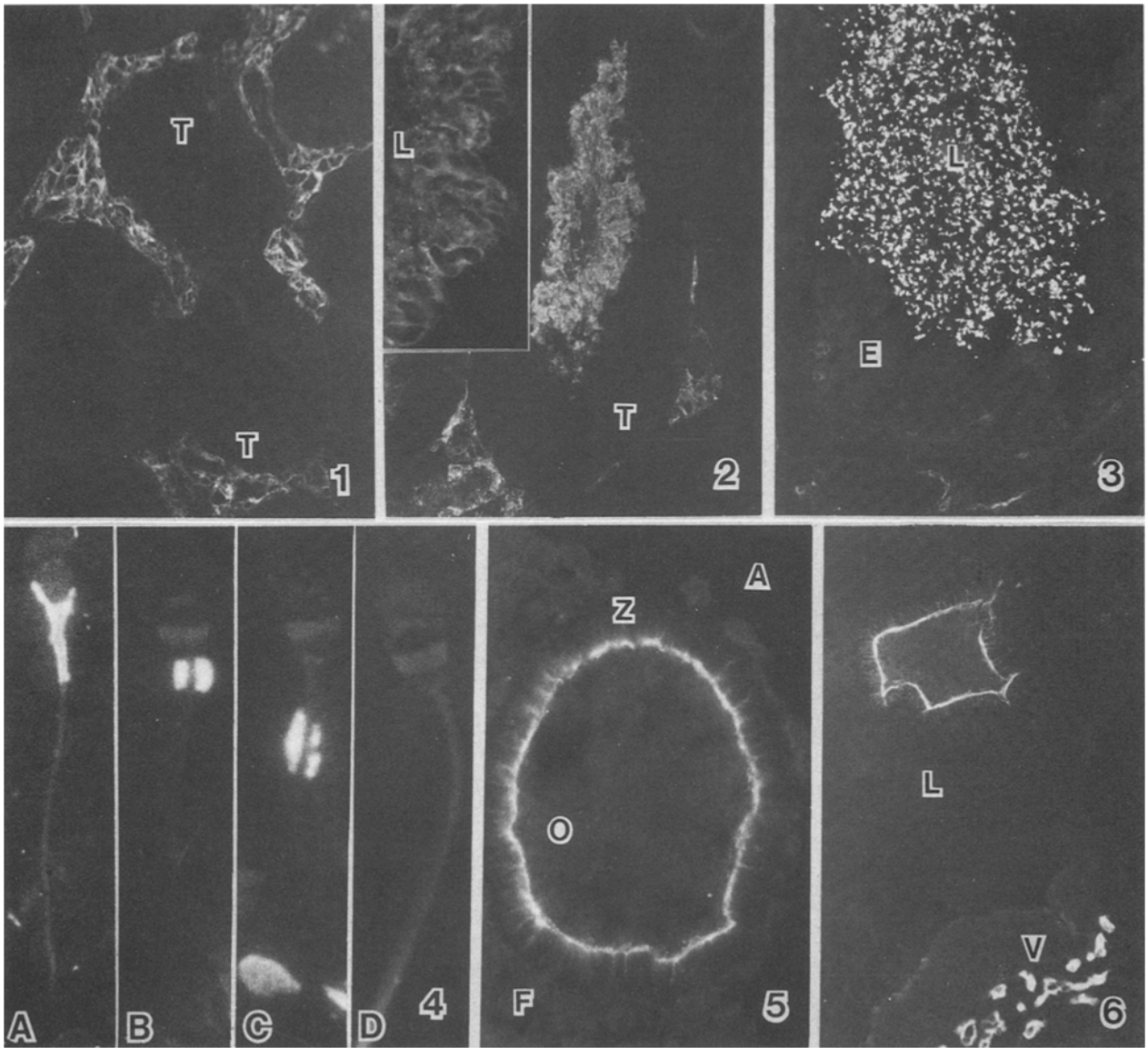
*Summary.* The localization of angiotensin converting enzyme (ACE) in the gonads of the normal rabbit was studied by immunofluorescence and immunoelectron microscopy. The enzyme is present in the cytoplasm of testicular spermatids and of epididymal and ejaculated spermatozoa, and on the surface of follicular and tubal cocytes. These findings support the hypothesis that ACE has a role in gamete maturation and in fertilization.

