Hormone Effects Upon Cyclic Nucleotide Excretion in Man[1] (36162)

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c-AMP[2] is important in stimulating the secretion and action of hormones (1, 2). There is considerable specificity in the effect of many hormones upon the c-AMP levels of tissues, due to a specific receptor for each hormone. Hormones stimulate the following sequence: increased adenyl cyclase activity → increased c-AMP → increased protein kinase activity → increased action of other enzymes. In turn, these enzymes influence the metabolism of carbohydrates, lipids, proteins, nucleotides, and other body constituents. Another cyclic nucleotide in the body, c-GMP (3), probably also is very important in many metabolic reactions, but data about it are meager.

It has been difficult to measure the levels of c-GMP and c-AMP in plasma and especially in tissues of man. However, by using a simple and dependable assay (radioimmunoassay) of these cyclic nucleotides in urine, we have obtained some indirect information regarding the metabolism of these nucleotides. In this paper, we report effects of hormones and of other factors upon the excretion of c-GMP and c-AMP in normal man.

Materials and Methods. The major supplies were as follows: uniformly tritiated c-GMP and c-AMP labeled in the 8-position (New England Nuclear); c-AMP and c-GMP (Boehringer); aminophylline (Searle); ACTH, epinephrine, and vasopressin (Parke-Davis); crystalline glucagon and parathyroid hormone (Lilly); isoproterenol (Winthrop); propranolol (Ayerst); phentolamine (Ciba); hydrocortisone hemisuccinate (Upjohn); triiodothyronine (Smith, Kline and French).

Experimental design. Normal healthy male subjects, aged 20–30, were used. No food, coffee, smoking, or much exercise were permitted from midnight until completion of the experiment. Urine voided at 6:00 a.m. was discarded, but subsequent specimens were saved until infusion of the test compound was begun, 3 hr later, and at 45-min intervals thereafter; a few exceptions are presented later. Water was given to help assure prompt urine collections: 180 ml at 6:00, 7:00, and 8:00 a.m.; 330 ml was given at the start of the infusion and after each urine collection. The subjects were kept in bed in the Clinical Research Center, from 8:00 a.m. until all urine was collected. Each compound was infused intravenously during 30 min at a constant rate in 250 ml of 0.9% saline, using a Harvard pump. Further details concerning the infusions and urine collections are given in Results. After measuring the volume, urine specimens were centrifuged (2500 rpm) for 10 min. An aliquot of the supernatant was kept at −20° until assays for cyclic nucleotides were performed.

C-AMP and C-GMP assays. In most previour reports, the measurements of c-AMP and c-GMP have utilized enzyme assays (3–14). We have used radioimmunoassay, applying essentially the method of Steiner *et al.* (15), with some modifications indicated below. Adult New Zealand rabbits were injected monthly, in multiple subcutaneous sites, with 0.6 mg of succinylated c-AMP or c-GMP

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[2] Abbreviations: c-AMP, c-GMP, c-UMP, c-IMP, and c-CMP are abbreviations for the 3′,5′-monophosphate, respectively, of adenosine, guanosine, uridine, inosine, and cytosine. ATP and GTP are the triphosphates of adenosine and guanosine. AMP is the 5′-monophosphate of adenosine; PTH is parathyroid hormone; cpm is counts per minute.

bound to bovine serum albumin, suspended in Freund's adjuvant. Rabbits were bled by cardiac puncture at 6-week intervals. One week before each bleeding, 0.12 mg of antigen (without adjuvant) was injected intradermally as a "booster."

^{3}H-c-AMP (1 ng diluted 1:500) and sufficient 0.02 *M* Tris buffer were added to 50 μl of the c-AMP antibody serum (diluted 1:5) and varying dilutions of urine to give a final reaction volume of 0.5 ml and 30,000 cpm; the pH was adjusted to 7.9. After the solution was mixed and incubated at 4° for 2 hr, absolute ethanol (2 ml) was added. After 30 min the mixture was centrifuged (4000*g*) at 4° for 8 min. The supernatant was placed in a plastic scintillation vial containing 15 ml of Bray's Omnifluor, and the radioactivity was counted in a Packard Tri-carb liquid scintillation spectrometer. Using a semilogarithmic scale, the amount of antibody-bound ^{3}H-cyclic nucleotide was linearly related inversely to the total amount of cyclic nucleotide present; these responses served as reference standards. Each urine specimen was tested in duplicate in 4 different dilutions. In all instances, the dilutions chosen were ones that yielded reactions linearly related to the amount of dilution.

