Lactogenic Activity of Mammalian Growth Hormones *in vitro*. (31871)

E. M. RIVERA,* I. A. FORSYTH,† AND S. J. FOLLEY
Physiology Department, N.I.R.D., Shinfield, Reading, England

The lactogenic activity of human pituitary growth hormone (GH) preparations has been recognized for some time(1,2). Whether this activity is intrinsic to the GH molecule or due to a chemically distinct component in the preparation is still an open question.

Growth hormones from other mammalian species, from which prolactin has been isolated as a separate hormone, do not appear to be lactogenic in standard prolactin assays or in experimental studies of the mammary gland(3,4). One exception, however, has been described by Nandi(5-8), who found that bovine GH could replace prolactin in hormonal combinations causing mammary growth and lactogenesis in hypophysectomized-ovariectomized-adrenalectomized mice of certain inbred strains. Other mouse strains were less responsive or completely refractory to the lactogenic activity of bovine GH(6-8).

These differences in mouse strain sensitivity to bovine GH *in vivo* are also manifested in organ cultures of prelactating mammary tissues. It was found, for example, that 2 μg/ml of bovine GH initiated milk secretion in mammary tissues from C3H mice (responsive to GH *in vivo*) but this dose was without lactogenic effect in tissues from A mice (refractory to GH *in vivo*(9,10). Strain-specific responses can still be obtained with increasing levels of GH up to 10 μg/ml(10), but at 15 μg/ml and above, bovine GH initiates secretion in mammary tissues regardless of strain of origin(9). It is obvious that the concentration of GH in the medium plays an important role in determining the specificity of its lactogenic effect *in vitro*.

Prop(11, private communication) found that bovine GH could also cause alveolar development in cultures of immature mouse mammary glands, but noted that preparations from different laboratories differed in their mammotropic activity. Whereas a preparation from Professor C. H. Li was effective at 6 μg/ml, Organon and NIH bovine GH were ineffective even at 100 μg/ml. Inasmuch as the bovine GH preparations used in the *in vivo* work of Nandi(5,6,8) and in organ culture studies(9,10) had all been obtained from Professor Li, it was conceivable that the lactogenic effects ascribable to bovine GH in these studies were unique and confined to Li preparations. This possibility has now been investigated, and the results are reported here.

In addition, the results of the studies with bovine GH indicated that mammary organ cultures could be utilized to investigate the lactogenic activity of growth hormones from other mammalian species. Accordingly, the present report is concerned with the ability of bovine, ovine, porcine, simian, and human growth hormones to induce mammary secretion *in vitro*. Comparison is made of their activities in C3H mice (sensitive to Li bovine GH) and in A mice (refractory to Li bovine GH).

Materials and methods. The details of the growth hormones studied *in vitro* are listed in Table I. Seven preparations, including the 3 human GH's studied were assayed for prolactin (lactogenic) activity by the rabbit mammary intraductal test of Chadwick(12). The method may, in appropriate circumstances, be used to obtain potency estimates(13). The human GH preparations were also assayed by the local pigeon crop test. Mixed pigeons of unknown age were all that could be obtained for assay purposes. Difficulties which arose from their extreme variability in response were overcome by use of a balanced incomplete block design, a block consisting of the right and left crop sacs. This enabled allowance to be made for variability between birds (Forsyth & Hosking, unpublished). Thirty pigeons were used in a 4-point assay. Hor-

* Aided by Am. Cancer Soc. Grant PF-203. Present address: Dept. of Zoology, Michigan State Univ., East Lansing.

† Aided by a grant from British Empire Cancer Campaign for Research.

TABLE I. Growth-Promoting and Prolactin Activities of the Hormones Studied.