*Key words.* Angiotensin converting enzyme; rabbit, gametes; rabbit, Leydig cells.

Angiotensin converting enzyme (ACE) (kininase II; EC 3.4.15.1) is a dipeptidyl carboxypeptidase that catalyzes the conversion of angiotensin I to angiotensin II, a vasopressor, inactivates bradykinin, a vasodepressor, and is important in homeostasis of blood pressure. The enzyme is located on the luminal membrane of endothelial cells of vessels throughout the body[4,5]. In addition, ACE has been demonstrated on the brush border of renal and intestinal epithelial cells[6,7] and in neuroepithelial cells of the brain[8,9]. However, measurement of ACE activity in a variety of animal species and in man indicates that the highest concentration of ACE is present in the testis, the epididymis, and the seminal fluid[10,11]. In the rat the rise in ACE activity in the testis parallels sexual maturation[10,11], while in man a correlation has been observed between the activity of ACE in seminal plasma and semen quality, such as density and motility[12]. Recently it has been demonstrated by three different techniques that in rat[13,14], and swine[15] ACE is associated with male gametes. In one report[15], the precise localization of ACE in male swine

gonads was investigated by using the immunofluorescence microscopy technique. The enzyme was shown to be present in spermatids and in the cytoplasmic droplets of epididymal spermatozoa. In the present study immunohistochemical techniques were used to identify the localization of the enzyme in rabbit testis and ovary and in extragonadal gametes.

*Materials and methods.* An antiserum against ACE purified from rabbit lungs was prepared in goats[16,17]. The monospecificity of this antiserum has been documented previously[16,17]. Although pulmonary and testicular ACE are not identical but appear to be isoenzymes, antipulmonary ACE antibodies recognize all the antigenic determinants of testicular ACE[18,19]. In immunofluorescence microscopy studies the following normal specimens were used: frozen sections from testes and epididymides of sexually immature (n = 3) and mature rabbits (n = 14); smears of testicular and epididymal tubular contents (n = 6); semen smears (n = 2); and frozen sections of ovaries (n = 10) and of oviducts (the latter collected from two animals induced to




All figures are immunofluorescence (figs 1–6) or immunoelectron (figs 7 and 8) micrographs showing the localization of ACE.

Figure 1. Testis of a sexually immature rabbit. ACE is observed in cells present in the interstitium surrounding the seminiferous tubules (T). ×70.

Figure 2. Testis of a sexually mature rabbit. ACE is seen not only in interstitial cells but also in the lumen of the seminiferous tubules (T). ×70. Inset: At higher magnification ACE is observed surrounding the heads of developing spermatozoa in the lumen (L) of the seminiferous tubules. × 565.

Figure 3. Epididymis of a sexually mature rabbit. The tubular lumen (L) contains numerous discrete granules of ACE. E: tubular epithelium. ×70.

Figure 4. A) Spermatozoon in a testis smear. ACE is seen in the residual cytoplasm surrounding part of the head and the tail. ×565. B and C) Spermatozoa in epididymis smears. ACE-containing cytoplasmic droplets are seen at different positions along the sperm tails. ×565. D) No ACE can be demonstrated in an epididymal spermatozoon that has lost its cytoplasmic droplet. ×565.

Figure 5. Rabbit antral follicle. ACE is localized in the oolemma in a diffuse pattern and in the zona pellucida in striations radiating from the oocyte surface. A: antrum; F: follicular cells; O: oocyte; Z: zona pellucida. ×375.