The assay for c-GMP was like that for c-AMP, except that the c-GMP antibody dilution was 1:10, the ^{3}H-c-GMP dilution was 1:750, and the incubation was for 1 hr. The binding of c-GMP to its antibody was not influenced by adding 2–1000 pmoles of c-AMP or 40–20,000 pmoles of ATP. Although c-IMP reacted significantly with this antibody, other studies indicated that no c-IMP was in urine (6, 12). In some of our studies, no c-GMP values were obtained, because we had not developed our c-GMP assay. Using c-AMP antibody, cross-reactivity was 0.001% with AMP or GTP, 0.004% with ATP, and 0.01% with c-GMP or adenosine. Reactions with other cyclic nucleotides, which apparently do not exist in man, were: c-UMP, 0.01%, c-IMP, 0.1%, c-CMP, 0.2%. Therefore our c-GMP and the c-AMP antibody sera could be used satisfactorily for measuring the respective nucleotide.

Statistical analysis of data. The immunoassay data were placed upon computer cards; standard curves were computed, and the linearity of responses was tested, using *F* values for 95% levels of confidence. In instances of rejection of linearity, the points at each end of the curve were tested and the ones with the greatest deviation from regression were rejected. The process was repeated until the curve became acceptable. The *F* values and values for rejected points were printed. The end points of the curves were used as limits for acceptable values for the unknown curves. Using Finney's method (16), the common regression coefficient was determined; then the concentration of unknown cyclic nucleotides and the 95% confidence limits were computed. Analyses of variance were conducted for comparing the parallelism of the standard and unknown curves no corrections were applied when parallelism was rejected.

TABLE I. Cyclic Nucleotide Urine Levels Before, During, and After Saline Infusion.[a]

	(n moles/mg of creatinine)		(n moles/hr)	
(hr)	Wat	Kir	Wat	Kir
c-GMP —3–0	0.341	0.277	26.3	22.5
0–1	0.510[b]	0.291	46.0[b]	16.0
1–1.75	0.682[b]	0.452[b]	52.7[b]	76.8[b]
1.75–2.5	0.516	0.216	35.5	22.9
c-AMP —3–0	3.6	4.9	273	404
0–1	4.6	3.9	414	347
1–1.75	4.8	5.3	367	905[b]
1.75–2.5	6.8[b]	4.7	470[b]	502

[a] 250 ml of 0.9% saline were infused at a constant rate during 30 min starting at 0 hr.
[b] Significant increase above the initial level (base line).

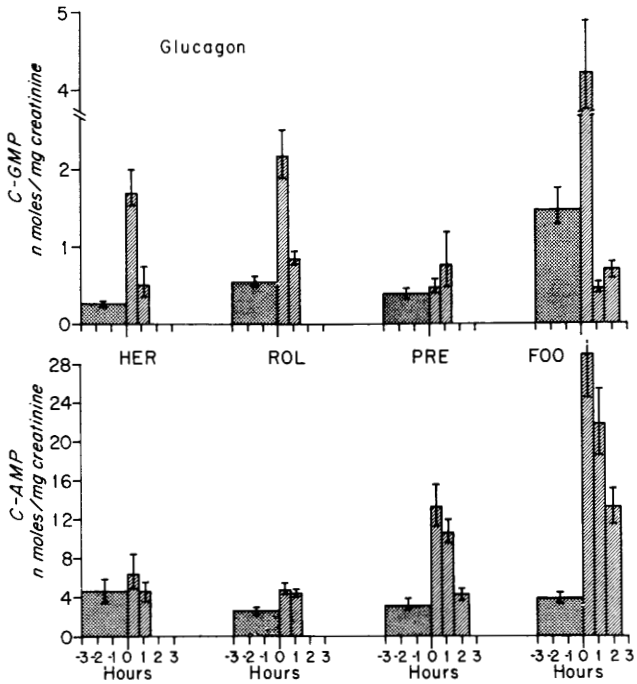

FIG. 1. After collection of a basal urine specimen within 3 hr, glucagon was infused intravenously throughout 30 min: Subjects "Her" and "Rol" received 0.5 mg, while "Pre" and "Foo" were given 1 mg. In Figs. 1, 2, and 3, infusion of the test compound was initiated at time "0." (elongated vertical line) The 95% confidence range for the assays.

