ABSTRACT

James Nixon Barnes. GIBBERELLIC ACID-MEDIATED CHANGES IN CHOLESTEROL IN ALBINO RATS. Under the direction of Dr. Graham J. Davis, Department of Biology, May, 1974.

Gibberellic acid (GA) is a plant hormone that affects stem elongation and often flowering, germination, and leaf shape change. Indirect evidence from the work of others led us to suspect that GA may affect sterol metabolism. Cycocel, a plant growth regulant, blocks production of GA in plants and synthesis of cholesterol in rat liver extracts. Smith, Kline, and French compound 7997, an animal steroid inhibitor, has been found to block floral induction and elongation from exogenous GA. The albino rat was chosen for these studies because of simple and well-established analytical procedures and similarities in sterol metabolic pathways to those of higher plants.

Total serum cholesterol levels were assayed colorimetrically 2, 4, and 6 hours after IP injections of citric acid-phosphate buffer, pH 6.9. Dosages of GA were 1, 2, and 3 mg GA/500 gm body weight; control buffer solutions were injected at a volume equal to the volume that would be required for GA treatments.

Buffer alone resulted in increased cholesterol levels while all GA treatments depressed these high levels to below normal. Results from the treatments were significant at the 0.05 level, no significance with time or interaction of time and treatment were found.

GIBBERELLIC ACID MEDIATED CHANGES IN CHOLESTEROL LEVELS IN THE ALBINO RAT

A Thesis

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In Partial Fulfillment

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Master of Arts in Biology

by

James Nixon Barnes

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GIBBERELLIC ACID MEDIATED

CHANGES IN CHOLESTEROL LEVELS

IN THE ALBINO RAT

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The author wishes to express his gratitude to Dr. Graham J. Davis for his support and direction of this thesis and especially for his willingness to work on a project that crossed the line between botany and zoology.

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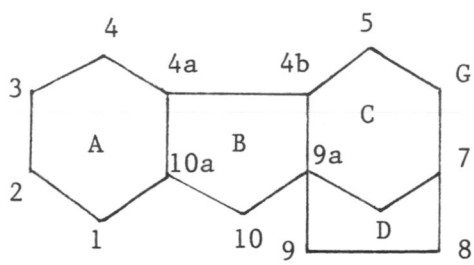
INTRODUCTION

Gibberellic acid (GA) is a naturally occuring plant hormone. Study of GA was begun in Japan during the 1920's in association with studies of bakanae (foolish seedling) disease. Bakanae causes rice seedlings to grow excessively tall. The chemical nature of the disease was demonstrated by Kurosawa in 1926 who showed that extracts from the fungus Gibberella fujikuori would cause symptoms identical with bakanae. Yabuta and Hayashi, working in the 1930's, isolated and identified the active compound from Gibberella fujikuori extracts. The active compound was named gibberellin.

The active substances isolated and identified as gibberellin have received several names - gibberellic acid, GA, GA_3. The term gibberellin now is used to designate any compound having a gibbane skeleton (Fig. 1) and producing effects similar to those of the original gibberellin.

Figure 1. GIBBANE SKELETON



The gibberellins are named by numbers (GA_1-GA_n) and often from the plants from which they were first isolated. To the present 29 gibberellin-like compounds have been discovered (20). (A gibberellin-like compound is one that produces effects similar to gibberellins but has an unidentified structure.)

Gibberellins are known to function in germination, stem elongation, leaf shape change and flowering (31, 32, 16, 28). Knowledge of the physiological effects of gibberellins has naturally led to speculations as to the mode of biochemical action of gibberellins.

Gibberellins and other plant hormones appear to interact in the control of stem elongation. Gibberellins have been shown to stimulate indoleacetic acid oxidase which catalyzes the conversion of indoleacetic acid to methoxyindole. Some researchers suggest that this is the area for the mode of action of the gibberellins (32).

