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A unique *in vivo* stimulation of labeled amino acid incorporation into protein by fusidic acid in the rat*

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Fusidic acid, an antibacterial steroidal antibiotic, produced by *Fusidum coccineum*, was first isolated by Godtfredsen *et al.* [1] and tentatively characterized by Godtfredsen and Vangedal [2]. The three antibacterial steroid antibiotics, helvolic acid, fusidic acid and cephalosporin P_1, are chemically related.

Preliminary work by Harvey *et al.* [3] and Yamaki [4] showed that fusidic acid inhibits protein synthesis in whole cells and synthesis directed by both polyuridylic acid and endogenous messenger in cell-free extracts of various bacteria. Further work by Harvey *et al.* [5] has shown that fusidic acid affects the final polymerization of amino acids after formation of the ternary complex (polyribosomes with bound phenylalanyl S-RNA). Fusidic acid stops the movement of aminoacyl- or peptidyl-tRNA from the acceptor site to the donor site even if the donor site is empty [6]. Fusidic acid also stabilizes both prokaryotic [7] and eukaryotic [8] ribosome—translocation factor—GDP complexes while allowing a single round of GTP hydrolysis and translocation. Okura *et al.* [9] and Willie *et al.* [10] have reported that sodium fusidate and the sodium salt of 24,25-dihydrofusidic acid, respectively, inhibit polypeptide chain elongation by binding to the ribosome—elongation factor-G—GDP complex, thereby preventing its dissociation.

Active cation transport across the cell membrane is a function of Na^+, K^+-ATPase. This complex enzyme system can also be inhibited by fusidic acid [11]. Furthermore, steroids of the fusidane family structurally resemble the bile salts, which act as alimentary biodetergents [12, 13]. Several derivatives of fusidic acid are similar in chemical and biophysical properties to bile salts [14, 15]. This surface activity and micelle formation are similar to those found for the interaction of a drug with receptor sites, serum proteins or membrane components *in vivo* [16].

Similarities in the structure of some steroid anabolic hormones (testosterone, estrogen, etc.) to fusidic acid suggest that the mode of action of these hormones on protein synthesis in eukaryotes might be elucidated through the use of fusidic acid *in vivo* and *in vitro*.

MATERIALS AND METHODS

Male and female Sprague–Dawley strain and female Germ-Free (Axenic) Sprague–Dawley rats of various weights, fed or fasted, were injected i.p. with saline, sodium fusidate, deacetylated cephalosporin P_1, cephalosporin P_1, cephalothin P_1, or one of the six fusidic acid analogues and a radioactive amino acid at arbitrarily determined concentrations and times as noted in the table, figure or results.

Bilateral orchidectomy, ovariectomy, adrenalectomy or thyroidectomy and hypophysectomy were performed under ether anesthesia. After thyroidectomy, rats that gained little or no weight during a 30-day period were used. Hypophysectomy was at least 1 month prior to experimental use and all other surgically altered rats were used 1 week later. Adrenalectomized rats were given access to 1% (w/v) NaCl solution rather than water. Hypophysectomized rats were fed ground Purina Lab Chow containing 30% sucrose (w/w) moistened with evaporated milk. Sodium fusidate, 16-epideacetylfusidic acid-potassium salt (WG-551 K), tetrahydrofusidic acid-sodium salt (WG-553 Na), 24,25-dihydrofusidic acid-sodium salt (WG-559 Na), 3-*O*-acetyl-24,25-dihydrofusidic acid-sodium salt (WG-593 Na), 3-*O*-acetyl-16-epifusidic acid-sodium salt (WG-598 Na) and 3-*O*-acetyl-16-epi-24,25-dihydro-fusidic acid-sodium salt (VD-1163 Na) were a gift of Dr. W. O. Godtfredsen and Dr. W. von Daehne, Leo Pharmaceutical Products, Ballerup,

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Denmark. Sodium fusidate, cephalosporin P_1 and cephalothin P_1 were gifts of Eli Lilly & Co., Indianapolis, IN, U.S.A. Deacetylated cephalosporin P_1 was prepared according to Godtfredsen and Vangedal [2].