Results. We present the effect of some compounds upon c-GMP and c-AMP excretion. Since the excretion of these nucleotides usually depends largely upon glomerular filtration (11), and some of the test agents influence this, we have also related their excretion to creatinine excretion. Table I shows the excretion by normal resting, fasting subjects, infused only with 250 ml of 0.9% saline. There is about 10 times as much c-AMP as c-GMP. A few increases in each nucleotide occurred within the 5.5 hr of observation. These are discussed below.

Glucagon. When 0.5 mg of glucagon was infused during 30 min, there was a significant increase in the ratio of c-GMP excretion to creatinine excretion (Fig. 1); the increase in c-AMP was relatively much less. While the larger dose (1 mg) of glucagon caused more of an increase in c-AMP than did the smaller amount, it promoted less of an increase in c-GMP excretion. This latter phenomenon (less response to higher dosage) presumably resulted from stronger counteraction stimulated by the larger dosage. The highest rate of c-GMP and c-AMP excretion was during the infusion. Glucagon caused proportionately more increase in the total excretion of the cyclic nucleotides (Table II) than in their ratios to creatinine excretion (Fig. 1). A possible basis for this is discussed below.

Parathyroid hormone. This hormone increased c-GMP and c-AMP excretion. The increase in their total amounts (Table II) was comparable to their increase as related to creatinine excretion (Fig. 2). In 2 of 3 subjects, the maximal excretion rate of c-GMP was after cessation of hormone infusion, but distinctly the maximal rate of c-AMP excretion occurred during infusion; then there was a rapid decline.

Isoproterenol. During infusion of isoproterenol (0.09 mg), there were significant increases in c-GMP and c-AMP excretion. There were somewhat greater increases in

TABLE II. Effect of Glucagon, Parathyroid Hormone and Isoproterenol upon c-GMP and c-AMP (n moles/hr.)

	c-AMP				c-GMP			
				Glucagon				
Time (hr)	Her	Rol	Pre	Foo	Her	Rol	Pre	Foo
—3–0	202	122	208	248	12	26	26	95
0–0.75	1178[a]	595[a]	2255[a]	5211[a]	317[a]	274[a]	83[a]	762[a]
0.75–1.5	500[a]	713[a]	758[a]	2220[a]	60[a]	141[a]	54[a]	46
1.5–2.5			370[a]	885[a]				46
				Parathyroid hormone				
	Whi	Hed	Shu	Wal	Whi	Hed	Wal	
—3–0	189	320	350	326	51	34	15	
0–0.75	6547[a]	>14,600[a]	4220[a]	11,161[a]	85[a]	269[a]	78[a]	
0.75–1.5	1990[a]	1260[a]	1425[a]	3430[a]	170[a]	315[a]	74[a]	
1.5–2.5	345	360	388	410	55	30	16	
				Isoproterenol				
	Wil	Rob	Bar		Wil	Rob	Bar	
—3–0	373	108	388		16	10	8	
0–0.75	662[a]	712[a]	334[a]		81[a]	127[a]	21[a]	
0.75–1.5	240	373[a]	293[a]		34[a]	86[a]	16[a]	
1.5–2.5	351	353[a]	337[a]		38[a]	106[a]	63[a]	

[a] $p < .05$, compared with preinfusion levels.

their total amounts (Table II) than as related to creatinine excretion (Fig. 3). When related to creatinine excretion, the increase in c-GMP was greater than that of c-AMP (Fig. 3). The high levels of excretion persisted throughout the observation periods.

Epinephrine, with and without propranolol or phentolamine. Epinephrine, 0.18 mg, was infused within 30 min into 6 subjects, beginning at 0 time. Between 135 and 140 min, 2.5 mg of propranolol were infused into 3 of these subjects and another 2.5 between 140 and 170 min, along with another dose (0.18 mg) of epinephrine. Between 135 and 140 min, 5 mg of phentolamine were infused into the other 3 people, and between 140 and 170 min, 15 mg were given. Urine collections were within the following (min): —180–0, 0–45, 45–90, 90–135, 135–180, 180–225, 225–270. Excretion of c-AMP, total and as related to creatinine excretion, was measured in each specimen. In 5 of 6 subjects, a very small increase in c-AMP excretion followed epinephrine infusion; and in 3 persons, when phentolamine was administered before the second infusion of epinephrine, there was a slight increase in c-AMP. Detailed results are not presented because the changes are not significant compared with results obtained with only saline infusion (Table I).

