But in the case of chronic treatment, the hepatic metabolic alterations described by some authors[13] had apparently reached a stage of at least partial adaptation, since hepatic excretion of cholesterol suffers no alteration and the lipid excretion is only diminished by the action of PGE_1. As regards cholesterolemia, a difference between PGE_1 and PGE_2 is observed, the former increasing and the latter not modifying that parameter. We would tend to interprete the rise of blood cholesterol caused by PGE_1 as a direct effect on its processes of bio-synthesis, since the exogenous contribution of cholesterol would be inhibited by a lesser contribution of intestinal biliary salts due to the clear anticholeretic effect.

As regards the effect on lipids, the difference between PGE_1 and PGE_2 in chronic treatment appears again. With respect to the former, our results agree with those of other authors[14], who found the antilipolytic effect of this prostaglandin. We found no variations in the blood values of total lipids. PGE_2, on the other hand, increases the blood values of total lipids without altering their biliary excretion. According to the low dosage employed for the present study, a lipolytic effect may be observed, which had been already described in another animal species administered in vivo[14]. On dealing with prostaglandins and their effects on organs and tissues, results are contradictory and even more controversial in the field of the lipid metabolism. Endogenous mechanisms, as well as the exogenous or intestinal reabsorption contributions, are disturbed by the direct effect of the prostaglandins on the intestinal muscular system[15,16].

Large contradictory contributions of experimental data attribute the effect of prostaglandins both to direct mechanisms acting on the intramural neuronal plexus[17] and to indirect mechanisms which are either dependant on oxygen or mediated by the ATP-cAMP system[18]. Disparity and variety contributions increase the interest and excite attention to this field, justifying the need of deeper studies to reach more established conclusions.

[13] R. A. Levine, Gastroenterology *64*, 867 (1973).
[14] P. Nestel and S. Bergstrom, Biochem. Pharmac. *12*, 764 (1963).
[15] A. Bennett, K. G. Eley and G. B. Scholes, Br. J. Pharm. *34*, 639 (1968).
[16] B. Fleshler and A. Bennett, J. Lab. clin. Med. *74*, 872 (1969).
[17] J. Sanner, Ann. N. Y. Acad. Sci. *180*, 396 (1971).
[18] R. K. Goyal and S. Rattan, J. clin. Invest. *52*, 337 (1973).

Fluorometric Study of Interaction between ACTH Fragments and Bovine Adrenocortical Membranes[1]

T. Muraki, E. Saito and Y. Ichikawa

Departments of Pharmacology and Internal Medicine, Keio University School of Medicine, Shinanomachi, Shinjuku, Tokyo (Japan), 30 April 1976.

Summary. Corticotropin$_{1-24}$ and [Gly^1]corticotropin$_{1-18}$ amide increased the fluorescence of 1-anilinonaphthalene-8-sulfonate which bound to the bovine adrenocortical membranes. The two ACTH fragments interacted with the protein of the membranes and increased the net positive charge of the membranes.

1-Anilinonaphthalene-8-sulfonate (ANS) is a fluoroprobe which emits strong fluorescence when it binds to biological membranes, and its fluorescence is a sensitive indicator of changes in membrane charge[2]. If ACTH changes the charge of adrenocortical membranes when it binds to them, it would be possible to detect the changes of membrane charge by investigating changes in ANS fluorescence. The present study was planned to investigate whether ACTH fragments affect the net positive charge of bovine adrenocortical membranes, using ANS as a probe.

Materials and methods. Corticotropin$_{1-24}$ was obtained from Ciba, Basel, [Gly^1]corticotropin$_{1-18}$amide, [Gly^1]-corticotropin$_{1-14}$ and [Gly^1]corticotropin$_{1-10}$ were obtained from Shionogi, Osaka. Pig ACTH was purchased from Sigma. Corticotropins were dissolved at a concentration of 1 mg/ml 0.01 *N* HCl for titration. ANS (Tokyo Chemical Industry) was used as the sodium salt. Phosphatidylcholine cholinephosphohydrolase [E.C.3.1.4.3.] (phospholipase C from *Cl. welchii*) was purchased from Sigma, pronase (grade E) from Kakenkagaku, Tokyo. The plasma membranes of the bovine adrenal cortex were prepared according to the method of Finn et al.[3], and were characterized by electronmicroscopy. Membranes were suspended in 20 m*M* *Tris*-HCl, pH 7.4 containing 1 m*M* EDTA. EDTA was omitted in case of titration with $CaCl_2$. Fluorescence measurements were carried out in a Hitachi 203 spectrofluorometer. Determination of the number of binding sites for ANS and the apparent binding constants were made by Scatchard plots constructed according to Weidekamm et al.[4]. Protein concentration was determined by the method of Lowry et al.[5]. Phospholipids

