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Stimulation of Protein Synthesis in Mouse Liver by Ecdysterone

Tadahiko Otaka, Seiichi Okui,[1] and Mitsuru Uchiyama

Pharmaceutical Institute, Tohoku University School of Medicine[2]

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The effect of insect-moulting steroid, ecdysterone, on protein synthesis in mouse liver was studied.

The administration of ecdysterone caused an early stimulation of protein synthesis, as indicated by the marked increase in the incorporation of ^{14}C-chlorella hydrolysate into hot-acid insoluble protein, as early as 2 hr after the treatment as well as does 4-chlorotestosterone.

Ecdysterone was able to exert its stimulatory effect on the protein synthetic ability of microsomes or polysomes, but not S-105 fluid, soluble components for protein synthesis, except in microsomal system.

The stimulation induced by ecdysterone was partly insensitive to actinomycin, inhibitor of DNA-dependent RNA synthesis, but the stimulation by 4-chlorotestosterone was completely repressed by it.

In 1963 Karlson showed that moulting in insects is induced by the hormone, ecdysone, prepared from the prothoratic glands.[3] An early effect of this hormone to change the activity of specific gene loci has been found in studies on the puffing phenomenon in salivary gland chromosomes of *Chironomus tetans*.[4] Pupatation of blowfly larvae, which is controlled by ecdysone, can be delayed considerably by the injection of inhibitors of RNA and protein synthesis and, at the same time, the induction of the enzyme, DOPA-decarboxylase, is inhibited by these metabolic inhibitors.[5]

Experiment *in vivo* showed that ecdysone stimulates the formation of nuclear RNA with increase in an amino acid incorporation into protein.[6] Moreover, RNA isolated from nuclei which had been incubated with the hormone was found to contain increased template activity over that of appropriate controls[7].

Recently it has become generally recognized that insect-moulting steroids are widely distributed in the plant kingdom: isolation of the ponasterones, ecdysterone, inokosterone, pterosterone and cyasterone has been reported.[8] Since these substances have a steroid-skelton such as adrenal corticoid, sex hormones and so on, and since the plants containing these steroids are generally acceptable as oriental remedy and dietary source for mammalia, it became interest to investigate the effect of these steroids on mammalian metabolic systems.

In the present paper, it will be reported that the administration of ecdysterone to mice can elevate the protein synthetic activity in the livers as 4-chlorotestosterone, a potent anabolic steroid. Such effect was observed as early as 2 hr after the administration. Stimulation by these steroids of protein synthetic activity was not associated with cell sap, enzymes

1) Deceased March 18th, 1967.
2) Location: *No. 85, Kita-4-bancho, Sendai.*
3) P. Karlson, *Angew. Chem.*, **75**, 257 (1963).
4) U. Clever and P. Karlson, *Exptl. Cell. Res.*, **20**, 623 (1960).
5) C. E. Sekeris and P. Karlson, *Arch. Biochem. Biophs.*, **105**, 483 (1964).
6) C. E. Sekeris, N. Lang, and P. Karlson, *Z. Physiol. Chem.*, **341**, 36 (1965).
7) P. P. Dukes, C. E. Sekeris, and W. Schmid, *Biochim. Biophys. Acta*, **123**, 126 (1966).
8) *cf.* T. Takemoto, Y. Hikino, A. Arai, M. Kawahara, C. Konno, S. Arihara, and H. Hikino, *Chem. Pharm. Bull.* (Tokyo), **15**, 1816 (1967).

for protein synthesis and *t*-RNA, but microsomes and polysomes. A part of this work was preliminarily reported.[9]

Experimental

Materials——L-^{14}C-alanine with a uniformly labelled (specific radioactivity of 39.6 mCi/mmole) and ^{14}C-chlorella hydrolysate with a uniformly labelled (specific radioactivity of 4.2 mCi/mg) were obtained from the Daiichi Chemical Co., Japan. Actinomycin D was kindly given by Merck Sharp and Dohme. ATP, GTP, creatine phosphate and creatine kinase [EC 2.7.3.2.] were obtained from Boehringer und Soehe G.m.b.H., Manheim, Germany. Ecdysterone was kindly given by Dr.S. Ogawa, the research laboratories of Rhoto Pharmaceutical Co., Ltd.