ACTH, hydrocortisone, triiodothyronine, vasopressin, aminophylline. No definite changes in c-AMP excretion were found with the following compounds infused intravenously into each of 4 subjects within 30 min: (a) ACTH, 40 units; urine was collected for three 2-hr intervals thereafter; (b) hydrocortisone hemisuccinate, 10.0 mg; urine was collected for three 2-hr intervals; (c) aminophylline, 500 mg; urine was collected during three 45-min periods; (d) vasopressin, 0.2 units; urine was collected during three 45-min intervals. After ingestion of 0.1 mg of triiodothyronine by four people, no significant changes in c-AMP levels were found in urine collected during four 2-hr intervals. Each of the designated urine collections began with the drug administration.

Vigorous exercise. Strenuous exercise (2- or

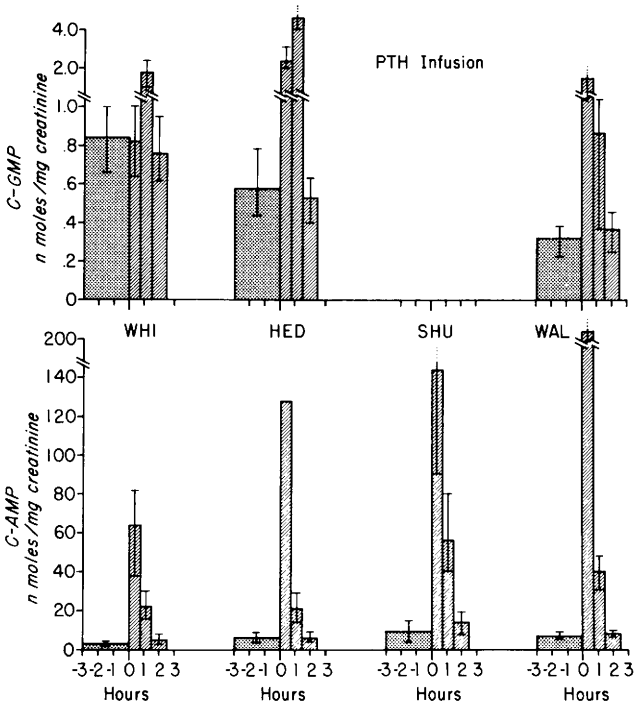

FIG. 2. Parathyroid hormone, 250 units, was infused throughout 30 min: Subject "Shu" differed from the others in that she was a lady with moderate obesity.

3-mile race) by 4 subjects who engaged in an interuniversity track meeting did not significantly increase c-GMP excretion. It did cause a slightly greater increase in total c-AMP excretion than was found in control subjects.

Discussion. We found that radioimmunoassay for c-GMP and c-AMP in urine was a simple, rapid, and dependable method. Excellent linear relationships were demonstrated with known increments of cyclic nucleotides. No significant amount of cross-reactivity occurred between c-AMP and the serum with antibodies for c-GMP and vice versa. The levels of excretion in normal subjects in a basal state were comparable to ones determined by enzyme assay (11). Small increases in both nucleotides were found in some urine specimens during, or after, saline infusion. The changes were probably not due to the saline infusion *per se,* but to diurnal changes in hormonal and metabolic activities occurring during the 5.5 hr experiments. Based upon studies of others (8, 11) the large fluid intake (1780 ml) would not increase cyclic nucleotide excretion. The c-GMP excretion rates were constant during infusion of hypertonic or hypotonic sodium chloride, despite changes in the urinary concentration of the nucleotide and of urine volume of as much as 80-fold (8). It has been reported (11, 14) that c-GMP was excreted entirely by glomerular filtration; plasma was considered to deliver all c-GMP excreted. The kidneys rapidly clear cyclic nucleotides. For example, over 40% of c-GMP injected into the renal artery was in the urine within 10 min (8). About 85% of the clearance of both cyclic nucleotides is extrarenal (11).

