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Stimulatory Effect of Ecdysterone on RNA Synthesis in Mouse Liver

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The administration of ecdysterone to mice is accompanied by the incorporation of orotic acid into ribonucleic acids in the liver. This effect was completely repressed by actinomycin D.

Judging from the template activity of RNA in an amino acid-incorporating system and the results of sucrose gradient analysis, it is obvious that the rate of synthesis of messenger RNA is regulated by ecdysterone as well as other RNA in mouse liver.

The stimulating effect of ecdysterone on protein synthesis reported previously can be explained at least partially in terms of its influence on messenger RNA synthesis.

It was reported previously that various insect-metamorphosing steroids obtained from plant kingdom can stimulate the amino acid incorporation into protein in mouse liver,[2] and the cause of which may be explained in terms of its effect on the ability of ribosomes to assemble amino acids into polypeptide chains.[3]

The present paper describes some results which suggest that ecdysterone, one of the insect-metamorphosing steroids, acts on protein synthesis through regulating the synthesis of messenger and other types of RNA.

Materials and Methods

Animals and Treatment——Male mice (*dd*-strain) weighing 18—22 g were used throughout the experiment. Ecdysterone was dissolved in 0.9% saline and injected intraperitoneally in a dose of 10 μg per mouse. Actinomycin D was dissolved in propylene glycol and administered by intraperitoneal injection. ^{14}C-Orotic acid (30 mCi/mM) and ^{14}C-chlorella hydrolysate (4.2 mCi/mg) were obtained from Daiichi Chemical Company Ltd. The orotic acid was dissolved in dilute alkali saline.

Preparation of Cell-free Fraction——The mice were killed by decapitation and the livers were homogenized in 3 volumes of medium K_1 (0.25M sucrose; 0.01M $MgSO_4$; 0.025M KCl; 0.05M tris-HCl, pH 7.6)[4] and 4 volumes of medium H (0.05M tris-HCl, pH 7.4; 10^{-4}M $MgSO_4$; 10^{-4}M EDTA; 4 μg/ml polyvinyl sulfate) for protein synthesis and RNA preparation, respectively. Cytoplasm fraction used was prepared by the centrifugation of homogenate at $600\times g$ for 10 min or at $12000\times g$ for 10 min, depending on a purpose of experiment. Ribosomes used for assay of template activity were isolated as follows: Livers obtained from mice starved for 24 hours were homogenized. After removal of mitochondria, nuclei and debris by centrifugation at $20000\times g$ for 15 min at 0°, the supernatant was treated with one-ninth of its volume of ice-cold 10% (w/v) sodium deoxycholate freshly dissolved in deionized water. Resulted mixture was laid on a top of the centrifugation-tube containing medium K_1 in 1M sucrose to prepare the total ribonucleoprotein particles by centrifugation at $105000\times g$ for 3 hours. Cell sap was prepared by centrifugation of the $20000\times g$ supernatant (without the addition of deoxycholate) at $105000\times g$ for 3 hours.

Preparation and Analysis of Ribonucleic Acid——Cytopoasm and nuclei were treated at room temperature and at 60°, respectively, by shaking with equal volume of water-saturated phenol containing 0.5% sodium dodecyl sulfate. After repeated treatment with phenol and RNA in the aqueous layer was precipitated with 2% (final conc.) sodium acetate and 2 volume of ethanol. The resulted precipitation was again dissolved in water, and reprecipitated with ethanol. Finally, the RNA dissolved in water and analysed by

1) Location: *Aobayama, Sendai.*
2) S. Okui, T. Otaka, M. Uchiyama, T. Takemoto, H. Hikino, S. Ogawa and N. Nishimoto, *Chem. Pharm. Bull.* (Tokyo), **16**, 384 (1968).
3) T. Otaka, S. Okui and M. Uchiyama, *Chem. Pharm. Bull.* (Tokyo), **17**, 75 (1969).
4) K. Koike, T. Otaka and S. Okui, *J. Biochem.*, **59**, 201 (1966).
5) A. Hadjivassiliou and G. Brawerman, *Biochemistry*, **6**, 1934 (1967).