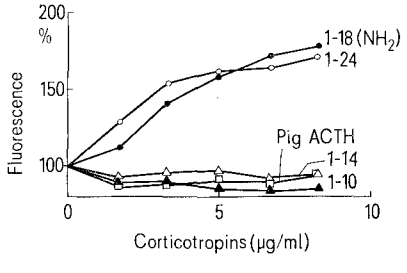

Fig. 1. Effect of corticotropin analogs on the fluorescence of ANS-adrenocortical membrane complexes. 63 µg membrane protein/ml; ANS, 76 µ*M*. The fluorescence without corticotropin analogs was set at 100%. 1–24, Corticotropin$_{1-24}$; 1–18(NH$_2$), [Gly^1]corticotropin$_{1-18}$ amide; 1–14, [Gly^1]corticotropin$_{1-14}$; 1–10, [Gly^1]corticotropin$_{1-10}$.

[1] We thank Prof. Dr. M. Kikuno for his stimulating criticism. This work was partly supported by a grant from Keio University School of Medicine.
[2] G. K. Radda and J. Vanderkooi, Biochim. biophys. Acta *265*, 509 (1972).
[3] F. M. Finn, C. C. Widnell and K. Hofmann, J. biol. Chem. *247*, 5695 (1972).
[4] E. Weidekamm, D. F. H. Wallach and H. Fischer, Biochim. biophys. Acta *241*, 770 (1971).
[5] O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, J. biol. Chem. *193*, 265 (1951).

Effect of corticotropin$_{1-24}$ and [Gly^1]corticotropin$_{1-18}$ amide on the ANS-adrenocortical membrane interaction

	Binding sites (nmoles ANS/mg protein)	Binding constant (mM^{-1})
Adrenocortical membranes	27.6	27
Adrenocortical membranes with corticotropin$_{1-24}$	46.3	28
Adrenocortical membranes with [Gly^1]corticotropin$_{1-18}$ amide	51.4	34

Titration with ANS or with adrenocortical membranes was done in 3 ml of 20 m*M* *Tris*-HCl, pH 7.4 containing 1 m*M* EDTA with or without 2.3 μM corticotropin fragments.

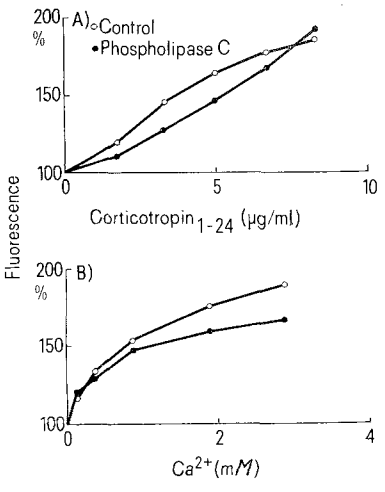

Fig. 2. Effect of treatment of adrenocortical membranes with phospholipase C on the enhancement of ANS fluorescence by corticotropin$_{1-24}$(A) or calcium ion(B). 42 μg membrane protein/ml; ANS, 6.7 μM. Phospholipase C, membranes treated with phospholipase C; control, membranes incubated without enzyme. The fluorescence without corticotropin$_{1-24}$ or $CaCl_2$ was set at 100%.

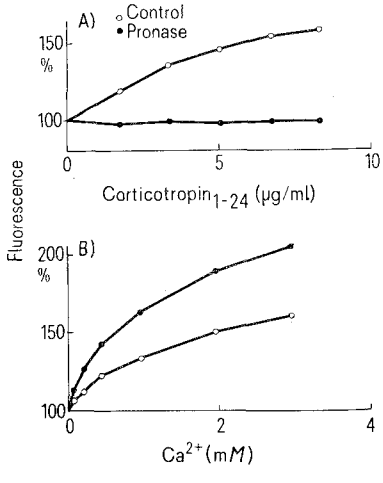

Fig. 3. Effect of treatment of adrenocortical membranes with pronase E on the enhancement of ANS fluorescence by corticotropin$_{1-24}$ (A) or calcium ion(B). 37 μg membrane protein/ml; ANS, 6.7 μM. Pronase, membranes treated with pronase E. The other conditions are similar for Figure 2.

were extracted by the method of BLIGH and DYER[6], and phosphorus was determined by the method of CHEN et al.[7]. For digestion of membranes by phospholipase C, 0.36 mg of phospholipase C (*Cl. welchii*) was added to 4.5 mg membrane protein in 1 ml of 20 m*M* *Tris*-HCl, pH 7.4 containing 10 m*M* $CaCl_2$ and the mixture was incubated at 37 °C for 30 min. At the end of incubation, 0.1 ml of 100 m*M* EDTA was added to the mixture and membranes were washed twice by suspension in 5 ml of 20 m*M* *Tris*-HCl, pH 7.4 and centrifugation. In case of treatment with pronase E, 10 mg of pronase E was added to 4.5 mg of membrane protein in 1 ml of 20 m*M* *Tris*-HCl, pH 7.4. The mixture was incubated at 37 °C for 30 min and the membranes were washed twice by suspension in 5 ml of 20 m*M* *Tris*-HCl, pH 7.4 and centrifugation. Controls were treated identically but without enzyme.

