Histone Acetylation and Hormone Action

EARLY EFFECTS OF OESTRADIOL-17 β ON HISTONE ACETYLATION IN RAT UTERUS

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The effect of oestradiol treatment on the acetylation of histones of the immature rat uterus has been studied. A 10 μ g dose of oestradiol causes a 70% increase at 5 min and a 140% increase at 10 min after administration in the labelling of the histone fraction F2 + F3. No effect of oestradiol is seen on the labelling of histones F1 or acidic non-histone chromatin proteins. The oestradiol stimulation is seen in animals pretreated with either cycloheximide or actinomycin D. The stimulation of labelling caused by oestradiol is completely abolished by pretreatment of the animals with the anti-oestrogen, nafoxidine. The stimulation is given by lower doses of oestradiol, by stilboestrol and oestriol, but is not given by testosterone. These results suggest that stimulation of histone acetylation in the uterus is the earliest known effect of the hormone on its target tissue.

An increase in the acetylation of certain histones in chromatin has been demonstrated as an early result of a stimulus which leads to either increased growth or biosynthetic capacity. Pogo *et al.* (1966) demonstrated that the exposure of human lymphocytes to phytohaemagglutinin caused an increase in histone acetylation which preceded other changes in the cell, such as stimulation of RNA synthesis and protein synthesis. This stimulation has been confirmed by Mukherjee & Cohen (1969), and by Ono *et al.* (1969), although Ono *et al.* (1969) showed that stimulation of histone acetylation could be dissociated from stimulation of RNA synthesis by cortisol. In contrast, Monjardino & MacGillivray (1970) have shown that the stimulation of histone acetylation is not a property of all preparations of phytohaemagglutinin, and have suggested that the stimulation is due to a contaminant of the phytohaemagglutinin.

Several other systems which respond to a stimulation by an increase in histone acetylation have been reported: these include the remaining liver of partially hepatectomized rats (Pogo *et al.*, 1968), liver and thymus of adrenalectomized rats after treatment with cortisol (Allfrey *et al.*, 1966), the spleen of mice treated with erythropoietin (Takaku *et al.*, 1969), and the kidneys of adrenalectomized rats treated with aldosterone (Libby, 1972a; Liew *et al.*, 1972; Trachewsky & Congote, 1972).

Since rat uterus contains an enzyme system which acetylates exogenous histones, and which is stimulated by oestradiol-17 β , it was of interest to study the effect of oestradiol on uterine histone acetylation *in vivo*. This report shows that a very early effect of

oestradiol is to stimulate the acetylation of the uterine histone F2a1.

Preliminary reports of some of this work have appeared (Libby, 1971, 1972b).

Materials and Methods

Reagents

Sodium [3 H]-acetate was purchased from New England Nuclear Corp. Boston, Mass., U.S.A., and used at a specific radioactivity of 100 mCi/mmol. Cycloheximide was from Sigma Chemical Co., St. Louis, Mo., U.S.A., and actinomycin D was purchased from Calbiochem, Los Angeles, Calif., U.S.A. Steroids were from Steraloids, Flushing, N.Y., U.S.A. Ethylene diacrylate was from Borden Chemical Co., Philadelphia, Pa., U.S.A. Nafoxidine {1-[2-[*p*-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)-phenoxy]ethyl]pyrrolidone hydrochloride} was a gift from Dr. Herbert Jacobson. Sepraphore III was purchased from the Gelman Co., Ann Arbor, Mich., U.S.A.

Animals

Female Sprague-Dawley rats were usually used before they were 28 days old. In a few cases, 29-day-old rats were oophorectomized 6 days before use. Animals were injected intraperitoneally with 1 ml of 0.9% NaCl containing 1 mCi of sodium [3 H]acetate. Hormones were dissolved in ethanol and 0.01 ml of the

ethanol solution was added to the saline before injection, 0.01 ml of ethanol being added to the solution injected into control animals.

Preparation of histones

Animals were killed by cervical dislocation at various intervals after injection. The uteri were removed, frozen on solid CO₂ and stored at -20°C. After thawing, three or four uteri were pooled, minced, and homogenized in an all-glass Potter-Elvehjem homogenizer with 5 ml of 0.075 M-NaCl-0.024 M-EDTA, pH 8.0 (Bonner *et al.*, 1968). All procedures were carried out at 0-4°C unless otherwise stated. The homogenate was forced through nylon gauze and centrifuged at 3500 rev./min for 10 min in a Sorvall SM-24 rotor. The pellet was washed three more times with the above buffer. The pellet was extracted two times with 0.3 ml of 0.1 M-H₂SO₄, the first extraction for 1 h, and the second overnight. The histones in the extracts were precipitated with acetone, recovered by centrifugation, and the histone pellet dried in air.