centrifugation for 3 or 3½ hours at 39000 rpm on a linear 5—20% sucrose-gradient containing tris-HCl, pH 7.6 (0.05M) and KCl (0.025M) with the RPS-40 rotor of Hitachi ultracentrifuge. Following the centrifugation the bottom of the tube was punctured with a hypodermic needle, and successive three drop-samples were collected. From each fraction 0.1 ml was separated for determination of radioactivity by subsequent addition of albumin (2 mg) as a carrier and precipitation with 6% $HClO_4$. The radioactvie precipitates were dissolved in 90% (v/v) formic acid, plated in planchettes, dried under an infrared lamp and measured with a 2π gas flow counter. The rest of each fraction was diluted with 3 ml of water and the content of RNA was calculated by absorbance at 260 mμ (1 mg RNA=21.6 $A_{260\ m\mu}$ Unit). Alkali-extracted RNA was prepared by the procedure of Hadjivassiliou and Brawerman.[5] High molecular RNA was prepared according to LiCl method: An equal volume of 4M LiCl solution was added to total RNA solution to make a final concentration of 2M. The solution was kept at 0° and the white flocculant precipitate was used as high molecular RNA.

Assay for Template Activity of RNA——The reaction mixture for the incorporation of amino acid into protein was as same as described previously.[3] RNA prepared from control and ecdysterone-treated animals was dissolved in 0.1 ml of water as indicated in Table IV and added in the reaction mixture. Incubation was carried out under air for 30 min at 37°. Procedures for preparation of acid-insoluble protein and the measurement of radioactivity were reported previously.[3] Protein was determined by Biuret method.[6]

Results

Stimulatory Effect of Ecdysterone on Orotic Acid Incorporation into RNA *in Vivo*

Table I summarizes the labelling of nuclear and cytoplasmic RNA in normal and ecdyste-

Table I. Incorporation of ^{14}C-Orotic Acid into Liver Nuclear and Cytoplasmic Ribonucleic Acid of Normal Mice Given Ecdysterone

Treatment		cpm/mg RNA	%
(A) Cytoplasm			
Control		121±10	100
Ecdysterone	2.0 hr[a)]	364±13	301
	2.5 hr	261±12	216
	3.5 hr	235±11	194
(B) Nuclei			
Control		491±15	100
Ecdysterone	2.0 hr	1243±17	253
	2.5 hr	1017±11	207
	3.5 hr	998±18	203

Ecdysterone (10 μg per mouse) was administered to normal mice. In this experiment, cytoplasmic fraction is 12000×*g* supernatant. Values are the mean±standard error for six mice used.

a) ^{14}C-orotic acid (1 μCi per mouse) was injected 1.5, 2 and 3 hr after ecdysterone administration and 30 min later the animals were killed and the livers removed.

Table II. Influence of Ecdysterone Administration on ^{14}C-Orotic Acid Incorporation into High-molecular RNA of Cytoplasmic and Nuclear Ribonucleic Acid from Liver

Treatment		cpm/mg RNA	%
(A) Cytoplasm			
Control		71± 3	100
Ecdysterone	2 hr	205±12	289
(B) Nuclei			
Control		276±16	100
Ecdysterone	2 hr	768±20	278

Experimental details are given in the experimental section. Values are the mean±standard error for six mice used.

6) A.G. Gornall and M.M. Bardawill, *J. Biol. Chem.*, **177**, 751 (1949).

rone–treated mouse liver with ^{14}C-orotic acid. A single injection of ecdysterone into normal mice remarkably enhanced the incorporation of radioactivity both into nuclear RNA and cytoplasmic RNA. The increment was rather great at early time after the administration. As shown in Table II, the label in high molecular RNA obtained from nuclear and cytoplasmic RNA was also significantly elevated at 2 hours after the administration, which indicates the increased incorporation of orotic acid into the other RNA as well as low molecular RNA.