We found significant differences in the effects of hormones upon patterns of excretion of c-GMP and c-AMP. Glucagon increased the total excretion of the cyclic nucleotides much more than it elevated their ratio to creatinine. Isoproterenol augmented the total excretion of these nucleotides only slightly more than their concentration per creatinine level. Parathyroid hormone increased their

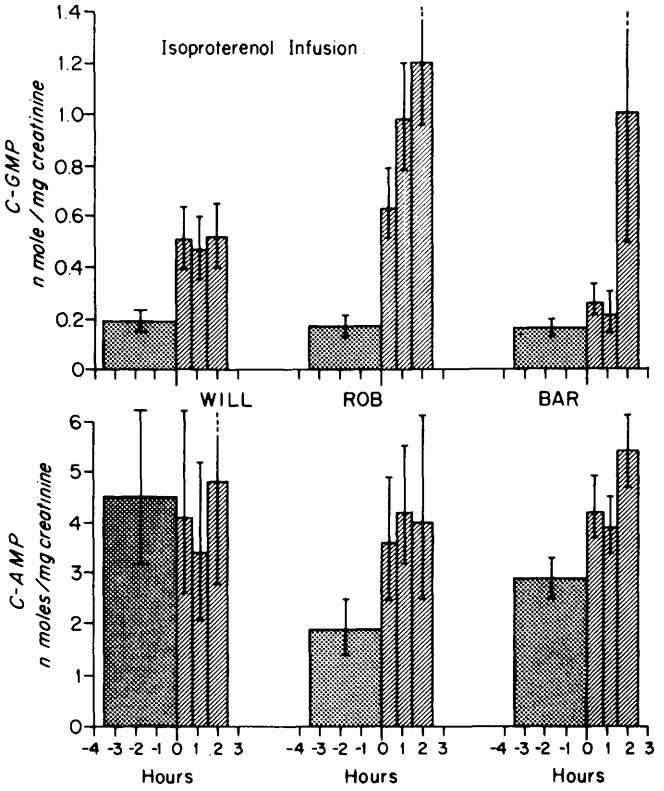

FIG. 3. Isoproterenol, 0.09 mg, was infused throughout 30 min.

total levels comparably to the increment in cyclic nucleotide:creatinine. Presumably, an increase in total cyclic nucleotide excretion per hour results from increase in one or more of the following: (a) glomerular filtration rate; (b) renal tubular release; and (c) contribution to the plasma by nonrenal sources. An increase in the cyclic nucleotide: creatinine is supposedly due to increase in (b) or (c). These considerations suggest that: (a) glucagon increased the release of c-AMP and c-GMP from the kidney tubules and/or nonrenal sources, and accelerated its glomerular filtration rate; (b) isoproterenol acted similarly, but caused less augmentation of glomerular filtration; (c) PTH increased c-GMP and c-AMP supply from nonrenal tissues and/or from renal tubules, but did not change glomerular filtration rate.

Isoproterenol caused a more prolonged increase in c-GMP and c-AMP excretion than did glucagon or PTH. The effect of isoproterenol was in excess of 2 hr, despite a very short half-life of it and the cyclic nucleotides in the plasma. Possibly it increased the level of some hormone(s) or other compound which continued to generate and/or release the cyclic nucleotides.

Others reported (12) that glucagon markedly increased c-AMP excretion, but caused no increase in c-GMP, even when used in amount equal to that used by us. The only major difference in the two studies seems to be in the assay method–enzyme procedure vs immunoassay. When 250 units of PTH was infused, others also found that c-GMP excretion was increased (13). After PTH, the increment in c-GMP was longer than that of c-AMP. The marked effect of PTH upon c-AMP excretion is due chiefly to the increment from the kidneys (7, 13, 17); normally, about one-third of c-AMP is added by the kidneys (11, 14). Patients with hyperparathyroidism excrete hypernormal quantities of c-AMP, and those with hypoparathyroidism have subnormal levels (10).