Species	Code number	Growth-promoting activity (i.u./mg)*	Prolactin as measured by: Pigeon crop (i.u./mg)*	Rabbit mammary intraductal test (i.u./mg)*	Immunodiffusion % cross-reacting material by wt
Growth hormone					
Bovine	61A (Li)	1.51 (1.26-1.81)			<1.5
	B2 (NIH)	1.53 (.82-2.76)	.33	<1	
	59859-II (Organon)	.58 (.31-1.03)			<1.5
	42157-III (Organon)	1.50 (.84-2.71)			<1.5
Ovine	S646A (NIH)	.96 (.39-2.12)		<2	
Porcine	P476C (NIH)	1.0 (.64-1.80) .6 (.37- .89)	<.3	<1	
Simian	M629B (NIH)	1.0†			
Human	MY-61 (Hartree)	.89 (.48-1.58)	1.5 (.5-2.4)	17 (11-24)	
	HS 269 (Wilhelmi)	1.0 (.50-1.30)	3.7 (2.3-5.0)	25 (16-39)	
	HS 612A (Wilhelmi)	1.10 (.73-1.70) 1.70 (1.10-2.60)	7.1 (4.4-9.9)	42 (31-56)	
Prolactin					
Ovine	P-S-4 (NIH)	.0045 (.001-.019)	21.0 (17.4-24.7)	24 (15-40)	

* 95% fiducial limits in parentheses. † *Idem* not available.

mone was administered intradermally over the crop sacs once daily on 3 consecutive days in a daily volume of 0.1 ml. The total doses of standard were 5 μg and 10 μg. The birds were killed on the 4th day and the response was measured by cutting out and taking the fresh weight of the stimulated area of the crop sac (Kanematsu & Sawyer(14)). The standard for all bioassays was ovine prolactin, either NIH-P-S-4, 21 i.u./mg or the Second International Standard Prolactin, 22 i.u./mg.

Three of the bovine GH preparations were available only in small quantities, inadequate to perform an assay in the rabbit or pigeon. Therefore, to determine whether they contained any material cross-reacting with bovine prolactin they were studied by an immunodiffusion method, the results of which correlated well with bioassay data in the case of human GH(15). The antiserum used was prepared in guinea pigs against purified ovine prolactin, NIH-P-S-4. The standard was bovine prolactin, prepared by Dr. K. A. Ferguson, Ian Clunies Ross Animal Research Laboratory, Australia, with rabbit lactogenic activity of approximately 15 i.u./mg. The results are expressed as percentage cross-reacting material by weight. Polyacrylamide gel electrophoresis (in a borate buffer containing 6 M urea) was kindly performed on 2 of the bovine GH preparations (Organon 58959-II and 42157-III) by Dr. A. J. Parcells, Upjohn Co. Neither preparation showed components corresponding to ovine prolactin, NIH-P-S-4.

The growth-promoting activities of the preparations were provided by the source of each hormone except for the Organon preparations, which were kindly assayed by Dr. J. Evans, Upjohn Co. The results of pigeon crop assays on B2(NIH) and S646A(NIH) were kindly provided by Professor A. E. Wilhelmi.

The basal maintenance medium was chemically-defined synthetic medium 199 (Glaxo Laboratories, Greenford, Middlesex) supplemented with insulin (5 μg/ml, kindly provided by Dr. O. K. Behrens, Eli Lilly) and either aldosterone (1 μg/ml, kindly provided by Ciba Pharmaceutical Products) or corticosterone (1 μg/ml, kindly provided by Merck, Sharp & Dohme). This medium maintains the viability of prelactating mammary explants without inducing secretion(4,9). Penicillin G, 50 i.u./ml, was usually added to the medium. Stock solutions of growth hormones were prepared by dissolving the preparations in small amounts of 1×10^{-4} N NaOH before addition of medium 199 to yield the desired stock concentration. Aliquots of stock solution were then added to the

basal medium to yield final concentrations of 0.5, 1, 2, 5, 10, 15 μg/ml. In some cases, 0.25 μg/ml concentrations were also prepared. Ovine prolactin (NIH-P-S-4) was used as the standard for the *in vitro* experiments. Solutions of all protein hormones were sterilized by passage through millipore filters (average porosity 0.45 μ).

The inbred strains of C3H and A mice in the N.I.R.D. colony were obtained from the stock of the Chester Beatty Research Institute, London. The mice were in the second week (10-13 days) of their first pregnancy. The technique for removal of tissues (alveolar lobules) for explantation and the organ culture method used have been described(16,17). Two mice of the same strain were used for each experiment. About 70 explants were dissected from each and these were distributed to all concentrations of growth hormone and standard being studied. The experiment was usually repeated with tissues from 2 other mice of the same strain, and was carried out similarly with tissues from 2-4 mice of the second strain.