Figure 6. Rabbit oocyte after ovulation in the lumen (L) of an oviduct. The expression of ACE on the oviductal oocyte is similar to that of ACE on the ovarial oocyte shown in figure 5. ACE is also present on the endothelium of capillaries of an oviductal villus (V). ×70.

ovulate following gonadotropin administration). All specimens were fixed at 4 °C in acetone for 15 min. The direct and indirect immunofluorescence technique utilizing the IgG fraction of the anti-ACE serum and the appropriate control experiments were performed as described previously[20]. Localization of ACE by immunoperoxidase electron microscopy was done in epididymal spermatozoa and ovary tissue, using the technique described[21]. In one rabbit ACE activity was measured in a pellet of epididymal spermatozoa, in sperm-free fluid of epididymal tubules, in washed epididymal tissue fragments, and in lung tissue according to the method described by Cushman and Cheung[22].

*Results.* By immunofluorescence microscopy ACE was identified along the vascular endothelium in testis, epididymis, ovary and oviduct. In the testis of immature and mature animals the enzyme was also found on the Leydig cells (figs 1 and 2). ACE was not detectable in the seminiferous tubules of any of the sexually immature rabbits (fig. 1). In contrast, in all of the mature testes extensive localization of ACE was observed at the luminal side of the seminiferous tubules (fig. 2), especially around the heads of spermatids and spermatozoa (fig. 2, inset). Whereas, in the epididymis of sexually immature rabbits ACE was found only along endothelia, in all sexually mature animals the epididymal tubular lumina were filled with sperm cell-associated accumulations of the enzyme (fig. 3). The study of testicular, epididymal, and semen smears revealed the presence of the enzyme in the sperm cytoplasmic droplets (figs 4A, B, and C), and its absence in spermatozoa that had shed their residual cytoplasm (fig. 4D). Thus, the association of ACE with male gametes followed strictly the formation and normal cycle of the cytoplasmic droplet[23,24]. While immunoelectron microscopy showed that ACE was localized in the cytoplasm surrounding the vesicular elements of the droplets (fig. 7), we could not establish whether the enzyme occurred also at the surface of the droplets because the handling of live spermatozoa resulted in the separation of the droplets from the cells and their subsequent disintegration[23,24]. The specific activities of ACE in epididymal sperm, sperm-free epididymal tubular fluid, washed epididymal tissue, and homogenized lung were 0.19, 0.11, 0.04, and 0.10 U/mg protein respectively. In double diffusion tests, a single immuno-precipitin band was obtained between the anti-ACE serum and the sonicated sperm pellet, tubular fluid preparation or homogenized lung.

In the ovary ACE was observed in a diffuse pattern on the surface of follicular oocytes as well as in the zona pellucida in striations radiating from the oocyte surface (fig. 5). The expression of ACE was most pronounced on oocytes present in large antral follicles and did not change after ovulation as shown by the presence of the enzyme in oviductal eggs (fig. 6). Immunoelectron microscopy revealed that ACE was distributed in a diffuse pattern along the oolemma and the plasma membrane of cumulus cell processes extending through the zona pellucida (fig. 8)[21].

*Discussion.* The demonstration of ACE in the cytoplasm of testicular spermatids and of epididymal and ejaculated spermatozoa confirms an earlier report[15] and explains the results of studies showing the existence of a strict correlation between male gonadal ACE activity and sexual maturity[10-12]. Furthermore, the high concentration of ACE in seminal plasma is most likely the consequence of the disintegration of cytoplasmic droplets shed by the mature spermatozoa[23,24]. The immunohistochemical demonstration of ACE in Leydig cells is in agreement with an earlier biochemical study showing ACE activity in these cells[25]. The finding of ACE on the plasma membrane of egg cells seems to represent the first documentation of oolemma-associated enzyme activity.