Using the enzyme assay method, even when isoproterenol was infused in larger dosage than we used, it was not found to increase the plasma c-GMP level (14), but did increase plasma c-AMP. We found, in the urine, that it produced more increase in c-GMP than c-AMP. A larger dose of epinephrine than we used has been reported (12) to cause an increase in plasma c-AMP and c-GMP. Upon infusion of epinephrine or norepinephrine with propranolol (14), there was an increase in plasma and urine c-GMP, but no change in plasma c-AMP. Conversely, these catecholamines infused with phentolamine caused no change in plasma or urine c-GMP, but increased plasma c-AMP.

Very little or no change in c-AMP excretion followed administration of ACTH, hydrocortisone, triiodothyronine, vasopressin, or aminophylline. Vasopressin has been reported not to increase (14) c-AMP excretion, but other investigators found (5, 7, 10) that it increased c-AMP excretion when large doses were used. Aminophylline was stated to decrease c-AMP (5) and theophylline to increase it (7). Purified ACTH did not affect c-AMP excretion (7). In certain conditions, corticosteroids or thyroxine increased c-AMP and c-GMP (9) excretion.

Urine and blood are the only commonly and readily available specimens for measuring cyclic nucleotides in man. The levels in urine are much more simply, rapidly, and accurately determined than are those in blood. In normal subjects, most of the cyclic nucleotides in plasma are derived from tissues other than blood cells. The plasma and kidneys serve chiefly as transport agencies. Since all tissues that have been tested contain adenyl cyclase and guanyl cyclase, it seems likely that each tissue could contribute some c-AMP and c-GMP to blood and urine. However, despite a large amount of information about these nucleotides, recently summarized (18), we know relatively little about the amounts of c-AMP and c-GMP that different tissues contribute to the plasma, or take from it, and of factors influencing them. Experience has demonstrated the importance of obtaining specific data in this area, because some "reasonable" surmises are erroneous. For example, although PTH and vasopressin increase c-AMP in certain kidney cells, PTH markedly increases its level in urine, but vasopressin has essentially no effect. ACTH also causes enormous increases in adrenal c-AMP, but does not increase the urine level. c-AMP is formed in cell membrane and can be rapidly removed from some cell types by washing (19), but in other cells it is more firmly bound.

Despite these and many other variable factors, information that is helpful in certain clinical problems has been derived, and much more can be projected. For example, urine c-AMP is markedly increased with hyperparathyroidism, but is normal or subnormal with other etiologies of hypercalcemia. The levels fail to respond significantly to PTH in pseudohypoparathyroidism. There are hypernormal levels with mania and subnormal ones with depression (20), presumed to be associated, respectively, with excess and deficient brain catecholamines and c-AMP. These observations have added interest in view of the extensive actions of catecholamines throughout the body and the fact that with depression the plasma catecholamine levels tend to be hypernormal (21). Thus, in depression the net decrease in brain-released c-AMP appears to exceed the excess release from other parts of the body. Since we have shown that a β-adrenergic (isoproterenol) increases c-AMP in urine, and since there are many examples of β-adrenergic action paralleling adenyl cyclase activity (18), measurement of urine c-AMP may give useful information concerning this type of nervous tissue activity.

Although glucagon has been shown to increase c-AMP levels in liver, heart, and adipose tissue, most of the increased plasma and urine c-AMP that it produces is from the liver (12). Therefore, the response in urine c-AMP to glucagon probably could be helpful in evaluating liver function.

We showed that PTH, glucagon, and isoproterenol significantly increased c-GMP excretion in urine. Measurements of its excretion in urine are simple and accurate; whereas tissue analyses have been difficult. However, limitations in interpreting the results will persist until much more information is ob-

tained regarding the functions of c-GMP and the influence of various hormones upon these.

Summary. Using a radioimmunoassay for measuring guanosine 3,5′-phosphate (c-GMP) and adenosine 3,5′-phosphate (c-AMP), the effect of intravenous infusion of hormones and other compounds upon the urinary excretion of these cyclic nucleotides was tested in young healthy adult males. Glucagon, parathyroid hormone, and isoproterenol increased the excretion of c-GMP and c-AMP. The action of isoproterenol was more prolonged than that of the other two hormones. No definite effect was produced by epinephrine, ACTH, hydrocortisone hemisuccinate, vasopressin, triiodothyronine, or aminophylline. Vigorous exercise slightly increased total c-AMP excretion.

The methodologic advantages of measuring c-AMP and c-GMP in urine are presented, and some implications of the significance of our studies and others are discussed.

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