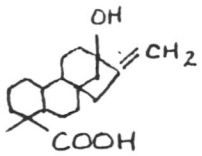
In seed germination GA stimulates the production of alpha amylase, a hydrolytic enzyme, and this action could be taken to show that GA action is through the de novo synthesis of hydrolytic enzymes (23). There are suggestions that GA functions through control of nucleic acid synthesis. Stimulation of alpha amylase production is accompanied by production of proteases and ribonucleases (8, 12). Gibberellic acid has also been found to stimulate RNA synthesis and affect DNA synthesis (30).

The mode of action for GA has not been determined as of this time and it appears it could be involved in several pathways. Another possible biochemical level of GA action is in steroid metabolism and this possibility is the concern of this paper. Very little is known about possible relationships between steroid metabolism and the gibberellins, and steroid metabolism is a very fertile area for research. Steroids have a broad spectrum of physiological effects in animals and it appears that such a class of compounds affecting so much in animals should be non-functional in plants. The effects of animal steroids are in the same general area as are the gibberellin mediated effects - growth and development.

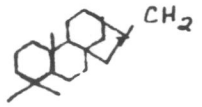
Cholesterol is the parent compound for the animal steroids, so any effects on steroid metabolism may be exhibited in the concentration of cholesterol. Plants contain cholesterol but the amounts are low (28). The steroid pathways are the same for both plant and animal (Figs. 2, 3, 4) and any effect found in the animal system could be postulated for the plant system.

Work with floral initiation showed that 2-chloroethyl trimethylammonium chloride (CCC) blockage of initiation in Pharbitis nil Chois, Japanese morning glory, could be overcome by GA treatment. The blockage of floral initiation by CCC was found only when treatment was prior to the inductive photoperiod (33). Experiments using known steroid biosynthesis inhibitors also showed blockage of floral initiation in Pharbitis (9).

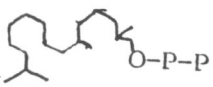
The information from the floral initiation experiments suggested a link between GA and sterol metabolism. A research project was undertaken to gather more information about the interaction of GA and CCC. Leaf shape in Proserpinaca palustris is photoperiod dependent (15). Plants maintained under long days form lanceolate-serrate (adult) leaves while plants maintained on short days form highly divided (juvenile) leaves. It was later shown that plants maintained on short days and treated with GA would form adult-type leaves (16). The project was to observe the effects of the plant growth regulator CCC on GA-mediated leaf shape changes. It was found that CCC blocked the formation of adult type leaves in the presence of GA (2). One effect of CCC on plant growth is by blockage of biosynthesis of GA at (-) kaurene (Fig. 2) (1). Blocking the biosynthesis of GA should not cause a reversal of action by applied