Results. When ANS was added to bovine adrenocortical membranes, the fluorescence intensity of ANS excited at 380 nm increased and the emission maximum shifted from 490 nm (ANS in 20 m*M* *Tris*-HCl, pH 7.4) to a shorter wavelength, 470 nm, which was used for measurement of ANS fluorescence throughout the present study. This change in ANS fluorescence seems to indicate that ANS bound to bovine adrenocortical membranes. Addition of corticotropin$_{1-24}$ or [Gly^1]corticotropin$_{1-18}$amide increased the fluorescence of ANS-adrenocortical membrane complexes without further shift of emission maximum (Figure 1). [Gly^1]corticotropin$_{1-10}$, [Gly^1]corticotropin$_{1-14}$ and natural pig ACTH did not increase the fluorescence of ANS-adrenocortical membrane complexes and the titration curves with these corticotropin analogs did not differ from that with 0.01 *N* HCl. When membranes were excluded from the titration mixture, ANS did not fluorescence upon addition of corticotropin$_{1-24}$ or [Gly^1]corticotropin$_{1-18}$amide up to 17 μg/ml. The mixture of corticotropin fragments and adrenocortical membranes without ANS did not show fluorescence at 470 nm when excited at 380 nm. As shown in the Table, both corticotropin$_{1-24}$ and [Gly^1]corticotropin$_{1-18}$amide at the concentration of 2.3 μM increased the number of binding sites for ANS rather than the apparent binding constant for ANS.

In order to compare the effect of ACTH analogs on ANS fluorescence with the known effect of calcium ions[8], we incubated the adrenocortical membranes with phospholipase C or pronase E and examined how these enzyme treatments influence the effects of calcium or corticotropin fragments on the fluorescence of ANS-adrenocortical membrane complexes. The phosphorus content of adrenocortical membranes without enzyme treatment (control) was 13.6 μg P/mg protein. In contrast, the phospholipid phosphorus contents of adrenocortical membranes treated with phospholipase C or pronase E were 8.1 and 19.9 μg P/mg protein respectively. This indicates that treatment with phospholipase C decreased the phospholipid content of membranes and treatment with pronase E increased the phospholipid content by digestion of membrane protein. It was shown that calcium ions increased the fluorescence of ANS-adrenocortical membrane complexes also (Figures 2B and 3B). Figure 2B shows that the calcium effect was diminished on phospholipase C-treated membranes, while the effect of corticotropin$_{1-24}$ was not affected (Figure 2A). Treatment of membranes with pronase E enhanced the calcium

[6] E. G. BLIGH and W. J. DYER, Can. J. Biochem. Physiol. *37*, 911 (1959).

[7] P. S. CHEN, JR., T. Y. TORIBARA and H. WARNER, Analyt. Chem. *28*, 1756 (1956).

[8] D. H. HAYNES, J. Membrane Biol. *17*, 341 (1974).

effect (Figure 3B) but abolished the effect of corticotropin$_{1-24}$ (Figure 3A). [Gly^1]Corticotropin$_{1-18}$amide showed a similar effect on the digested membranes to corticotropin$_{1-24}$.

Discussion. The active site of ACTH was postulared to be located in the NH_2 terminal region (positions 1–13) and the sequence Lys Lys Arg Arg (positions 15–18) was thought to be a likely binding site[9]. The present investigation shows that ACTH analogs bearing binding sequences increased the fluorescence of ANS-membrane complexes, but those without binding sequences did not. This result suggests that the ACTH fragments with binding sequences increased the membrane charge, and therefore enhanced the binding of anionic ANS. The basic amino acids composing binding sequences probably caused the increase of the net positive charge of membranes. The presence of the active site was not requisite for the fluorescent effect. Inability of pig ACTH to increase the ANS fluorescence may be different from that of shorter ACTH fragments which lack binding sequences. A possible explanation is that pig ACTH does not confer enough positive charge on the membranes to increase the bound ANS, owing to the acidic amino acids in the part of the peptide chain over the 25th amino acid.