Animals and Treatment——Male mice (18—22 g) of *dd*-strain were used with no restriction on their food intake throughout the experiments. Ecdysterone dissolved in 0.9% saline solution was administered intraperitoneally in a dose of 0.05 mg per 100 g of body weight at a.m. 9. 4-Chlorotestosterone was also suspended in saline and injected in a dose of 1 mg per 100 g body weight at a.m. 9. Actinomycin D dissolved in ethanol–saline solution was intraperitoneally injected in a dose of 40 μg per a mouse.

Preparation of Cell-free Fraction——The mice were decapitated at the indicated time after treatment. Livers were removed rapidly and rinsed in an ice-cold 1.15% KCl, weighed and homogenized with 1.5 volumes of Medium K_1 (0.25M sucrose; 0.01M $MgSO_4$; 0.025M KCl; 0.05M tris–HCl, pH 7.6)[10] by Potter–Elvehjem teflon homogenizer. The homogenate was centrifuged at 20000$\times g$ for 15 min at 0° to remove mitochondria, nuclei and cell debris and the supernatant fluid is designated as S–20 fluid. The microsomal fraction were prepared by centrifuging S–20 fluid, 3 volumes of Medium K_1 was used in this case, for 60 min at 105000$\times$ g by Hitachi ultracentrifuge (RP 40 rotor) and yielded pellets were suspended in Medium K_3 (0.01M $MgSO_4$; 0.025M KCI; 0.05M tris–HCI, pH 7.6)[10] for use. Preparation of polysomes was performed by the method of Wettstein, *et al.*[11] as follows: The S–20 fluid was treated with one–seventh of its volume of an ice–cold 10% (w/v) solution of sodium deoxycholate. Five ml of deoxycholate-treated S–20 fluid was layered over 2 ml of 0.5M sucrose solution in Medium K_3, which was layered itself over 3 ml of 2.0M sucrose sulution in Medium K_3. After centrifuging for 3 hr at 105000$\times$ g, the supernatant fluid was sucked up and the sides of the centrifuging tube were cleaned and wiped to dryness with filter paper. The pellet was rinsed in Medium K_3 and suspended in Medium K_3 by gentle agitation. The resultant fluid was used as polysome fluid. S–105 fluid used as enzyme source was obtained as the supernatant after separation of microsomes described above and, if necessary, treated with Sephadex G–25 to remove low molecules.

Incubation System——The reaction mixture consisted of 50 μmoles of sucrose, 25 μmoles of Tris–HCl (pH 7.6), 5 μmoles of $MgSO_4$, 12.5 μmoles of KCl, 2.0 μmoles of ATP, 0.2 μmole of GTP, 10 μmoles of phosphocreatine, 10 μg of creatinephosphokinase [EC 2.7.3.2], 0.1 μCi of ^{14}C-labelled chlorella hydrolysate, or 0.2 μCi of ^{14}C-alanine, 0.1 ml of polysome fluid or 0.1 ml microsomal fluid and 0.1 ml S–105 fluid (approx. 2.5 mg of protein) as the polysomal or microsomal system, and 0.2 ml of S–20 fluid (approx. 10 mg of protein) as the S–20 system in total 0.5 ml. Incubation was carried out in test tubes under air at 37° for 25 min and for 40 min in the S–20 system and in the microsomal or polysomal system, respectively.

Assay of the Protein Synthetic Activity *in Vivo*——One μCi of ^{14}C-chlorella hydrolysate was injected 15 min before sacrificed to mice which were pretreated with steroid. The livers were homogenized in 3 volumes of Medium K_1 and fractionated as described in the legend of Fig. 1.