The mammary explants were cultivated for a period of 5 days with a medium change at the end of 3 days. The tissues were fixed in Carnoy's fluid, embedded in paraffin, sectioned serially at 7 μ, and stained with hematoxylin and eosin. The histological preparations were assigned arbitrary numbers to avoid biased evaluation, and the secretory response was rated as follows(9,10): — = nonsecretory; no change over initial appearance; + = submaximal secretion; less than 50% of the alveoli contain cells with lipid vacuoles; alveolar lumina with scanty secretion; alveoli moderately distended; ++ = maximal secretion; more than 50% of the alveoli contain cells with lipid vacuoles; alveolar lumina with abundant secretion; alveoli greatly distended.

Results and discussion. The results are summarized in Tables II to IV. The activity of each growth hormone was compared with that of prolactin in C3H and A mammary tissues. Tables II and III show the distribution of responses to the several levels of growth hormone employed. It can be seen that explants vary in responsiveness, but that at certain levels and above, all C3H explants show maximal responses. A tissues, however, showed variable responses to some of the hormones even at the highest concentrations employed. Table IV lists for both strains the lowest levels of each growth hormone, where known, causing maximal responses in all explants. For prolactin these concentrations were 1 and 5 μg/ml in C3H and A mice, respectively, and are consistent with the minimal doses giving maximal response in all explants previously reported for these strains (9).

Non-primate growth hormones. As indicated in Tables II and IV, the 4 preparations of bovine GH were equally effective in inducing secretion in C3H mammary tissues. Their lactogenic potency was 50% that of ovine prolactin. Thus, the lactogenic activity of bovine GH is not unique to Li preparations. Furthermore, in accord with earlier results, this hormone had comparatively little effect in strain A mammary tissues, although some secretion was sometimes obtained with the higher concentrations used.

The activity of ovine GH was similar to that of bovine GH in both strains of mice (Tables II and IV). The porcine GH induced secretion in C3H tissues at a minimal dose of 5 μg/ml, indicating a lactogenic potency of about 20% that of prolactin.

Despite restriction of activity to strain C3H mice, it is apparent that bovine, ovine and porcine GH are capable of initiating mammary secretion *in vitro*; on a weight basis they appear to possess 20-50% the activity of purified ovine prolactin. Whether this lactogenic activity is due to contamination by prolactin or is intrinsic is unknown. Components corresponding to prolactin were not found in polyacrylamide gel electrophoresis of 2 of the bovine GH preparations used in this study (see *Materials and methods*). However Wallis and Kovačic(18) found that bovine GH preparations with low luteotropic content did show components in starch gel electrophoresis corresponding to bovine prolactin. This led them to postulate that prolactin is only a contaminant of bovine GH and that the growth-promoting and luteotropic activities of bovine pituitary preparations

TABLE II. Lactogenic Activity of Non-Primate Growth Hormone in Mouse Mammary Cultures.

Species and code No.	Response†	No.* of explants showing response indicated: C3H tissues, Hormone concentration (μg/ml) 0.25	0.5	1	2	5	10	15	A tissues, Hormone concentration (μg/ml) 0.25	0.5	1	2	5	10	15
Growth hormone															
Bovine 61A	—		2							16	16	15	11	3	1
	+		10	8								1	5	7	3
	++		2	8	16	15	13	15						5	12
			(14)	(16)	(16)	(15)	(13)	(15)		(16)	(16)	(16)	(16)	(15)	(16)
B2	—		1							16	16	14	4	2	1
	+		13	5									10	7	4
	++		2	11	16	16	14	12						7	10
			(16)	(16)	(16)	(16)	(14)	(12)		(16)	(16)	(14)	(14)	(16)	(15)
59859-II	—		4	1						15	15	16	10	2	
	+		10	6									6	9	8
	++		2	7	14	16	14	8						5	8
			(16)	(14)	(14)	(16)	(14)	(8)		(15)	(15)	(16)	(16)	(16)	(16)
42157-III	—		1							16	16	12	5	1	
	+		14	6									7	7	3
	++		1	10	16	15	16	8						6	5
			(16)	(16)	(16)	(15)	(16)	(8)		(16)	(16)	(12)	(12)	(14)	(8)
Ovine S646A	—									14	14	10	2	3	
	+		7	14							2	6	10	5	7
	++		1	2	16	16	16	8					2	4	9
			(8)	(16)	(16)	(16)	(16)	(8)		(14)	(16)	(16)	(14)	(12)	(16)
Porcine P476C	—		9	2						16	16	15	8		2
	+		7	4	4								6	8	4
	++			8	12	16	16	8						6	2
			(16)	(14)	(16)	(16)	(16)	(8)		(16)	(16)	(15)	(14)	(14)	(8)
Prolactin															
Ovine P-S-4	—	6							16	12	6	1			
	+	8	5							4	8	4			
	++	10	19	23	24	23	16	16			10	18	23	21	15
		(24)	(24)	(23)	(24)	(23)	(16)	(16)	(16)	(16)	(24)	(23)	(23)	(21)	(15)