L[U-^{14}C]leucine (250 mCi/m-mole) and L[2-^{14}C]glycine (5.5 mCi/m-mole) were obtained from New England Nuclear, Boston, MA, U.S.A. L[U-^{14}C]- and [3-^{14}C]serine (135 mCi/m-mole and 30 mCi/m-mole) were a gift from International Chemical and Nuclear Corp., Irvine, CA, U.S.A. L[U-^{14}C]leucine (311 mCi/m-mole, 324–348 mCi/m-mole and 222 mCi/m-mole) was obtained from Schwarz/Mann, Orangeburg, NY, U.S.A., Amersham, Arlington Heights, IL, U.S.A., and Calatomic, Los Angeles, CA, U.S.A. respectively. L[4,5-^{3}H]leucine (46 Ci/m-mole) and L[^{35}S]methionine were purchased from Amersham.

Liver perfusions were as described by Fuhremann *et al.* [17].

Liver and kidney (3–5 vol.), and brain and muscle (5 vol.) were prepared with 0.25 M sucrose TKM (50 mM Tris–Cl–2.5 mM KCl–10 mM $MgCl_2$, pH 7.5). Mitochondria, microsomes and the post-microsomal supernatant (PMS) were prepared according to Sottocasa *et al.* [18]. Endoplasmic reticulum and polyribosomes were prepared from the post-mitochondrial supernatant fraction according to Sunshine *et al.* [19], except that the $MgCl_2$ content of the TKM was increased from 5 to 10 mM [20].

Isolation, washing and determination of specific radioactivity of labeled proteins were performed as described by Hochberg *et al.* [21] and Mans and Novelli [22].

Blood, liver and kidney were extracted as described by Godtfredsen and Vangedal [23]. The extracts were chromatographed on silica gel 60-F-254 (0.25 mm) precoated plates (E. Merck, Darmstadt, Germany) using a solvent containing 10% methanol and 90% chloroform. Compounds were located by exposure to iodine vapor.

RESULTS AND DISCUSSION

Various radioactive amino acids, such as leucine (Figs. 1, panels A, B and C), glycine (Table 1), serine (not shown) and methionine (not shown), showed enhanced incorporation into washed proteins isolated from liver, kidney, brain and muscle (not shown) of both male and female rats under the influence of sodium fusidate *in vivo*. Not only were the specific radioactivities of these proteins increased by sodium fusidate but the uptake of these amino acids into liver organelle fractions of the female rat was also stimulated in several experiments (Table 1). The increase in uptake of amino acids may represent an indirect action of sodium fusidate on amino acid transport systems [24, 25]. In contrast to the *in vivo* action, sodium fusidate added to the medium of the perfused liver of the female rat failed to stimulate uptake of radioactive amino acid. This suggests that some intrinsic *in vivo* factor is required for stimulation of incorporation of amino acids into protein by sodium fusidate. The potentiation of the amino acid transport systems *in vivo* might be through the action of sodium fusidate on receptor sites, serum proteins and/or membrane components [16]. In contrast, sodium fusidate acts as an inhibitor of incorporation of labeled amino acid into protein in an *in vitro* incubation system utilizing a hepatic cell suspension (E. Ziv and F. W. Stratman, unpublished data).

Similar response curves, determined by the incorporation of ^{14}C from L[U-^{14}C]leucine, were obtained when 5, 10 or 30 mg of sodium fusidate was injected i.p. into female rats at the same time as the labeled amino acid. If the dose was reduced to 1 mg, there was no apparent stimulation of incorporation into protein. In most experiments the incorporation rate of ^{14}C into washed proteins from the crude liver homogenate and liver cell organelles by sodium fusidate was greater than