Analysis of histones

Histones were subjected to cellulose acetate electrophoresis by the method of Pogo *et al.* (1966). Staining was carried out by a modification of the method of Tidwell *et al.* (1968). The strips were immersed in saturated picric acid for at least 2 min, rinsed in tap water, and in 0.05 M-sodium barbital buffer, pH 8.2, and then immersed in Fast Green solution (2 mg/ml in 0.05 M-sodium barbital buffer, pH 8.2) for at least 3 min. Excess of Fast Green was washed out of the strips by several rinses in methanol.

As previously reported (Libby, 1968; Pogo *et al.*, 1966; Tidwell *et al.*, 1968), the front band contained histones F1, and the rear band contained all the histones F2 and F3 (Johns *et al.*, 1960; Johns, 1967b). Comparison with chromatin non-histone proteins and with proteins extracted with 0.1 M-H₂SO₄ from a uterine microsomal pellet showed no contamination of histones with either ribosomal proteins or chromatin non-histone proteins. Quantitative determination of histones and radioactivity was carried out as previously described (Libby, 1968) with changes as follows: the bands were dissolved in dioxan containing 4% (v/v) 2 M-HCl, and the acid was neutralized in the scintillation vials with 4 M-KOH. Results were calculated as pmol of acetate incorporated/mg of histone. The statistical significance of the difference between groups was determined by Student's *t* test.

Polyacrylamide-gel electrophoresis was carried out by the method of Panyim & Chalkley (1969) with the following modifications: ethylene diacrylate was substituted for methylene bisacrylamide, and 75 mm

tubes were used. Under these conditions, Amido Schwartz staining (Panyim & Chalkley, 1969) revealed four major bands, corresponding to histones F1, F3, F2b+F2a2 (unresolved) and F2a1 in order of increasing mobility. The sections of the gels containing these bands were cut out and dissolved in 1.0 M-NaOH. *E*₆₁₅ was determined and portions were counted for radioactivity. Results were calculated as d.p.m./*E*₆₁₅. Since Johns (1967a) has shown that different histone fractions bind different amounts of Amido Schwartz/μg, we cannot compare acetate incorporation into different histone fractions, but comparison of incorporation into two groups of the same histone fraction is valid.

Preparation of chromatin non-histone proteins

After acid extraction of the histones, the pellet was washed with ethanol, twice with ethanol-diethyl ether (3:1) and with ether. The pellet was then extracted twice with 0.1 M-NaOH. The NaOH solution was brought to 5% (w/v) trichloroacetic acid and redissolved in 0.1 M-NaOH. Protein concentrations were determined by the method of Lowry *et al.* (1951) and the ³H content by liquid-scintillation spectrometry.

Results

Time-course of incorporation of acetate into uterine chromatin proteins

Table 1 shows that incorporation of acetate into the two histone fractions is essentially complete in the first 2-5 min after administration. In contrast to the incorporation into histones, the incorporation into the chromatin non-histone proteins increases linearly over the 20 min of the experiment. Administration of 10 μg of oestradiol with the acetate had no effect on the incorporation of acetate into either the histone F1 (the apparent effect at 10 min is not statistically significant, and was not seen in another series) or the chromatin non-histone proteins. However, the administration of 10 μg of oestradiol caused a striking increase in the incorporation of acetate into the histone fraction F2+F3. This effect is maximum at 10 min (nearly 2.5 times control value) and has disappeared at 20 min, where experimental values were the same as controls.

Distribution of label among various histones

The histone fraction F2+F3 is composed of several different protein species, with at least four major components (Panyim & Chalkley, 1969). To determine the effect of oestradiol on acetylation of each component, the histones from another 5 min incorporation experiment were subjected to polyacryl-

Table 1. Incorporation of [³H]acetate into uterine chromatin proteins

Rats (28 days old) were injected with 1 mCi of [³H]acetate with or without 10 µg of oestradiol and killed 2min after injection. Rats (25 days old) were used for the 5 and 10min time-points and 26-day-old animals for the 20min time-point. Each group contained four pools of three uteri (2min time-point) or four uteri (5, 10 and 20min time-points). Values are given ±S.D. * *P*<0.02 from control value. ** *P*<0.01 from control value.