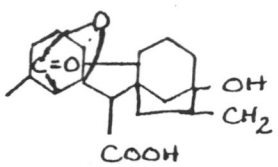
STEVIOL



(-)-KAURENE



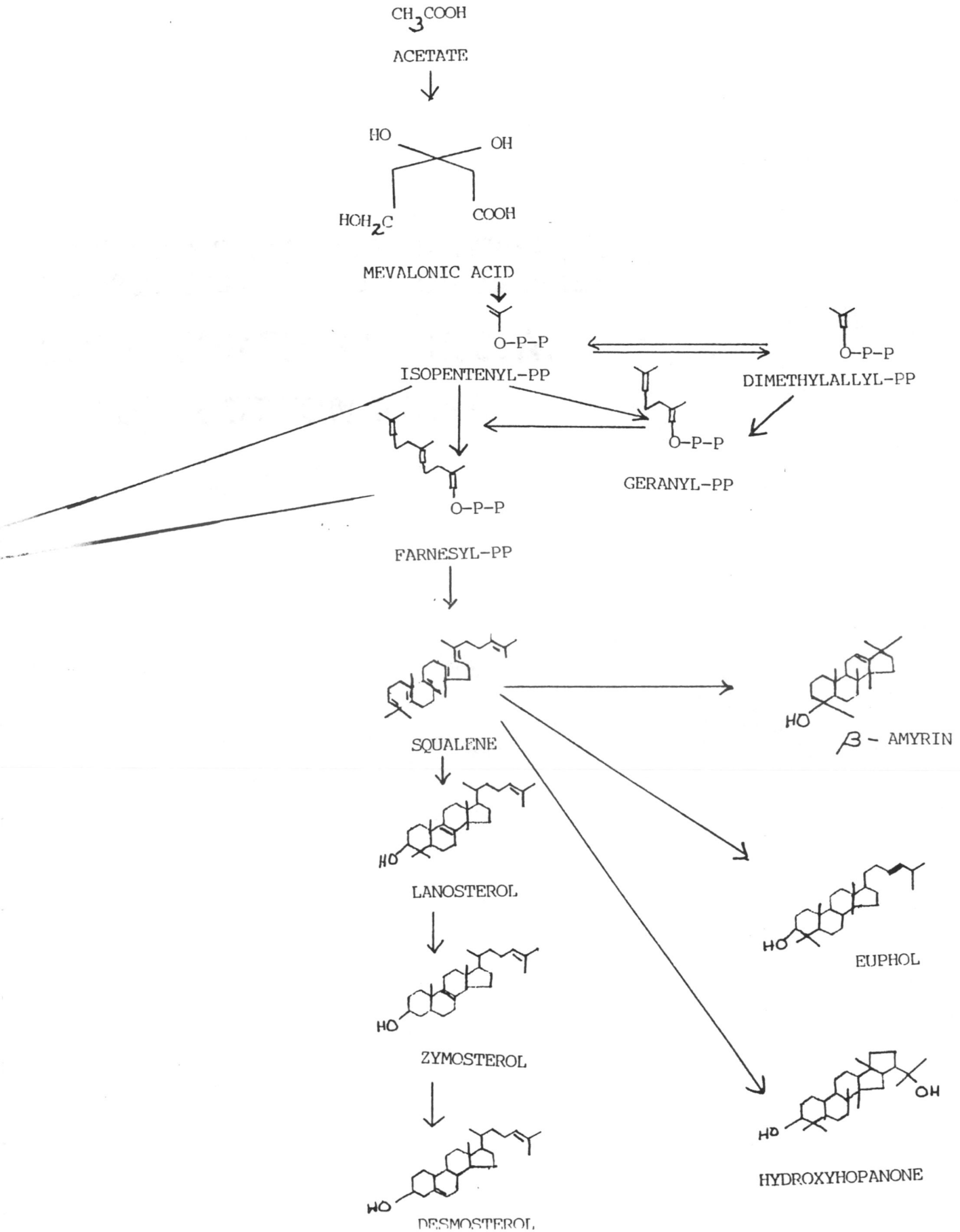
GERANYLGERANYL-PP



GIBBERELLIC ACID

FIGURE 2