Preparation and Counting of Radioactive Protein——After incubation the reaction was stopped by adding 4 ml of 6% perchloric acid. The precipitate was separated by centrifugation, washed three times with 6% perchloric acid and treated with the same reagent at 90° for 15 min and finally extracted twice with ether–ethanol–chloroform (2:2:1). The resulted labelled protein was dissolved in 90% (v/v) formic acid and plated on planchets and dried under an infrared lamp. The protein synthetic activity *in vivo* was also measured in the above–mentioned procedure. A 2π gas flow counter with a thin window was used for counting. Protein content was determined by a slight modification of the method of Gornall and Bardawill[12] or gravimetric analysis. The incorporation of ^{14}C-amino acids was calibrated after correction for self–absorption effect.

Gradient Analysis of the Products in S-20 System-Incubation——The products in S–20 system–incubation were analysed by sucrose–density–gradient centrifugation by layering 0.3 ml of it on a linear 5—20% sucrose gradient set up with Medium K_3. A cushion of 0.6 ml of 2 M sucrose solution in Medium K_3 was put at the bottom of the tube. After centrifugation for 3.5 hr at 39000 rpm by Hitachi ultracentrifuge (RPS 40 rotor),

9) Late S.Okui, T.Otaka, M. Uchiyama, T. Takemoto, H. Hikino, S. Ogawa, and N. Nishimoto, *Chem. Pharm. Bull.* (Tokyo), **16**, 374 (1968).
10) K. Koike, T. Otaka, and S. Okui, *J. Biochem.* (Tokyo), **59**, 201 (1966).
11) F.O. Wettstein, T. Staehelin, and H. Noll, *Nature*, **197**, 430 (1963).
12) A.G. Gornall and M.M. Bardawill, *J. Biol. Chem.*, **177**, 751 (1949).

each four drops was collected from the bottom of the tube. Two mg of albumin was added to each tube as carrier and the protein was precipitated by addition of 4 ml of 6% perchloric acid. The subsequent procedures were the same as described in the preparation for protein counting.

Result

Effect of Ecdysterone and 4-Chlorotestosterone on Amino Acid Incorporation into Liver Protein *in Vivo*

In view of the stimulatory effect of ecdysterone and 4-chlorotestosterone on protein synthesis in mouse liver, experiments were conducted to determine on which component in the cell treatment with these steroids produces stimulatory effect. Results presented in Fig. 1 show the incorporation of ^{14}C-amino acids into protein of the liver sub-fractions in normal and treated mice which were given a single injection of 10 μg of ecdysterone 1, 2, and 4 hours before death or of 200 μg of 4-chlorotestosterone 4 hr before death. Incorporation of ^{14}C-amino acids into protein *in vivo* was obviously enhanced by the treatment of the steroid in all subcellular fractions. The degree of stimulation by these steroids was almost same in three fractions, nuclei, mitochondria and microsomes. The amount of ^{14}C-amino acids incorporated into protein of mouse liver treated with

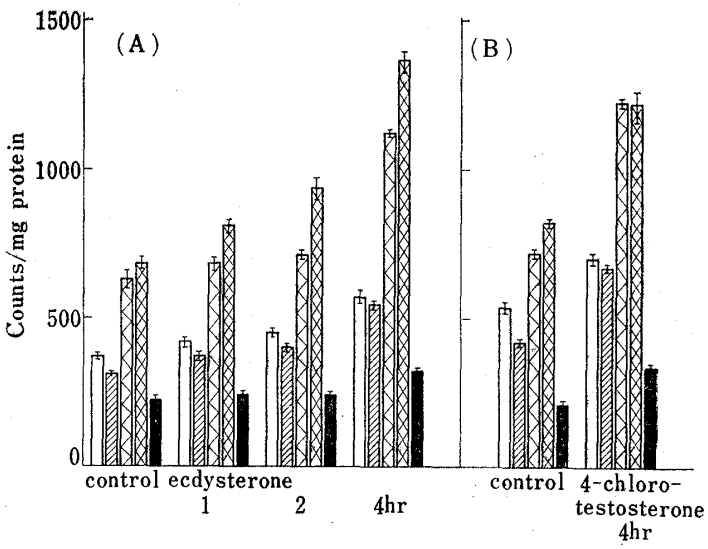

Fig. 1. Stimulatory Effect of Ecdysterone on Protein Synthetic Activity in Mouse Liver *in Vivo*