Time (min)	Incorporation (pmol of acetate/mg of protein)					
	Histone F1		Histone F2+F3		Chromatin non-histone proteins	
	Control	+Oestradiol	Control	+Oestradiol	Control	+Oestradiol
2	5.0±2.2	4.8±2.25	11.0±1.6	10.3±2.0	—	—
5	10.5±4.7	10.9±3.1	11.9±1.9	19.5±4.0*	16.7± 8.8	18.5± 4.9
10	5.5	11.9	9.7±1.8	23.8±6.3**	40.2±11.9	47.6±26.7
20	4.4	6.6	12.2±2.0	12.3±1.6	90.0±27.5	115.5±12.0

Fig. 1. [³H]Acetate labelling of histone fractions

Histone fractions were separated by polyacrylamide-gel electrophoresis as described in the text. Rats (24 days old) received 1 mCi of [³H]acetate. Experimental animals received 1 µg of oestradiol. Animals were killed 5 min after injection. There were four pools of three uteri in each group. The bars represent ±S.D. □, Control; ▨, +oestradiol.

amide-gel electrophoresis. Fig. 1 presents the results of this experiment, and it is apparent that oestradiol stimulates the incorporation of acetate into the histone F2a1.

Effect of oestradiol on the incorporation of acetate into histones of the rat liver

In another experiment, histones were isolated from the livers as well as from uteri of animals killed 5 min after injection of either acetate alone, or acetate plus oestradiol. Table 2 presents the results of this experiment. Whereas there was no effect of the oestradiol on the acetylation of the liver histones, the uterine histones showed a stimulation of acetylation similar to that previously described.

Effect of anti-oestrogen on the acetylation of uterine histones

Nafoxidine (Upjohn 11100) is an anti-oestrogen of high specificity and potency (Duncan *et al.*, 1963). It has been shown to inhibit the responses of the uptake of oestradiol by the rat uterus (Jensen *et al.*, 1966). Rats were pretreated with nafoxidine and 30min later were treated with the acetate, or acetate plus oestradiol. After a 5 min incorporation period *in vitro*, the animals were killed, and uterine histones were prepared.

The results of one such experiment are given in Table 3 with some data from Table 1 included for comparison. Nafoxidine apparently causes a decreased incorporation of acetate into uterine histones, although this decrease was not statistically significant either in group of histone F1 or in the histone F2+F3 from the non-oestrogen-treated group of animals. However, in the animals pretreated with the anti-oestrogen, oestradiol was unable to stimulate the incorporation of acetate into the histone fraction F2+F3, suggesting that the nafoxidine antagonized the oestradiol effect on histone acetylation.

Effect of antibiotics on uterine histone acetylation

To determine whether oestradiol exerted its stimulatory effects on histone acetylation through syn-

thesis of new enzymes, animals were pretreated with either actinomycin D or cycloheximide, and then given either acetate or acetate plus 10 µg of oestradiol. The animals were killed 5 min after the administration of acetate. Table 4 presents the results of these experiments. Although pretreatment with the antibiotics decreased the amount of acetylation of the histones compared with non-pretreated controls, both the actinomycin D- and the cycloheximide-treated animals showed a stimulation of acetate incorporation into histones in animals treated with oestradiol comparable with the stimulation caused by oestradiol in animals not pretreated.

Effects of different steroids and steroid doses

Lower doses of oestradiol than the 10 µg dose employed above are still in the physiological range and perhaps represent a more physiological dose, although this range has frequently been used to demonstrate short-term effects of the hormone on the uterus (Church & McCarthy, 1970; Means &

Hamilton, 1966; DeAngelo & Gorski, 1970; Mayol & Thayer, 1970). Fig. 2 shows that lower doses of oestradiol also cause stimulations of incorporation of acetate into the histone fraction F2+F3 similar to the 10 µg dose.

Stilboestrol and oestriol are also common examples of oestrogens, stilboestrol being considered as active as oestradiol, and oestriol being thought somewhat less potent. Fig. 2 also shows that 10 µg of these compounds also cause increases in the acetylation of the histone fraction F2+F3 comparable with the increases caused by oestradiol.