STEROID BIOSYNTHESIS



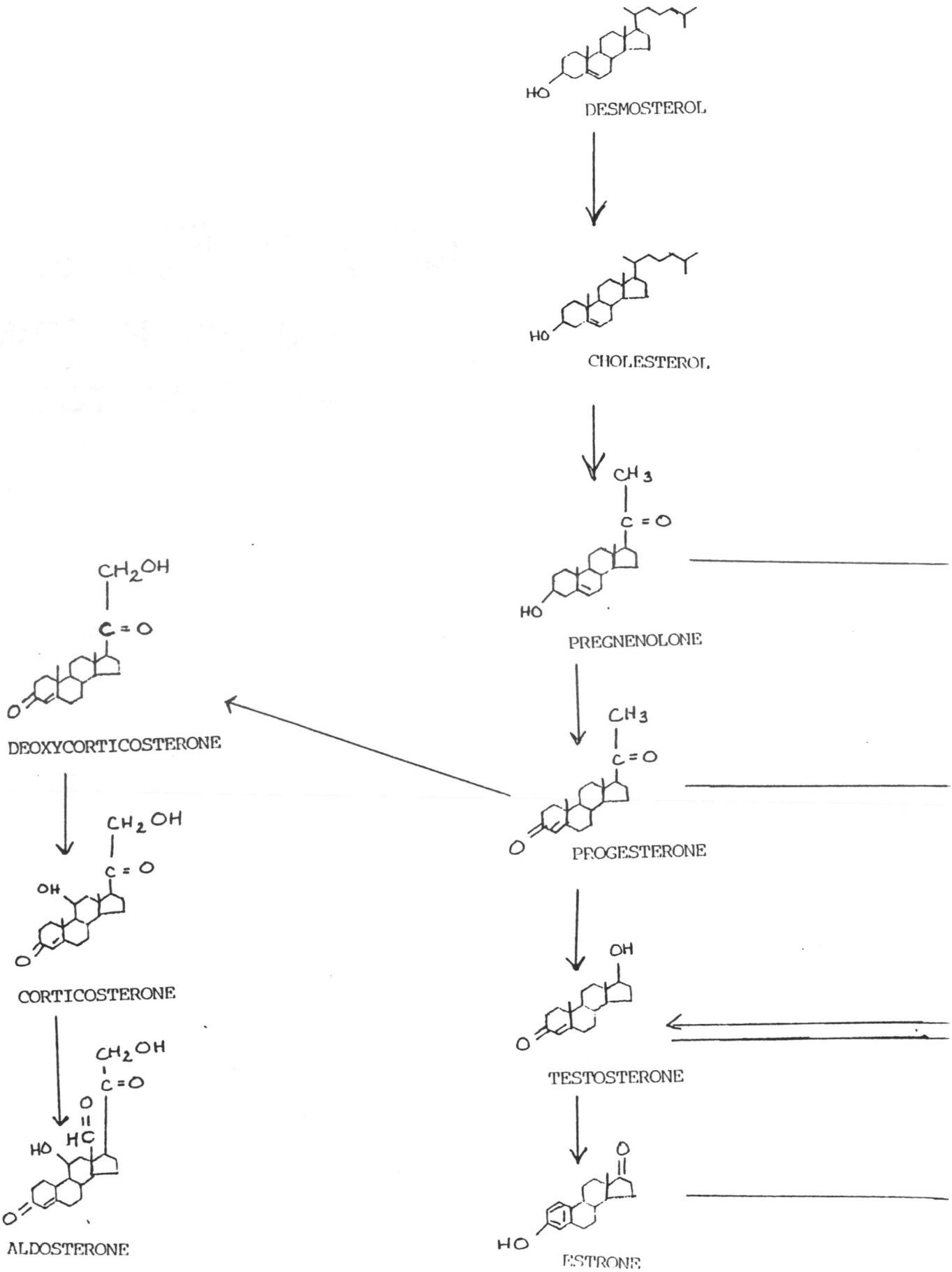
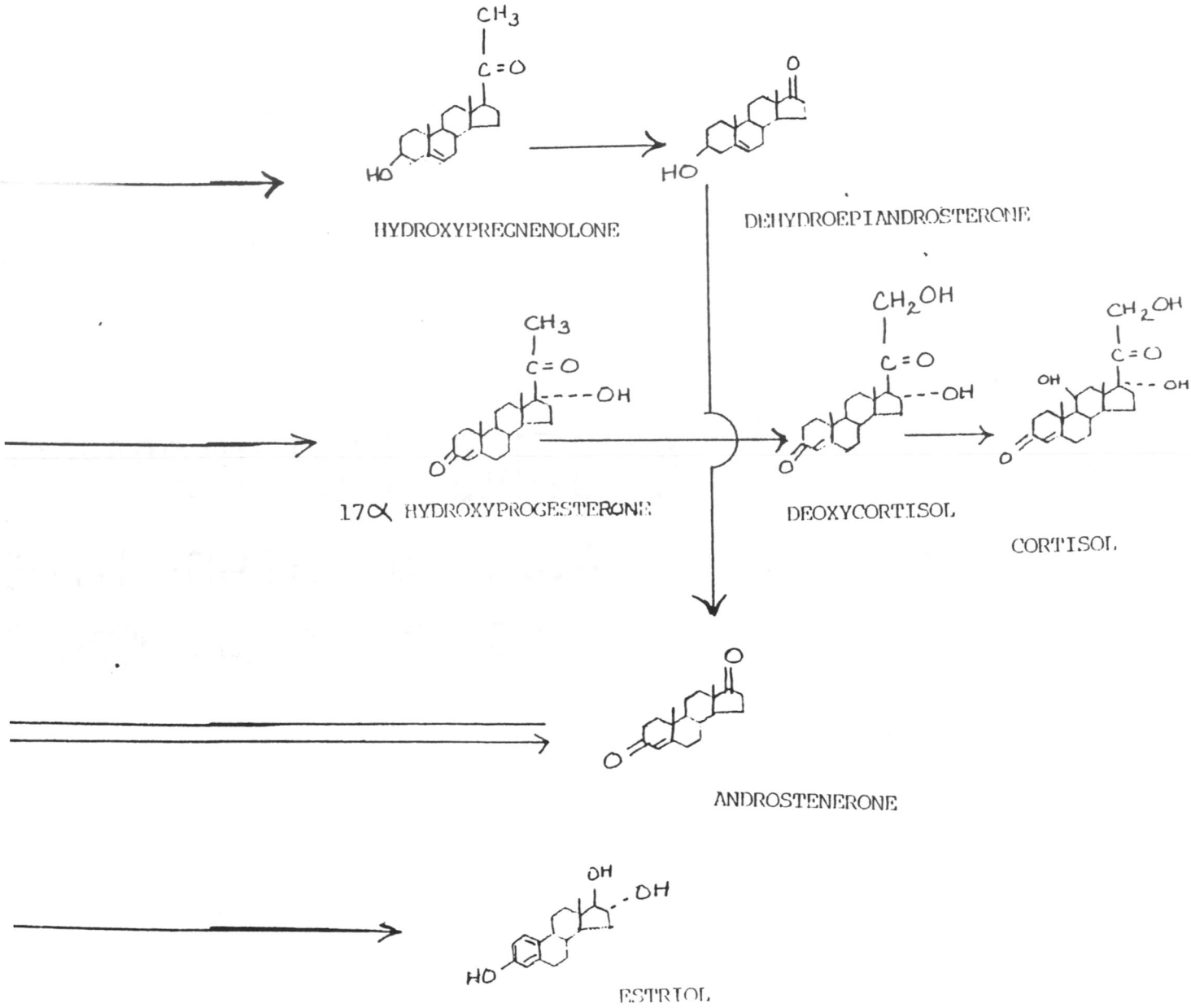
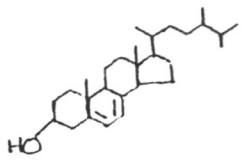