Ecdysterone was injected intraperitoneally in a dose of 0.5 mg per 100 g body weight and 1 μCi of ^{14}C-chlorella hydrolysate was also injected intraperitoneally 15 min before sacrifice. The livers were homogenized in 3 volumes of Medium K_1. Nuclei and cell debris were sedimented at $600 \times g$ for 10 min, mitochondria at $9000 \times g$ for 10 min, microsomes at $85000 \times g$ for 90 min, and supernatant was postmicrosomal fluid. The treatment of protein samples was given in the Experimental section.

whole homogenate
nuclei and cell debris
mitochondria
microsomes
supernatant

Table I. Stimulatory Effect of Ecdysterone and 4-Chlorotestosterone on the Incorporation of Amino Acids into Hot-acid Insoluble Protein Fraction *in Vitro*

	Treatment		cpm/mg protein	Stimulation (%)
(A)	Control		307±4	100
	Ecdysterone	1 hr	400±62	130
		2 hr	553±31	180
		4 hr	603±12	196
	4-Chlorotesterone	1 hr	398±8	130
		2 hr	580±16	189
		4 hr	610±20	199
(B)	Control		118±4	100
	Ecdysterone	2 hr	153±6	130

(A) ^{14}C-Chlorella hydrolysate was used as labelled amino acids. Each result is the mean ± standard error for ten mice used.
(B) ^{14}C-L-Alanine was used as a labelled amino acid. Each result is the mean ± standard error for four mice used.

ecdysterone increased in course of time and was not less than that with 4-chlorotestosterone.

Incorporation of Amino Acids into Protein with S-20 Fluid from Livers of Normal and Steroid-treated Mice

Table I (A) shows that the S–20 fluid obtained from the livers treated with ecdysterone or 4-chlorotestosterone is more active to incorporate amino acids than that obtained from normal livers. The incorporation increased significantly at 2 and 4 hr after the treatment with ecdysterone. The magnitude of the increment was almost equal to that in 4-chlorotestosterone treatment. When ^{14}C-alanine was used instead of ^{14}C-chlorella hydrolysate the similar result was obtained (Table I (B)). These results were consistent with that of amino acid incorporation *in vivo* (Fig. 1).

Kinetics of Amino Acids Incorporation in the S-20 System

As shown in Fig. 2, the similar time course of amino acid incorporation in the S–20 system was obtained in both normal and the steroid–treated livers and the incorporation of amino acids significantly increased already at 5 min after incubation. These facts indicate that the increment of amino acid incorporating activity by the steroids may be not due to stability of protein synthetic machine, but augmentation of its capacity.

Sucrose-Density-Gradient Analysis of Protein Produced in the S-20 System

Fig. 3 illustrates a typical result, by sucrose–density–gradient centrifugation, of the distribution of protein produced in the

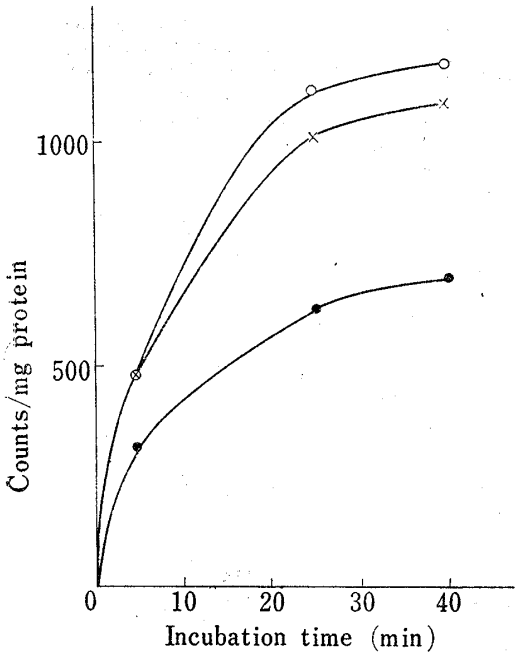

Fig. 2. Time Course of Incorporation of ^{14}C–Chlorella Hydrolysate into Hot–acid Insoluble Proteins in *in Vitro* System Consisting of S–20 Fluid from Normal and Steroid-treated Mice Livers

Ecdysterone or 4-chlorotestosterone was administered by intraperitoneal injection 2 hr prior to sacrifice.