The augmentation of ANS fluorescence bound to plasma membranes by corticotropin$_{1-24}$ or [Gly^1]corticotropin$_{1-18}$amide was not specific for adrenal cortex, since both corticotropin fragments increased the ANS fluorescence bound to bovine thyroid membranes or human erythrocyte ghosts which do not carry ACTH receptors (unpublished observation). This suggests that the changes of ANS fluorescence by the two ACTH fragments do not show the conformational changes of ACTH receptors, but indicate a rather nonspecific change of the charge of membranes.

Calcium ion is claimed to increase the fluorescence of ANS-membrane complexes through shielding the phospholipid phosphate group[2,8]. The mechanism of ACTH effect seems different, since reduction of phospholipid phosphorus content in the membranes did not affect the fluorescence effect of ACTH fragments, while it decreased the effect of calcium ion. Inability of ACTH fragments to increase the fluorescence of ANS bound to the pronase-treated membranes may suggest that ACTH fragments interact with the pronase-sensitive protein of adrenocortical membranes. On the contrary, membrane protein did not contribute the fluorescence effect of calcium ion so much.

Although the fluorescence effect of ACTH fragments was not related to their binding on ACTH receptors, the present study suggests the possibility that the synthetic, basic ACTH analogs may have a different pharmacological action from that of the natural ACTH by changing the electrostatic environment of the ACTH receptors.

[9] K. Hofmann, W. Wingender and F. M. Finn, Proc. natn. Acad. Sci., USA *67*, 829 (1970).

Conversion of Testosterone to Androstenedione by Liver Homogenates of Testicular Feminized Mice[1]

J. D. Daley, S. Ohno[2] and E. V. YoungLai

Department of Obstetrics and Gynecology, McMaster University Medical Centre, 1200 Main Street West Hamilton (Ontario, Canada L8S 4J9), and Department of Biology, City of Hope National Medical Centre, Duarte (California, USA 91010), *8 June 1976.*

Summary. Liver homogenates of testicular feminized (*Tfm*) mice carrying the protective (o^{hv}) gene were found to be less capable of converting testosterone to androstenedione than Tfm without the protective gene.

Testicular feminization in the mouse is genetically determined by the presence of a mutant gene (*Tfm*) on the *X*-chromosome[3]. Recently, a strain of mice has been developed with a further mutant gene (o^{hv}) on the *X*-chromosome, the position of which enables it to modify the expression of *Tfm* so as to restore a measure of androgen sensitivity to some of the target organs[4]. The relationship between the androgen receptor defect[5] and altered steroid metabolism in adult testicular feminized mice[6] is not clear. Differences in the metabolism of androstenedione (A) have been observed in the liver of the Stanley- Gumbreck male pseudohermaphrodite (PS) rat and found to be similar to that of females[7]. Here we report differences in the liver conversion of testosterone (T) to A in two strains of testicular feminized mice.

Mice of genotypes *Tfm*+(o^+)+/*Y*♀, *Tfm*+(o^{hv})*Blo*/*Y*♀, maintained at City of Hope Medical Center, California and normal male BALB/C mice (Health Research Inc.) used in this study ranged in ages from 53 to 190 days. Mice were anesthetized and liver tissue excised, weighed and homogenized in Medium 199 (Gibco). Co-factors were not added in order to simulate in vivo conditions as close as possible. Crude homogenates (1 ml, 11–14 mg wet wt. tissue) were incubated in 5 ml medium 199 with 0.4 μCi [7α-^{3}H] testosterone (25 Ci/m*M*; New England Nuclear) dissolved in propylene glycol, for 2 h in a Dubnoff metabolic shaker at 37 °C with air as the gas phase. Incubations were terminated by quick freezing. Medium was extracted with diethyl ether and the ether extract partitioned between toluene and 1 *N* NaOH. The neutral fraction was subjected to thin layer chromatography in chloroform: methanol (98:2) at 4 °C. Radioactive zones corresponding to T and A were eluted and rechromatographed on Whatman No. 1 paper in heptane:methanol: water (5:4:1) and then crystallized with carrier to constant specific activity. No correction for losses were made

[1] This work was supported by the Medical Research Council of Canada, No. MT 4192 and Ortho Pharmaceutical Company.

[2] Address: Department of Biology, City of Hope National Medical Centre, Duarte, California 91010, USA.

[3] M. F. LYon and S. G. Hawkes, Nature, Lond. *227*, 1217 (1970).

[4] U. Drews, S. R. Blecher, D. A. Owen and S. Ohno, Cell *1*, 3, (1974).

[5] B. Attardi and S. Ohno, Cell *2*, 5 (1974).

[6] C. W. Bardin and L. P. Bullock, J. clin. Invest. *51*, 75 (1974).

[7] K. Einarsson, J. -P. Gustafsson and A. S. Goldman, Eur. J. Biochem. *31*, 345 (1972).