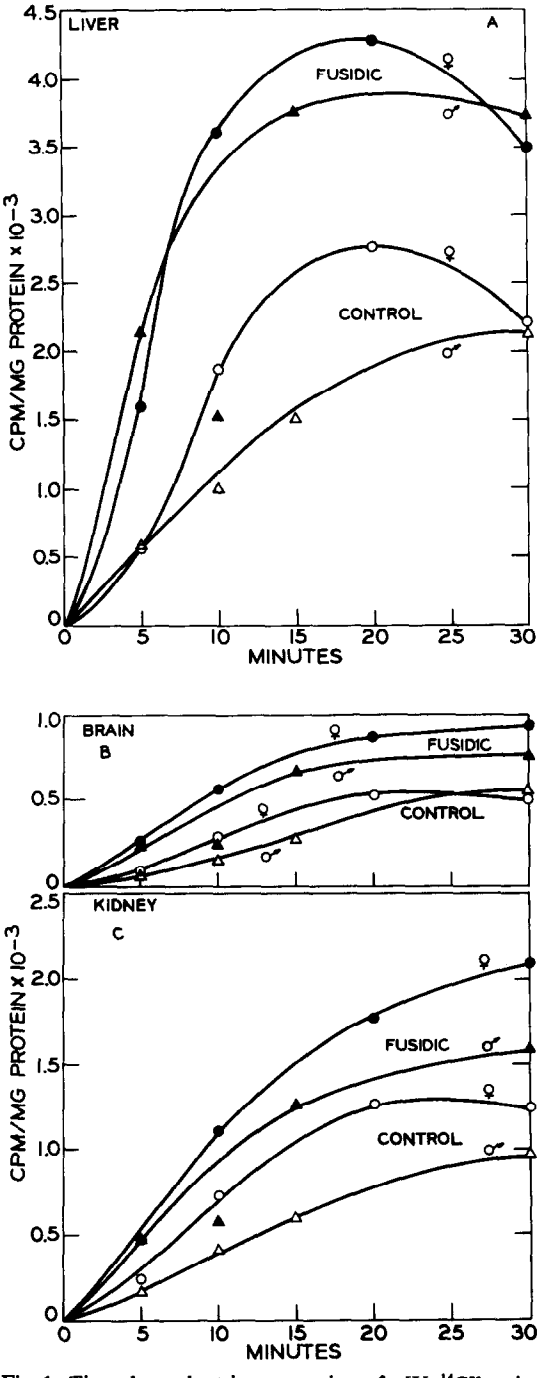

Fig. 1. Time-dependent incorporation of L[U-^{14}C]leucine into crude homogenates of liver (A), brain (B) and kidney (C) of male (♂) and female (♀) rats (160–175 g). Rats were injected i.p. with sodium fusidate (10 mg/0.5 ml saline/rat) or saline (0.5 ml/rat) and a pulse dose of L[U-^{14}C]leucine (1 ml/rat) in rapid sequence at zero time. Each point represents one rat. Key: male control, △—△; female control, ○——○; male sodium fusidate, ▲——▲; and female sodium fusidate, ●——●.

the controls at the end of a 90-min time span. Relatively constant elevated rates of incorporation into protein are maintained by sodium fusidate, compared to the controls, over a 150-min time interval in the female rat when the labeled amino acid was allowed to equilibrate with the rat for intervals of 30 min. These results suggest that sodium fusidate is metabolized relatively slowly,

Table 1. Effect of fusidic acid on uptake and incorporation of L[2-^{14}C]glycine into fractions and proteins of the liver of male and female rats*

	Male				Female			
	Saline		Fusidic acid		Saline		Fusidic acid	
Fraction	$\bar{x}$†	r‡	$\bar{x}$	r	$\bar{x}$	r	$\bar{x}$	r
	(Per cent of radioactivity injected)							
Homogenate	6.2	2.1	6.1	1.3	5.1	0.7	9.7	1.1
600 *g*	4.2	0.6	4.5	1.0	4.6	0.8	7.5	0.5
15,000 *g*	2.9	0.4	3.3	0.6	3.6	0.0	5.8	0.8
PMS§	2.0	0.1	2.5	0.6	2.7	0.4	4.1	0.3
Microsomes	0.6	0.2	0.7	0.1	0.6	0.1	1.0	0.1
	(Specific radioactivity, cpm/mg protein)							
PMS	1700	43	1670	340	1500	370	2930	310
Microsomes	3430	67	4150	720	3060	590	5440	1090

* Two Sprague–Dawley rats per treatment (males, 200–225 g; females, 175–200 g) were injected i.p. with either 1 ml saline or fusidic acid (Na salt) (10 mg/ml of saline) at zero time; L[2-^{14}C]glycine (5.5 mCi/m-mole), 75×10^6 cpm at 30 min; the rats were killed 2 hr after glycine.

† Mean value.

‡ Range or difference between highest and lowest values.

§ Post-microsomal supernatant.

thereby maintaining its activity over a long time span. Alternatively, sodium fusidate may activate some unknown mechanism having long term anabolic or reduced catabolic effects on protein synthesis even though it is rapidly degraded within the body.

Several closely related steroidal antibiotics—cephalothin P_1, cephalosporin P_1, deacetylated cephalosporin P_1 (10 mg/rat or 60 mg/kg, no other dose tested)—did not stimulate incorporation into protein above control levels. Six derivatives of fusidic acid (10 mg/rat or 60 mg/kg) with modifications in the A and B rings and/or side chain were as effective as sodium fusidate (10 mg/rat or 60 mg/kg) in stimulating incorporation. Therefore, molecular configuration may be a critical factor in the mechanism whereby *in vivo* protein synthesis is stimulated by sodium fusidate or its derivatives.