—●— normal —○— ecdysterone
—×— 4-chlorotestosterone

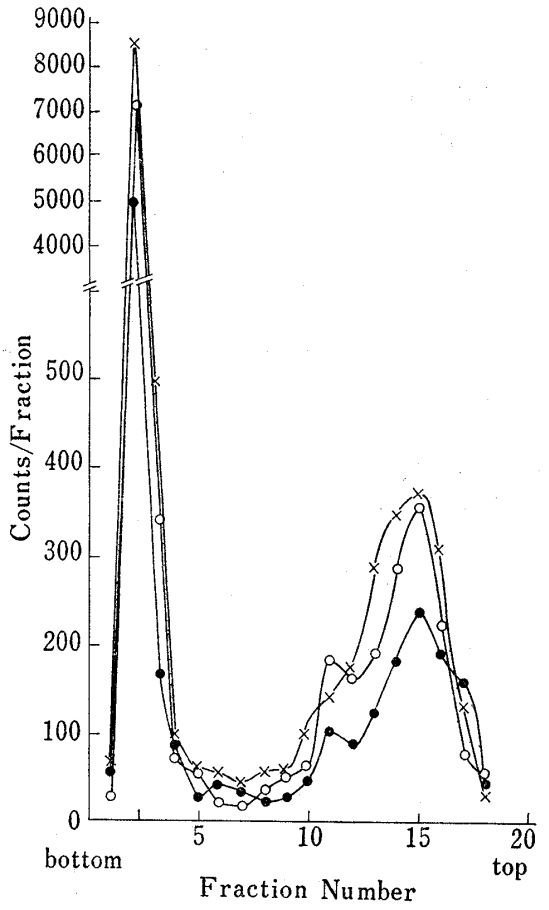

Fig. 3. Sucrose–Density–Gradient Analysis of Proteins Produced in the S–20 System

Experimental details are described in the text. Mice were injected 2 hr prior to sacrifice with ecdysterone or 4-chlorotestosterone. The arrow indicates the position of the interface between 2 M and 20% (w/v) sucrose layers.

normal 628 cpm/mg protein
ecdysterone 1012 cpm/mg protein
4-chlorotestosterone 1256 cpm/mg protein

—●— normal —○— ecdysterone
—×— 4-chlorotestosterone

S–20 system of normal, ecdysterone– or 4-chlorotestosterone–treated livers. It is clear that the incorporation of amino acids into protein increased equally in both interface, membranes and ribosomes, and supernatant (free protein).

Alteration of Protein Synthesis in Microsomes after the Administration of Ecdysterone or 4-Chlorotestosterone

Results presented in Fig. 4 show the incorporation of amino acids into protein in systems consisting of microsomes from treated mice livers plus S–105 fluid (without Sephadex G-25 treatment) from normal mice livers. When the activity of the microsomal fractions from normal and the steroid–treated liver was compared, it was apparent that the microsome particles from the steroid–treated liver are more active than those from normal liver, especially in lower amount of microsomal protein. The above results suggest that increment of protein synthetic activity may be due to change of protein synthetic machine.

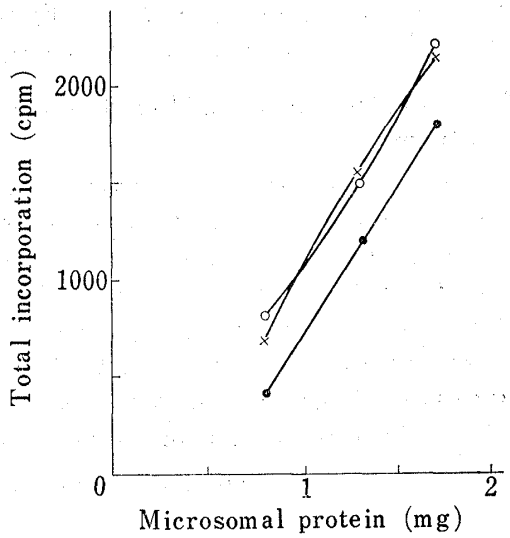

Fig. 4. Incorporation of ^{14}C-Chlorella Hydrolysate into Hot–acid Insoluble Proteins with a Fixed Quantity of Cell Sap from Normal Liver, and Increasing Amount of Microsomal Proteins