Testosterone, on the other hand, is not an oestrogen. Although it will cause increases in uterine weight, the characteristic histological changes caused by oestrogens are not present (Korenchewsky *et al.*, 1937). Fig. 2 shows that testosterone, at the same dose as the oestrogenic steroids, does not cause the increases in histone acetylation which were found with the oestrogens.

Discussion

The evidence presented above shows that a very early response of the uterus to oestradiol is an increase in the acetylation of certain histones of the uterine chromatin. Other early responses of the uterus to oestradiol are known; they include the stimulation of the ability of the uterine chromatin to act as a template for exogenous RNA polymerase (Church & McCarthy, 1970); the stimulation of uterine nuclear RNA synthesis (Means & Hamilton, 1966), and the induction of the synthesis of a new chromatin non-histone protein (DeAngelo & Gorski, 1970; Mayol & Thayer, 1970). The first two effects were found at 10 and 5 min, respectively, after administration of oestradiol, suggesting that they occur at about the same time as the stimulation of histone acetylation. Because the 10-min point was the earliest examined for the study on the effect of oestradiol on the efficiency of template activity, it is impossible to determine the relationship of increased

Table 2. *Effect of oestradiol on incorporation of [³H]-acetate into histones of uterus and liver*

Uteri and livers were taken from 23-day-old animals, injected with 0.83 mCi of [³H]acetate and 10 µg of oestradiol 5 min before being killed. Three livers were taken from each group and five uteri were pooled for each of three pools in each group. Values are given $\pm$ S.D. * $P < 0.05$ from control value.

Tissue	Incorporation (pmol/mg of histone F2+F3)
Uterus from control rats	6.6 $\pm$ 0.8
Uterus from oestradiol-treated rats	11.5 $\pm$ 2.7*
Liver from control rats	3.6 $\pm$ 0.8
Liver from oestradiol-treated rats	3.7 $\pm$ 0.8

Table 3. *Effect of nafoxidine on incorporation of [³H]acetate into histones of the uterus*

Animals were pretreated with nafoxidine as described in the text. Each group contains three pools of four uteri each. Results for animals not pretreated are taken from Table 1. Values are given $\pm$ S.D.

Pretreatment	Incorporation (pmol of acetate/mg of histone)			
	Histone F1		Histone F2+F3	
	Control	+Oestradiol	Control	+Oestradiol
Nafoxidine	7.6 $\pm$ 2.5	7.8 $\pm$ 3.3	7.5 $\pm$ 3.3	4.0 $\pm$ 1.0
None	10.5 $\pm$ 4.7	10.9 $\pm$ 3.1	11.9 $\pm$ 1.9	19.5 $\pm$ 4.0

Table 4. *Effect of antibiotics on [³H]acetate incorporation into histones of the uterus*

Animals (35 days old), oophorectomized at 29 days, were treated with cycloheximide (5mg/kg) 3h before the injection of acetate (experiment 1), or actinomycin D (2mg/kg) 2h before the injection of acetate (experiment 2). Animals were killed 5min after injection of 1mCi of [³H]acetate with or without 10µg of oestradiol. Four uteri were pooled and there were three pools for each group. Values are given ±s.d. * *P*<0.01. ** *P*<0.02.

Treatment	Acetate incorporation into histone (pmol/mg)	
	Histone F1	Histone F2+F3
Experiment 1		
Control	3.3 ± 1.0	15.5 ± 1.9
Control+oestradiol	1.8 ± 1.1	26.9 ± 0.6*
Cycloheximide	1.65 ± 0.8	10.0 ± 0.8
Cycloheximide+oestradiol	1.9 ± 1.6	19.1 ± 2.3*
Experiment 2		
Control	6.8 ± 1.9	22.0 ± 1.2
Control+oestradiol	6.6 ± 2.7	29.4 ± 1.5*
Actinomycin D	3.8 ± 0.8	14.9 ± 1.4
Actinomycin D+oestradiol	4.3 ± 0.4	21.9 ± 2.6**

Fig. 2. *Effect of different steroids on [³H]acetate labelling of uterine histones*

Animals (35 days old), oophorectomized at 29 days, were used. There were three pools of three uteri in each group. The bars represent ±s.d.

histone acetylation to increased template activity. Allfrey *et al.* (1964) suggested that increased acetylation of histones would lead to an increased template efficiency, although Clark & Byvoet (1969) found that *N*-terminal acetylated histones had similar

inhibitory action to histones with a free amino group at the *N*-terminal amino acid. To assess the relationship of histone acetylation to template activity in the uterus parallel studies will have to be carried out at very short times after oestradiol administration.