There is a lack of response of incorporation into protein in the perfused liver to sodium fusidate administered to the donor rat prior to liver removal and/or added to the perfusion medium. This suggests that sodium fusidate may be altered by metabolism, via some organ or tissue *in vivo*, to an active metabolite. The formation of an active metabolite of sodium fusidate could be similar to the conversion of another sterol, vitamin D, via several organs and tissues to its active metabolite [26]. Alternatively, removal of an organ or tissue for *in vitro* experiments might allow activation of an inhibitor protein as was noted for 25-OH-D_3-1-hydroxylase in the rat kidney [27]. Bilateral nephrectomy or ureter-ligation did not alter the *in vivo* stimulation of incorporation of labeled amino acid into protein by fusidic acid (P. Gachon, E. Ziv and F. W. Stratman, unpublished data). Chromatographic analyses of extracts of blood and tissue of male and female rats were not different and neither support nor reject this hypothesis at the present time.

The apparent sex difference in the activity of sodium fusidate *in vivo* may be a function of the endocrine state of the animal, in general, or it may be due to the release or activation of growth promoting hormone(s). Also, it could be related to drug turnover or sex differences in protein synthesis vs degradation. Alteration of the endocrine state by hypophysectomy, thyroidectomy, adrenalectomy, ovariectomy or orchidectomy, and several combinations of these surgical modifications failed to significantly reduce the stimulatory activity of sodium fusidate (2 hr after injection, 10 mg/rat or 60 mg/kg) on incorporation of amino acids. The inability of sodium fusidate to stimulate incorporation of amino acid (2 hr after injection) into kidney protein of the hypophysectomized rat as well as its increased effectiveness in liver and brain is noteworthy.

It has been suggested that toxins produced by bacteria in the gut of the normally fed rat might inhibit the maximum protein synthesis capability. Thus, fusidic acid, an antibacterial compound, would inhibit the production of bacterial toxins resulting in a release of inhibition of protein synthesis. This does not appear to be a valid hypothesis since there was no difference in incorporation into protein between normal and germ-free rats treated i.p. with fusidic acid.

In vivo phosphorylation of proteins, an important structural modification, is stimulated by fusidic acid in all of the hepatic cell fractions except mitochondria [28], even when incorporation of labeled amino acid is inhibited by cycloheximide. Thus, increased phosphorylation *per se* does not account for the action of fusidic acid on *in vivo* incorporation of labeled amino acids.

Several other actions of sodium fusidate have been observed, such as increased blood glucose concentration in fed or fasted rats, an increased blood concentration of cAMP with a concomitant decrease in cGMP [29], and increased acetylation of proteins (P. Gachon, E. Ziv and F. W. Stratman, unpublished data). Elevated insulin secretion could account for increased amino acid uptake and incorporation into proteins; however, this would require sex differences in the release of insulin in response to fusidic acid. No evidence exists at this time which supports the concept that fusidic acid may possess intrinsic hormone-like activity.

The rapid stimulation of incorporation of amino acid into protein by fusidic acid *in vivo* may reflect a receptor-independent mechanism involving post-transcriptional regulation of gene expression.

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2-Pyrrolidinone—A cyclization product of γ-aminobutyric acid detected in mouse brain

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The neurotransmitter candidate γ-aminobutyric acid (GABA) is structurally similar to glutamic acid. Cyclization of glutamic acid and GABA produces the lactams, 2 - pyrrolidinone - 5 - carboxylic acid (pyroglutamic acid, 5-oxoproline) and 2-pyrrolidinone respectively. While 2 - pyrrolidinone - 5 - carboxylic acid is known to occur in brain[1], studies on pyrrolidinone have been limited to exogenously administered compound. Pharmacological studies have indicated that pyrrolidinone in high doses exhibits anticonvulsant activity in animals, presumably by acting on the GABA system[2–4]. Other workers have been unable to verify these findings[5, 6]. Tower[7] has shown that cat cerebral cortex slices have the capacity to enzymatically convert [2-^{14}C]pyrrolidinone to [^{14}C]GABA. Thus, pyrrolidinone might potentially serve as a GABA precursor in the central nervous system. In this communication, mass spectral evidence is provided which suggests that pyrrolidinone is a natural constituent of mouse brain. Studies with labeled GABA are also presented which indicate that pyrrolidinone is not an artifact of the work-up process.

MATERIALS AND METHODS

Male ICR mice (25–30 g) were used for all the experiments. The animals were decapitated and the brains quickly removed, weighed and homogenized in cold