The functional significance of ACE in the male and female gametes is unknown. The observation that ACE persists in extragonadal gametes raises the possibility of a role for ACE not only in sperm and oocyte maturation but also in the fertilization process. The two known physiologic substrates of ACE are bradykinin and angiotensin I. Bradykinin enhances sperm motility, and kallikrein, the enzyme involved in bradykinin formation, is found in seminal plasma[26]. Leydig cells have been shown to contain renin[25,27] and angiotensins[25]. It should also be realized that ACE is capable of cleaving dipeptides from the C-terminal end of a variety of peptides and, as such, it may act on physiologic substrates other than angiotensin I or bradykinin. It is conceivable, therefore, that ACE influences gonadal and gamete physiology through yet non-defined, biologically active peptides. Experiments involving competitive antagonists of ACE should help to clarify the significance of ACE in reproductive physiology. Finally, we recently induced in situ formation of immune complexes in the zona pellucida of rabbit oocytes by systemic administration of anti-ACE antibodies[21]. Investigations on the effect of anti-ACE antibodies on the maturation and fertilizing competence of male and female gametes in vivo and in vitro seem warranted[28,29].

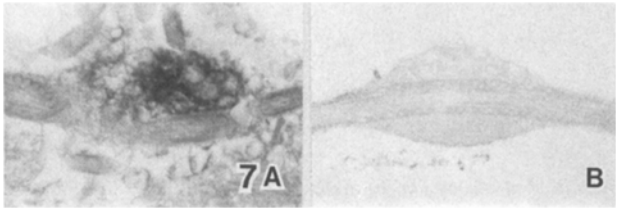



Figure 7. A) Demonstration by immunoperoxidase electron microscopy of ACE in a cytoplasmic droplet ×8200. B) Absence of peroxidase in a cytoplasmic droplet in a control experiment in which instead of goat anti-ACE IgG, non-immune goat IgG was used. ×8200.

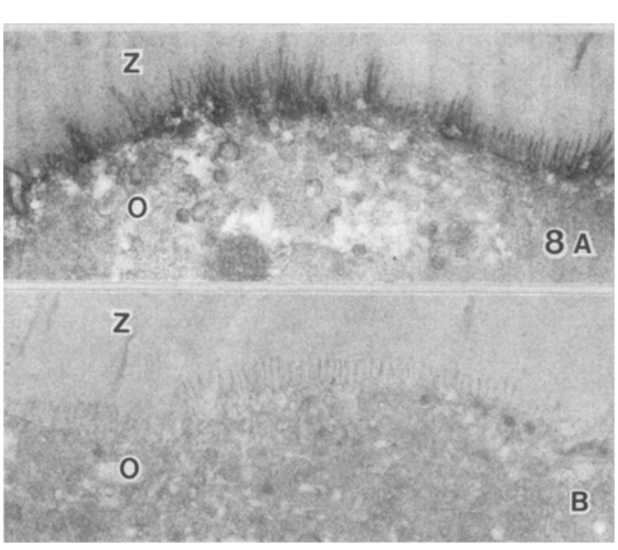



Figure 8. A) Demonstration by immunoperoxidase electron microscopy of ACE in a diffuse pattern along the oolemma. Shown is part of an oocyte (O) surrounded by the zona pellucida (Z). ×6500. B) Absence of peroxidase along the oolemma in a control experiment in which instead of goat anti-ACE IgG, non-immune goat IgG was used. ×6500.

1 Acknowledgments. This study was supported by NIH grant AM-36807 and by grants from the American Heart Association, the Joe and Emily Lowe Foundation, and the Dana Foundation. We thank L. Fernandez, B. Luders, I. Pawlowski, and K. Schwegler for technical assistance.