However, stimulation of histone acetylation does take place before either the stimulation of RNA synthesis occurs or the synthesis of the new acidic protein occurs. The experiments with actinomycin D show clearly that the stimulation of histone acetylation caused by oestradiol can occur in the absence of RNA synthesis. [Although the effect of actinomycin D on RNA synthesis in the uterus was not assessed in these experiments, Libby (1972a) showed that these conditions lead to a greater than 85% inhibition of RNA synthesis in the kidney.] Since the synthesis of the new chromatin non-histone protein has been shown to be inhibited by prior treatment of the animals with actinomycin D, it is obvious that the stimulation of histone acetylation is not dependent on prior synthesis of this protein either.

The increase in histone acetylation cannot be attributed to a stimulation of protein synthesis either, as the experiments with cycloheximide demonstrated. Since oestradiol increases the synthesis of uterine enzymes, it might be argued that I am seeing the rapid translation of a pre-existing messenger for a specific histone acetokinase. Cycloheximide, which causes an inhibition of protein synthesis in the mammary gland of over 90% (Libby, 1972b) and an inhibition of kidney protein synthesis of over 95% (Libby, 1972a), does not affect the stimulation of uterine histone acetylation caused by oestradiol.

It is also possible that oestradiol, by action on either the cellular or the nuclear membrane, could affect the pool size and thus the labelling of nuclear acetyl-CoA. I feel that this is unlikely because the acetylation of only one histone fraction is affected by oestradiol. Neither the acetylation of the remaining histone fractions (Fig. 1) nor the chromatin non-histone proteins (Table 1) are affected by oestradiol 5min after injection of the hormone. It is also possible that the difference in Michaelis constants of different acetokinases might mask such changes in pool size. Thus, all the enzymes except one would be saturated with acetyl-CoA under control conditions. This one enzyme, specific for the histone fraction F2a1, would not be saturated under control conditions, and thus the rate of acetylation would increase if the pool size were increased, giving rise to the observed effect. I have no direct evidence to bear on this point, as yet, but several separate histone acetokinases from calf uterus with different histone specificities all have approximately the same K_m value for acetyl-CoA, approx. $2 \times 10^{-6} M$ (S. Harvey & P. R. Libby, unpublished work). This suggests that these early effects of oestradiol on histone acetylation *in vivo* are not due to pool size effects.

It is of interest that one other report has appeared on histone acetylation in uterus. Anderson & Gorski (1971) studied histone acetylation under somewhat different conditions than were employed above. Oestradiol had no apparent effect on histone acetyla-

tion before 1 day after injection. However, their technique, injection of oestradiol, removal of uteri after various times and incubation of the uteri with labelled acetate *in vitro*, did not lend itself to studies of as short a time-period as the present studies did. Their earliest time-point was 20min, 5min *in vivo* and 15min *in vitro*, and they did not find any stimulation caused by oestradiol. This may be viewed as confirmation of the lack of any effect of oestradiol on histone acetylation 20min after injection which I found (Table 1).

The most likely mechanism of action whereby oestradiol can exert its effects on histone acetylation is by an activation *in vivo* similar to the one *in vitro* which increases the activity of the uterine histone acetokinase (Libby, 1968). This increase in enzyme activity would be expected to give results similar to those found above for the situation *in vivo*, since the effect of the oestradiol *in vitro* is to increase the acetylation of the histone fraction F2+F3, but not the acetylation of the histone fraction F1.

A second possibility for the action of oestradiol is an effect on the uterine histone de-acetylase. I have already shown, in the Novikoff hepatoma, that the turnover of histone *N*-acetyl groups seems to be controlled by the activity of this enzyme, rather than the activity of the histone acetokinase (Libby, 1970).

In addition to the action of oestradiol on histone acetylation being a very early action, it is obviously an 'oestrogenic' action and not a pharmacological action of oestradiol. This is shown by the effect of oestradiol on histone acetylation in the uterus, but not in the liver (Table 2), by the abolition of the oestradiol stimulation by prior administration of nafoxidine, a potent anti-oestrogen (Table 2), and by the demonstration of the effect with lower more nearly physiological doses of oestradiol, with stilboestrol and with oestriol, but not with testosterone. All this evidence led me to conclude that one of the earliest of effects of oestriol on the uterus is an increase in the acetylation of certain of the uterine histones.