* Number in parentheses indicates No. of viable explants at each dose level.
† See text for explanation of symbols.

TABLE III. Lactogenic Activity of Primate Growth Hormone in Mouse Mammary Cultures.

Species and code No.	Response†	No.* of explants showing response indicated — C3H tissues — Hormone concentration (μg/ml) 0.25	0.5	1	2	5	10	15	A tissues — Hormone concentration (μg/ml) 0.25	0.5	1	2	5	10	15
Growth hormone															
Simian M629B	—	4							16	14	7	2	1		
	+	5	7							2	9	14	9	2	
	++	7	9	15	16	16	8	8					5	10	15
		(16)	(16)	(15)	(16)	(16)	(8)	(8)	(16)	(16)	(16)	(16)	(15)	(12)	(15)
Human MY-61	—	3								14	10	8			
	+	4									6	5	8	3	
	++	9	14	15	12	15	8	8				3	4	13	15
		(16)	(14)	(15)	(12)	(15)	(8)	(8)		(14)	(16)	(16)	(12)	(16)	(15)
HS 269	—									15	12	10	2		
	+		9								4	5	8	3	
	++		7	15	15	14	8	8				1	2	11	16
			(16)	(15)	(15)	(14)	(8)	(8)		(15)	(16)	(16)	(12)	(14)	(16)
HS 612A	—								9	4					
	+	3							7	7	5				
	++	13	15	16	16	16	8	8		5	10	15	14	14	13
		(16)	(15)	(16)	(16)	(16)	(8)	(8)	(16)	(16)	(15)	(15)	(14)	(14)	(13)
Prolactin															
Ovine P-S-4	—	4							16	13	7	2			
	+	7	7							2	7	2			
	++	12	17	24	15	15					10	20	24	15	
		(23)	(24)	(24)	(15)	(15)			(16)	(15)	(24)	(24)	(24)	(15)	

* Number in parentheses indicates No. of viable explants at each dose level.
† See text for explanation of symbols.

TABLE IV. Lactogenic Activity of Growth Hormones in Mouse Mammary Cultures.

Species	Code No.	Lowest concentration (μg/ml) causing maximal secretion in all tissues from C3H mice	Lowest concentration (μg/ml) causing maximal secretion in all tissues from A mice
Growth hormone			
Bovine	61A (Li)	2	>15
	B2 (NIH)	2	>15
	59859-II (Organon)	2	>15
	42157-III (Organon)	2	>15
Ovine	S646A (NIH)	2	>15
Porcine	P476C (NIH)	5	>15
Simian	M629B (NIH)	1	15
Human	MY-61 (Hartree)	.5	15
	HS 269 (Wilhelmi)	1	15
	HS 612A (Wilhelmi)	.5	2
Prolactin			
Ovine	P-S-4 (NIH)	1	5

can be separated quite easily. Nevertheless the prolactin content of the preparations studied *in vitro* as measured by pigeon crop, rabbit mammary intraductal or immunodiffusion assay was less than 2%, except in one case (ovine GH) where it might have been as much as 10%. A low level of prolactin contamination in these GH preparations is thus possible. While this alone is not high enough to account for the *in vitro* lactogenic effects observed, these might be due to synergism between GH and a prolactin contaminant, rather than to an intrinsic property of GH itself.

It is important to note, however, that despite the effective separation of GH and prolactin in a number of mammalian species, there is overlap in some of their activities (19-21). An active core, common to all growth hormones has been postulated(19,22) and it is also possible that GH and prolactin share a common amino acid sequence, as do ACTH and MSH.