Table III. Effect of Actinomycin (Act.) D on Stimulation of ^{14}C-Orotic Acid Incorporation into Cytoplasmic and Nuclear RNA by Ecdysterone

Treatment	cpm/mg RNA	%
(A) Cytoplasm		
Normal	124± 2	100
Actinomycin D	56± 5	45
Act. D+Ecdysterone	59± 4	48
(B) Nuclei		
Normal	877± 3	100
Actinomycin D	269±17	31
Act. D+Ecdysterone	211±21	24

Actinomycin D (40 μg per mouse) and/or ecdysterone were administered to normal mice. 1.5 hr later, ^{14} C-orotic acid (1 μCi per mouse) was injected and the animals were killed 30 min. Values are the mean±standard error for six mice used.

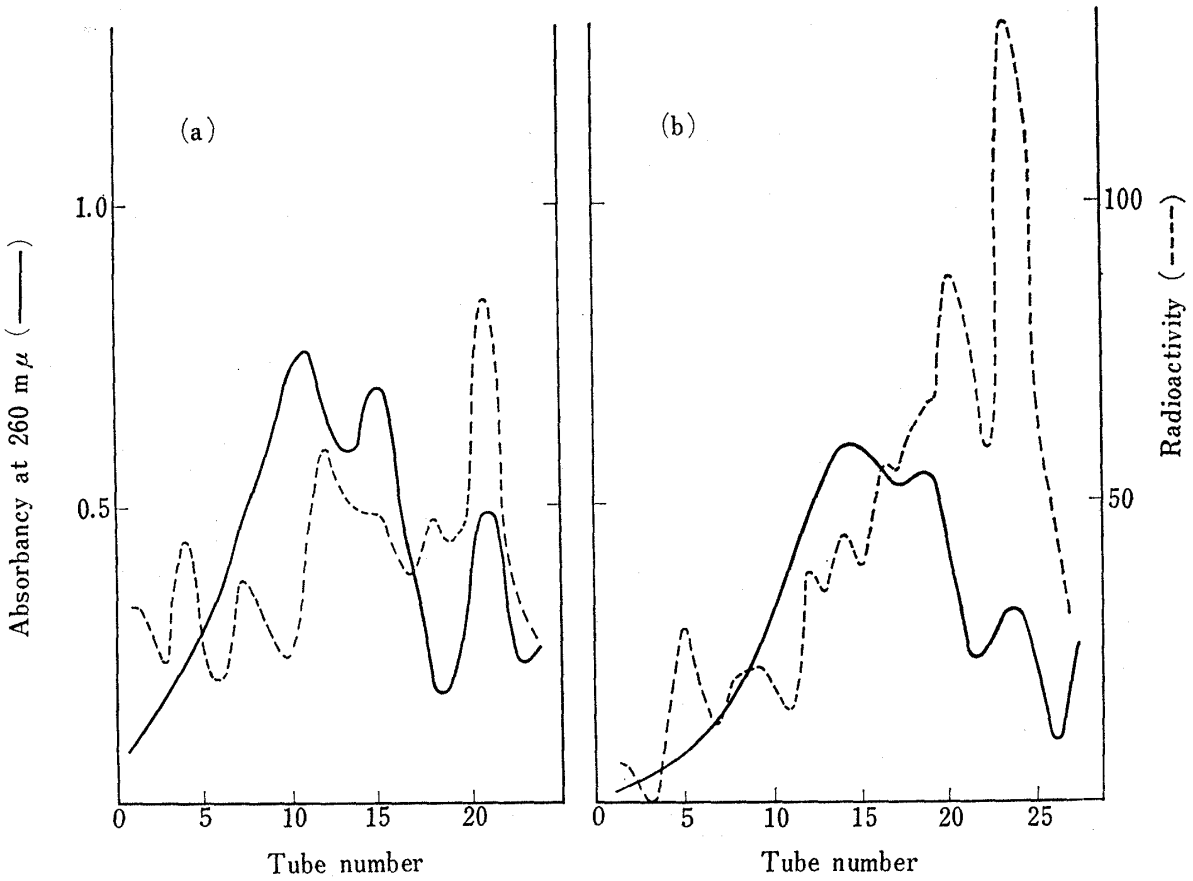

Fig. 1. Labelling Pattern of Cytoplasmic RNA influenced by Ecdysterone

Ecdysterone was administered normal mice 2 hr before the animals were killed (b). Saline was administered to the controls(a). ^{14}C-orotic acid was injected 30 min prior to sacrificing the animals. The liver cytoplasmic fraction (600×g supernatant) was isolated, the RNA extracted and centrifuged at 39000 rpm for 3½ hr on a linear surcose density gradient (5—20%) in a Hitachi RPS 40 rotor.

Actinomycin treatment of the mice caused the expected[7,8] inhibition of RNA synthesis either with or without ecdysterone (Table III).