2 Department of Medicine, Columbia University, New York, N.Y. 10032, USA.

3 Department of Pathology, Harbor-UCLA Medical Center, Torrance, CA 90509, USA.

4 Caldwell, P.R., Seegal, B.C., Hsu, K.C., Das, M.C., and Soffer, R.L., Science *191* (1976) 1050.

5 Ryan, U.S., Ryan, J.W., Whitaker, C., and Chiu, A., Tiss. Cell *8* (1976) 125.
6 Wigger, H.J., and Stalcup, S.A., Lab Invest. *38* (1978) 581.
7 Ward, P.E., Sheridan, M.A., Hammon, K.J., and Erdos, E.G., Biochem. Pharmac. *29* (1980) 1525.
8 Defendini, R., Zimmermann, E.A., Weare, J.A., Alheuc-Gelas, F., and Erdos, E.G., Neuroendocrinology *37* (1983) 32.
9 Strittmatter, S.M., Lo, M.M.S., Javitch, J.A., and Snyder, S.H., Proc. natn. Acad. Sci. USA *81* (1984) 1599.
10 Cushman, D.W., and Cheung, H.S., Biochim. biophys. Acta *250* (1971) 261.
11 Hohlbrugger, G., Schweisfurth, H., and Dahlheim, H., J. Reprod. Fert. *65* (1982) 97.
12 Kaneko, S., and Moriwaki, C., J. Pharm. Dyn. *4* (1981) 175.
13 Strittmatter, S.M., and Snyder, S.M., Endocrinology *115* (1984) 2332.
14 Velletri, P.A., Aquilano, D., Bruckwick, E., Tsai-Morris, C.-H., Dufau, M.L., and Lovenberg, W., Endocrinology *116* (1985) 2516.
15 Yotsumato, H., Sato, S., and Shibuya, M., Life Sci. *35* (1984) 1257.
16 Caldwell, P.R.B., Wigger, J.H., Fernandez, L.T., D'Alisa, R.M., Tse-Eng, D., Butler, V.P. Jr, and Gigli, I., Am. J. Path. *105* (1981) 54.
17 Barba, L.M., Caldwell, P.R.B., Downie, G.H., Camussi, G., Brentjens, J.R., and Andres, G., J. exp. Med. *158* (1983) 2141.
18 El-Dorry, H.A., Bull, H.G., Iwata, K., Thornberry, N.A., Cordes, E.H., and Soffer, R.L., J. biol. Chem. *257* (1982) 14128.
19 Velletri, P.A., Life Sci. *36* (1985) 1597.
20 Andres, G.A., Accini, L., Beiser, S.M., Christian, C.L., Cinotti, G.A., Erlanger, B.F., Hsu, K.C., and Seegal, B.C., J. clin. Invest. *49* (1970) 2106.
21 Matsuo, S., Caldwell, P.R.B., Brentjens, J.R., and Andres, G., J. clin. Invest. *75* (1985) 1369.
22 Cushman, D.W., and Cheung, H.S., Biochem. Pharmac. *20* (1971) 1637.
23 Wimsatt, W.A., Krutzsch, P., and Napolitano, L., Am. J. Anat. *119* (1966) 25.
24 Kaplan, M., Russell, L.D., and Martan, J., Tissue and Cell *16* (1984) 455.
25 Pandey, K.N., Misono, K.S., and Inagami, T., Biochem. biophys. Res. Commun. *122* (1984) 1337.
26 Schill, W.B., and Haberland, G.L., Hoppe-Seylers Z. physiol. Chem. *355* (1974) 229.
27 Parmetier, M., Inagami, T., Pochet, R., and Desclin, J.C., Endocrinology *112* (1983) 1318.
28 East, I.J., Mattison, D.R., and Dean, J., Devl. Biol. *104* (1984) 49.
29 Naz, R.K., Alexander, N.J., Isahakia, M., and Hamilton, M.S., Science *225* (1984) 342.