The experimental conditions are described in the text.
Mice were injected 2 hr prior to sacrifice with ecdysterone or 4-chlorotestosterone.
—●— normal —○— ecdysterone
—×— 4-chlorotestosterone

Alteration of Protein Synthesis in Polysomes after Administration of Ecdysterone or 4-Chlorotestosterone

Polysomes are aggregates of ribosomes held together by RNA, which are consisted to be functional units of protein biosynthesis by Wettstein, *et al.*[11] and Noll, *et al.*[13] This viewpoint is also supported by other worker.[14]

Results presented in Table II show the incorporation of ^{14}C-amino acids into protein in systems consisting of polysomes from normal or steroid–treated mice livers plus S–105 fluid from normal or steroid–treated mice livers. As shown in Table II (A), protein synthetic activity of polysomes from steroid–treated mice was more active than that from normal mice.

Table II. Effect of Ecdysterone or 4-Chlorotestosterone on the Incorporation of ^{14}C-Chlorella Hydrolysate into Protein in the Polysomal System

	Polysome	S–105	Total counts
(A)	Normal	normal	3679±87
	Ecdysterone	normal	4352±25
	4-Chlorotestosterone	normal	4395±44
(B)	Normal	normal[a]	9388±58
	Normal	ecdysterone[a]	9694±150
	Normal	4-chlorotestosterone[a]	9790±176

a) sephadex G-25 treatment
The experimental conditions are described in the text. Mice were injected 2 hr prior to sacrifice with ecdysterone or 4-chlorotestosterone.
Each result is the mean ± standard error for ten mice used.

13) H. Noll, T. Staehelin, and F. O. Wettstein, *Nature*, **198**, 632 (1963).
14) H.M. Goodman and A. Rich, *Nature*, **199**, 318 (1963).

Since there was no effect of S–105 fluid from steroid–treated mice on protein synthetic activity, both steroids may not activate enzymes for protein synthesis in the cell sap (Table II (B).

Effect of Actinomycin on Stimulation of Protein Synthetic Activity by Ecdysterone or 4-Chlorotestosterone

Since the above results suggested that stimulation of protein synthetic activity by ecdysterone or 4-chlorotestosterone was due to at least partly newly synthesized RNA, effect of actinomycin, inhibitor of DNA dependent RNA synthesis,[15] on its stimulation was investigated. As shown in Table III, difference of two steroids employed in inhibition of protein synthesis by actinomycin was observed: ecdysterone can partly stimulate the protein synthetic activity even under the presence of actinomycin, but actinomycin completely repressed the activation by 4-chlorotestosterone. These results indicate that stimulation of protein synthetic activity by 4-chlorotestosterone should be due to newly–RNA synthesis.

Table III. Effect of Actinomycin on Stimulation of Protein Synthetic Activity in S–20 System by Ecdysterone or 4-Chlorotestosterone

Treatment	Counts/mg protein	Treatment	Counts/mg protein
Normal	1220±20	Actinomycin D	1196±15
Ecdysterone	2271±43	Act. D+ecdysterone	1655±20
4-Chlorotestosterone	2538±30	Act. D+4-chlorotestosterone	1016±12

Mice were simultaneously injected 2 hr prior to sacrifice with ecdysterone or 4-chlorotestosterone and/or actinomycin.
Each result is the mean ± standard error for ten mice used.

Discussion

Effect of insect–moulting steroid, especially ecdysone, on larval epidermis of *Calliphora erythrocephala* Meig has been investigated in detail.[5–7] Our results suggested that these steroids can stimulate protein synthetic activity even in mouse liver as well as does 4-chlorotestosterone, a potent anabolic steroid.

Enhancement of incorporation of amino acids into protein in all subcellular components *in vivo* suggests that these steroids can not only stimulate the protein synthetic activity in microsomes, but in nuclei and mitochondria. In this experiment, stimulatory effect of ecdysterone on protein synthesis in microsomes was investigated in detail.