Primate growth hormones. By contrast with non-primate hormones, GH preparations from monkey and human showed activity equal to or greater than ovine prolactin in C3H tissues. With one exception, the responsiveness of strain A tissue to primate, as to non-primate, GH was considerably less than that of C3H tissues. The exception, a human GH, HS 612A, provided results of great interest. This preparation was obtained from Professor A. E. Wilhelmi, Emory University, Atlanta, Ga., and was made by him as an international standard for radioimmunoassay. Clinical grade human GH, of the type of HS 269, was further purified by chromatography on DEAE cellulose. HS 612A is A peak material, essentially homogeneous in disc gel electrophoresis(23). It shows relatively low pigeon crop stimulating activity, by comparison with ovine prolactin (Table I) or with B and C peak human GH preparations (23). However, in the rabbit its activity is 42 i.u./mg, higher than that of any other prolactin preparation so far tested(13). Its high mammalian lactogenic activity was further emphasized by the present experiments *in vitro*. HS 612A had twice the activity of ovine prolactin in tissues from both strains of mice and was the only GH tested showing high activity in strain A mammary tissues. Whereas prolactin had a minimal effective concentration of 5 μg/ml in this strain, HS 612A was effective at 2 μg/ml.

That a human GH fraction possessing low pigeon crop activity is highly lactogenic in mammals both *in vivo* and *in vitro* suggests that the structural basis for these biological effects may be quite distinct. It is possible that the pigeon crop stimulating activity resides in a separate entity or, alternatively, it may be determined at a site on the GH molecule distinct from that determining lactogenic activity. On the other hand, it is equally possible that the response elicited by a single molecule is determined by the nature of the responding tissue. However, the combination of high mammalian lactogenic activity with high growth-promoting activity exhibited by a highly purified preparation of human GH, which appears to be electrophoretically homogeneous, supports the view that human GH possesses intrinsic lactogenic activity.

Finally, it should be noted that although strain A mice are more responsive to human GH, HS 612A, than to ovine prolactin, this strain is consistently less reactive than C3H mice to both hormones. Whether this difference in hormonal sensitivity is a consequence of other dissimilarities in the two strains,

e.g., incidence of mammary tumors in virgin females, presence or absence of dual viral factors, has yet to be established(7).

Summary. Growth hormone preparations from several mammalian species were investigated for their ability to initiate secretion in organ cultures of prelactating mammary tissue from strain C3H and A mice. All the growth hormones studies showed some degree of activity in C3H mammary tissues. Bovine, ovine and porcine GH were 20-50% as active as ovine prolactin, although the prolactin content of the preparations was shown to be low. Simian and human GH, whose prolactin-like activity in other systems is well-known, showed activity equal to or greater than ovine prolactin in C3H mammary tissues. On the other hand, a single human GH preparation (one of three studied) was the only GH to show significant activity in strain A mammary tissues. These results suggest an intrinsic lactogenic activity of GH, particularly well-marked in the case of human GH, whose expression in mouse mammary gland cultures is in part determined by the genetic background of the tissues.

We are indebted to Dr. K. A. Ferguson, Dr. A. Stockell Hartree, Professor C. H. Li, Dr. K. W. Thompson, Professor A. E. Wilhelmi and the Endocrinology Study Section, National Institutes of Health, for the growth hormone and prolactin preparations used in this study. We are also grateful to Miss Z. D. Hosking for statistical analysis of bioassays, to Mr. A. Turvey for histological assistance and to Miss E. M. Grover for animal care.

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Received October 3, 1966. P.S.E.B.M., 1967, v124.

Antiviral Substances in Plants of the Mint Family (Labiatae). I. Tannin of *Melissa officinalis*.* (31872)

LOUIS S. KUCERA AND ERNEST C. HERRMANN, JR.
Mayo Clinic and Mayo Foundation, Section of Microbiology, Rochester, Minn.

Aqueous extracts of the plant *Melissa officinalis* (lemon balm), used medicinally in antiquity, have a variety of antiviral effects including prevention of the plaque formation, hemagglutination, and death of embryonated eggs induced by Newcastle disease virus (NDV). Because gelatin blocked certain of

* This investigation was supported in part by Research Grant AI-5603 from Nat. Inst. Health, USPHS.