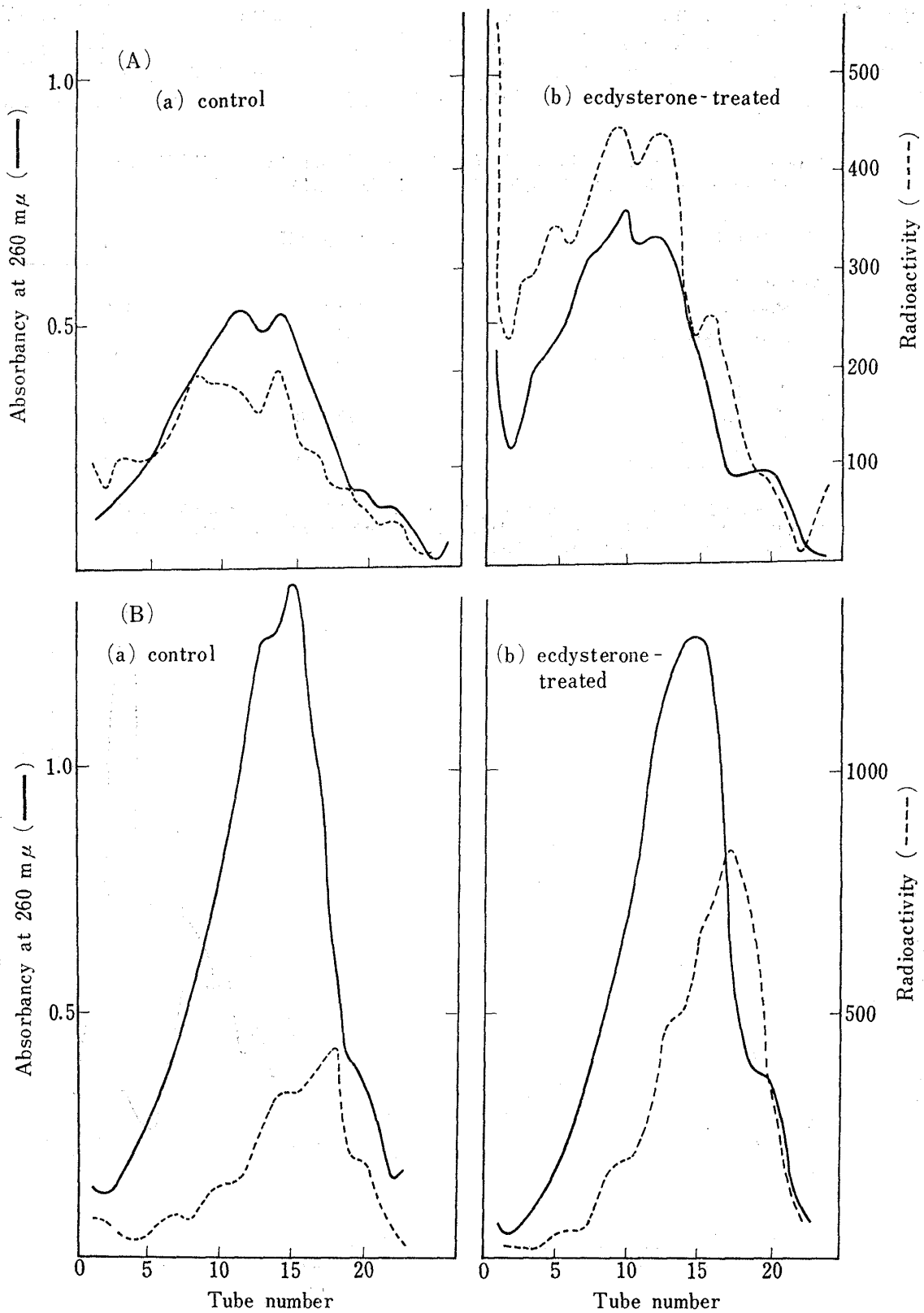

Fig. 2. Labelling Pattern of Nuclear RNA influenced by Ecdysterone

Experimental conditions are the same as that in Fig. 1. (A) and (B) are results of different experiments.

7) I. Meritz, *Biochem. Biophys. Res. Commun.*, **10**, 254 (1963).
8) R.M. Franklin, *Biochim. Biophys. Acta*, **72**, 555 (1963).

Sucrose Density Gradient Analysis of RNA——The density gradient analysis of the labelled cytoplasmic RNA obtained from normal and ecdysterone-treated mice was shown in Fig. 1.

The distribution of radioactivity in cytoplasmic RNA after a pulse labelling with orotic acid was heterogenous and not associated with the major peaks drawn by optical extinction. Treatment of normal mice with ecdysterone resulted in a clear increase in labelling of the RNA in 4S and 6-18S regions. The former can be designated as transfer RNA and the latter has some of the characteristics of messenger RNA.[9,10]

In the sucrose-gradient analysis of the nuclear RNA, as shown in Fig. 2, two types of pattern were obtained.

In Fig. 2 (A) the pattern of absorbance were similar to that obtained from cytoplasm. It was observed that ecdysterone stimulated incorporation of orotic acid into all types of RNA. On the other hand, another experiment showed the results as Fig. 2 (B), namely the absorbance apttern made a single peak around 19S region. In the latter case incorporation of orotic acid into 6-18S RNA was markedly stimulated. Although it is still not