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References

- Allfrey, V. G., Faulkner, R. & Mirsky, A. E. (1964) *Proc. Nat. Acad. Sci. U.S.A.* **51**, 787
- Allfrey, V. G., Pogo, B. G. T., Pogo, A. O., Kleinsmith, L. J. & Mirsky, A. E. (1966) *Ciba Found. Study Group Pap.* **24**, 42
- Anderson, N. O. & Gorski, J. (1971) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **30**, 361
- Bonner, J., Chalkley, G. R., Dahmas, M., Frebrough, D., Fujimora, F., Huang, R.-C., Huberman, J., Jensen, R., Marushige, K., Ohlenbusch, H., Olivera, B. & Widholm, J. (1968) *Methods Enzymol.* **12**, 3

- Church, R. B. & McCarthy, B. J. (1970) *Biochim. Biophys. Acta* **199**, 103
- Clark, P. R. & Byvoet, P. (1969) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **28**, 843
- DeAngelo, A. B. & Gorski, J. (1970) *Proc. Nat. Acad. Sci. U.S.* **66**, 693
- Duncan, G. W., Lyster, S. C., Clark, J. J. & Lednicer, D. (1963) *Proc. Soc. Exp. Biol. Med.* **112**, 439
- Jensen, E. V., Jacobson, H. I., Flesher, J. W., Saha, N. N., Gupta, G. N., Smith, S., Colucci, V., Shiplacoff, D., Neumann, H. G., DeSombre, E. R. & Jungblut, P. W. (1966) in *Steroid Dynamics* (Pincus, G., Nakao, T. & Tait, J. F., eds.), p. 133, Academic Press, New York and London
- Johns, E. W. (1967a) *Biochem. J.* **104**, 78
- Johns, E. W. (1967b) *Biochem. J.* **105**, 611
- Johns, E. W., Phillips, D. M. D., Simson, P. & Butler, J. A. V. (1960) *Biochem. J.* **77**, 631
- Korenchewsky, V., Dennison, M. & Eldridge, M. (1937) *Biochem. J.* **31**, 475
- Libby, P. R. (1968) *Biochem. Biophys. Res. Commun.* **31**, 59
- Libby, P. R. (1970) *Biochim. Biophys. Acta* **213**, 234
- Libby, P. R. (1971) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **30**, 361
- Libby, P. R. (1972a) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **31**, 294
- Libby, P. R. (1972b) in *Estrogens, Target Tissue and Neoplasia* (Dao, T. L., ed.), p. 85, University of Chicago Press, Chicago and London
- Liew, C. C., Suria, D. & Gornall, A. G. (1972) *Abstr. Int. Congr. Endocrinol.* **4th**. Abstr. 370
- Lowry, O. H., Rosebrough, N. H., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265
- Mayol, R. F. & Thayer, S. A. (1970) *Biochemistry* **9**, 2485
- Means, A. R. & Hamilton, T. H. (1966) *Proc. Nat. Acad. Sci. U.S.* **56**, 1595
- Monjardino, J. P. P. V. & MacGillivray, A. J. (1970) *Exp. Cell Res.* **60**, 1
- Mukherjee, A. B. & Cohen, M. M. (1969) *Exp. Cell Res.* **54**, 257
- Ono, T., Terayama, H., Takaku, F. & Nakao, K. (1969) *Biochim. Biophys. Acta* **179**, 214
- Panyim, S. & Chalkley, R. (1969) *Arch. Biochem. Biophys.* **130**, 337
- Pogo, B. G. T., Allfrey, V. G. & Mirsky, A. E. (1966) *Proc. Nat. Acad. Sci. U.S.* **55**, 805
- Pogo, B. G. T., Pogo, A. O., Allfrey, V. G. & Mirsky, A. E. (1968) *Proc. Nat. Acad. Sci. U.S.* **59**, 1337
- Takaku, F., Nakao, K., Ono, T. & Terayama, H. (1969) *Biochim. Biophys. Acta* **195**, 396
- Tidwell, T., Allfrey, V. G. & Mirsky, A. E. (1968) *J. Biol. Chem.* **243**, 707
- Trachewsky, D. & Congote, L. F. (1972) *Abstr. Int. Congr. Endocrinol.* **4th**. Abstr. 369