These stimulatory effects must be due to acceleration of detachment of newly–synthesized polypeptides from ribosomes or microsomes, based on the ratio of increment of amino acid incorporation into microsomes and cell sap proteins in both *in vivo* and *in vitro* systems (Fig. 1, 3). The time course of amino acid incorporation into proteins in S–20 system suggests that these stimulatory effects may not be due to stability of protein synthetic machine, but augmentation of its capacity.

By the reaction using microsomes from the steroid–treated liver plus S–105 fluid from normal liver, it is suggested that the stimulatory factor is associated with microsomes (Fig. 4). The preliminary study showed that the RNA/protein ratio of the microsomes was slightly higher in steroid–treated liver than in normal liver. Thus the enhanced rate of protein synthesis by microsomes from steroid–treated liver may be due to the presence of greater amounts of RNA including messenger RNA. The precise investigation on this problem is now under study.

In the system consisting of microsomes plus S–105 fluid, the slight stimulation of protein synthesis by S–105 fluid from steroid–treated liver was observed (Table IV). On the other

15) E. Reich, R.M. Franklin, A.J. Shatkin, and E.L. Tatum, *Science*, **134**, 556 (1961).

hand, in the system consisting of polysomes plus S–105 fluid, no stimulation by S–105 fluid was observed (Table II). These results suggest that microsomal membrane plays a certain roll in the stimulation by S-105 fluid.

Table IV. Effect of S–105 Fluid on the Incorporation of ^{14}C-Chlorella Hydrolysate into Protein in the Microsomal System

Microsome	S–105	Total counts
Normal	normal	1900±82
Normal	ecdysterone	2312±75
Normal	4-chlorotestosterone	2439±96

The experimental conditions are described in the text. Mice were injected 2 hr prior to sacrifice with ecdysterone or 4-chlorotestosterone.
Each result is the mean ± standard error for eight mice used.

In the system consisting of polysomes plus S–105 fluid, the enhanced rate of protein synthesis by polysomes from steroid–treated liver was observed, but its enhancement by S–105 fluid, enzymes for protein synthesis and tRNA, treated with Sephadex G–25 or not, was not, which was different of microsomal system. Increment of protein synthetic activity by steroid–treated polysome may be associated with its structure change from inactive form to active form,[17] and, at the same time, increment of polysome content itself can not be ruled out, because polysomal fraction may be contaminated with free ribosomes. Therefore increment of protein synthetic activity by the steroid–treated polysome may be caused by both increment of polysome content and its functional change.

There was significant difference in the effect of actinomycin on the enhanced incorporation of amino acids into protein in the S–20 system from liver of mice treated with two kinds of steroid. A part of the enhancement by ecdysterone was insensitive to actinomycin, but the stimulation by 4-chlorotestosterone was repressed completely. This result suggests that the stimulation by ecdysterone of protein synthetic activity is not partly dependent on newly–synthesized RNA. Further study on this phenomenon is in progress.

Burdette and Coda have reported that when the extracts of *Bombyx* which contains ecdysone activity was added to the postmitochondrial supernatant rate of liver protein synthesis *in vitro* was enhanced.[18] However, ecdysterone solution showed no enhanced rate of protein synthesis when added *in vitro* (Table V). This result indicates that something, not ecdysone, in extracts of *Bombyx* stimulate the protein synthesis.

Table V. Effect of Ecdysterone on the Incorporation of Amino Acids into Protein *in Vitro*

Addition	cpm/mg protein	Addition	cpm/mg protein
None	173±20	Ecdysterone 0.1 mg	182±16
Ecdysterone 0.005 mg	176±28	0.5 mg	163±23
0.01 mg	186±24		

The present findings give a reasonable explanation for the effective activation of protein synthesis in mammalian cells introduced by insect–moulting steroids which were derived from the plant kingdom.

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16) K. Koike, T. Otaka, and S. Okui, *J. Biochem.*, **61**, 679 (1967).
17) W.J. Burdette, R.C. Coda, *Proc. Soc. Exptl. Biol. Med.*, **112**, 216 (1963).