clear what kind of experimental condition produces such a different pattern, these results may be giving an account that the rapidly labelled nuclear RNA has a tendency to change easily to 6-18S RNA which in turn appeared in cytoplasm as shown in Fig. 1.

Template Activity of RNA Fractions——From the results mentioned above it is very likely that the RNA increased by ecdysterone treatment has a character of messenger RNA. To confirm the function of the RNA, template activity of both nuclear and cytoplasmic RNAs obtained from ecdysterone-treated mice was compared with those RNAs from normal mice. Table IV shows the significantly higher template activity produced by ecdysterone.

Table IV. Effect of RNA from Control and Ecdysterone-treated Mouse Liver nuclei and Cytoplasm on an Amino Acid Incorporation *in Vitro*

Treatment	Fraction	Amount (μg)	^{14}C-Amino acids incorporation (total counts)	$\Delta^{a)}$
—	—	—	414±11	
Control	cytoplasm	35	574±10	160
Ecdysterone	cytoplasm	35	736±12	322
Control	cytoplasm	70	536± 6	122
Ecdysterone	cytoplasm	70	666±10	252
Control	nuclei	40	590± 5	176
Ecdysterone	nuclei	40	718± 6	304
Control	nuclei	80	692± 3	278
Ecdysterone	nuclei	80	794± 2	380

a) Δ=net incorporation due to RNA added

The animals were killed 3 hr after ecdysterone administration. Nuclear or cytoplasmic ($600\times g$ supernatant) RNA was added, in the amounts indicated, to the ribosomal amino acid—incorporating system. Incubation procedure is given in experimental section.