0014-4754/86/040399-04$1.50 + 0.20/0


## A note on the use of protease inhibitors during chromatin fractionation on hydroxyapatite columns

M. Gaczyński

*Department of Cell Structures Biochemistry, University of Łódź, ul. S. Banacha 12/16, 90–237 Łódź (Poland), 14 May 1985*

*Summary.* It was found that $NaHSO_3$ present in the eluents enabled full and reproducible recovery of chromosomal proteins from a hydroxyapatite column. Another protease inhibitor, PMSF, did not have that effect.
*Key words.* Chromatin proteins; hydroxyapatite; protease inhibitors.

Hydroxyapatite (HAP) chromatography is a very widely used method of obtaining chromatin proteins. It makes it possible to obtain nonhistone proteins free from histones and nucleic acids by a single-column procedure, without long ultracentrifugation[1,2]. The major disadvantage of the method is the changing amount of proteins eluted from the column. Rickwood and MacGillivray[1] recommended washing nuclei with sodium deoxycholate for chromatin with totally 'elutable' proteins. Unfortunately, the nuclei treated with deoxycholate were found by the authors to be devoid of some proteins especially of high mol. wt.

*Materials and methods.* White Wistar male rats (120–150 g) were killed by decapitation and livers were quickly excised, perfused with ice-cold saline and immediately used for further experiment. All solutions used for obtaining cell nuclei and chromatin contained phenylmethylsulphonylfluoride (PMSF) added freshly as an 0.1 M solution in isopropanol[3]. All preparative and chromatographic work was carried out at 4 °C. Cell nuclei were obtained by the method of Widnell and Tata[4] with an additional washing with 0.5% Triton X-100 in isotonic sucrose. Chromatin was prepared according to Spelsberg and Hnilica[5]. The nuclei were washed twice with saline-EDTA and swollen in 1.5 mM NaCl – 0.15 mM sodium citrate. The chromatin was sonicated, adjusted to about 0.25 mg DNA/ml and applied onto a column. The column was washed with 1 mM potassium phosphate buffer (pH 7.5) and then the total chromatin proteins were eluted with a buffer containing 2 M KCl, 5 M urea, 80 mM potassium phosphate (pH 7.5). The solutions for washing and elution contained 5 mM $NaHSO_3$ (analytical grade, P.O.Ch.) or 1 mM PMSF (Sigma).

Protein concentration was estimated in the effluent by the dye binding method of Bradford[6] using bovine serum albumin as a standard.

*Results and discussion.* Samples of chromatin obtained in the same preparation cycle were run simultaneously on HAP columns with eluents containing one of the two protease inhibitors: $NaHSO_3$ or PMSF. The results given in the table showed that the amount of protein is 2.61–2.75 times higher when sodium bisulfite is added to the eluents.

Since Carter and Chae[3] showed that PMSF is a powerful protease inhibitor it has been widely used in chromatin studies ever since, though there has recently been critical discussion concerning its inhibitory properties[7]. We found that it cannot replace $NaHSO_3$ when total recovery of proteins from HAP is expected. Sodium bisulfite was used as an inhibitor of proteolytic activity by Chiu and his coworkers[8] during the removal of the bulk of nonhistone proteins with 5 M urea and by Fredericq and Hacha[9] in sequential elution of histones and nonhistone proteins from HAP columns. The eluents were composed of 2 M KCl and various concentrations of phosphate buffer only. The use of $NaHSO_3$ may have contributed to the successful separation without applying any strong solubilizing agents (urea or guanidine hydrochloride). Later, bisulfite was used by Bloom and Anderson[10] who reported a quantitative separation of chromatin

Total chromatin proteins (mg) recovered from an HAP column with 2 M KCl – 5 M urea – 80 mM potassium phosphate buffer (pH 7.5) supplemented with either 1 mM PMSF or 5 mM $NaHSO_3$. 1 mg chromatin DNA was applied onto a 2 ml column

| No. of sample | Eluent + PMSF | Eluent + $NaHSO_3$ |
|---|---|---|
| 1 | 0.68 | 1.79 |
| 2 | 0.71 | 1.85 |
| 3 | 0.65 | 1.79 |