Values are the mean±standard error for six mice used.

It is also noted that template activity of cytoplasmic RNA declined at higher concentrations, while that of nuclear RNA did not.

It has been reported that RNA having a template activity is to be extracted at alkaline solution.[5] As shown in Table V, incorporation of orotic acid into alkali-extracted RNA was markedly stimulated by ecdysterone administration, which is well compatible with the fact that the RNA formed by ecdysterone administration have a higher template activity.

9) A.J. Munro and A. Korner, *Biochem. J.*, **85**, 37P (1962).
10) A.C. Trakatellio, A.E. Axerlord and M. Montjar, *Nature*, **203**, 1134 (1964).

Table V. Effect of Ecdysterone on ^{14}C-Orotic Acid Incorporation into Alkali-extracted Ribonucleic Acids

Treatment	cpm/mg RNA	%
(A) Cytoplasm		
Control	136±13	100
Ecdysterone	205±11	152
(B) Nuclei		
Control	114± 7	100
Ecdysterone	487±20	427

The animals were killed 2 hr after ecdysterone administration.
Values are the mean±standard error for mice used.

Discussion

From the results obtained by the present experiments it is clear that the administration of ecdysterone to mice was attended by an increase in the incorporation of a precursor into both cytoplasmic and nuclear RNA of the liver. The stimulatory effect of ecdysterone on RNA synthesis was completely abolished by actinomycin which has been known to exert its effect on RNA synthesis by masking the guanine bases of the primer DNA[11] and thus stopping the polymerization of all DNA-primed RNAs.

Although Hiatt[12] has failed to detect any rapidly labelled RNA around 6-18S region in the cytoplasm of liver cells, Munro and Korner[9] and Trakatellis, *et al.*[10] reported the detection of rapidly labelled 6-18S RNA and presumed that is messenger RNA. Also in the present experiments, the labelling pattern of cytoplasmic RNA obtained by means of sucrose density gradient analysis indicated that ecdysterone enhanced the incorporation of orotic acid not only into structural RNA of ribosomes and 4-5S RNA, but also into 6-18S RNA.

Moreover, the extinction and labelling patterns obtained from nuclear RNA showed two distinct types, depending on an experimental condition: One was the spread labelling throughout the gradient and the other was a single labelling at about 6-18S. This phenomenon is likely to suggest that high density nuclear RNA may be unstable and easily degraded to 6-18S RNA, which may be transported into cytoplasm and show the characteristics of messenger RNA.

Most of the RNA of nuclei became labelled a short time after the injection of orotic acid and it is not easy to assign the name of 'Messenger RNA' to any of the nuclear RNA fractions. It is clear, however, the amount of RNA active as template *in vitro* was increased by ecdysterone. This could be relative increase of template RNA per cytoplasmic or nuclear 'bulk RNA.'

More likely, judging from RNA labelling and the stimulatory incorporation of orotic acid into alkali-extracted RNA, the above facts can be interpreted as a true increase of messenger RNA by (ecdysterone) hormone stimulation.

The resultant new messenger RNA may elevate the synthesis *de novo* of proteins as reported previously.[3] In fact, the long term administration of ecdysterone to mice brought the histological changes in liver, which showed stimulated metabolism and active regeneration of cells.[13]

It remains obscure what kind of proteins are produced and what is an actual roll of such proteins in physiological functions.

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11) I.H. Goldberg and M. Rabinowitz, *Science*, **136**, 315 (1962).
12) H.H. Hiatt, *J. Mol. Biol.*, **5**, 217 (1962).
13) H. Hikino, S. Nabetani, K. Nomoto, T. Arai, T. Takemoto, T. Otaka and M. Uchiyama, *Yakugaku Zasshi*, **89**, 253 (1969).