HO
DESMOSTEROL
HO
CHOLESTEROL
CH3
C=O
HO
PREGNENOLONE
CH2OH
C=O
O
DEOXYCORTICOSTERONE
CH3
C=O
O
PROGESTERONE
CH2OH
C=O
OH
O
CORTICOSTERONE
OH
O
TESTOSTERONE
CH2OH
C=O
O
HC
HO
O
ALDOSTERONE
O
HO
ESTRONE

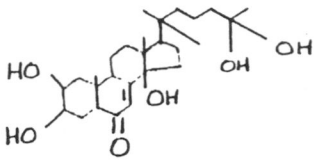
FIGURE 3

ANIMAL STEROID METABOLISM

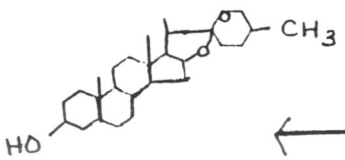




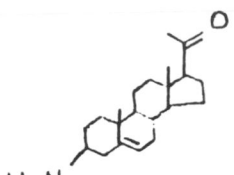
ERGOSTEROL



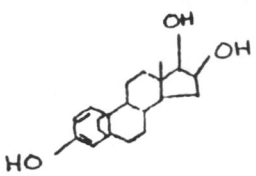
ECDYSTERONE



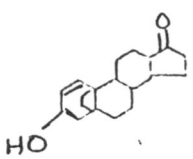
DIOSGENIN



HOLAPHYLLAMINE



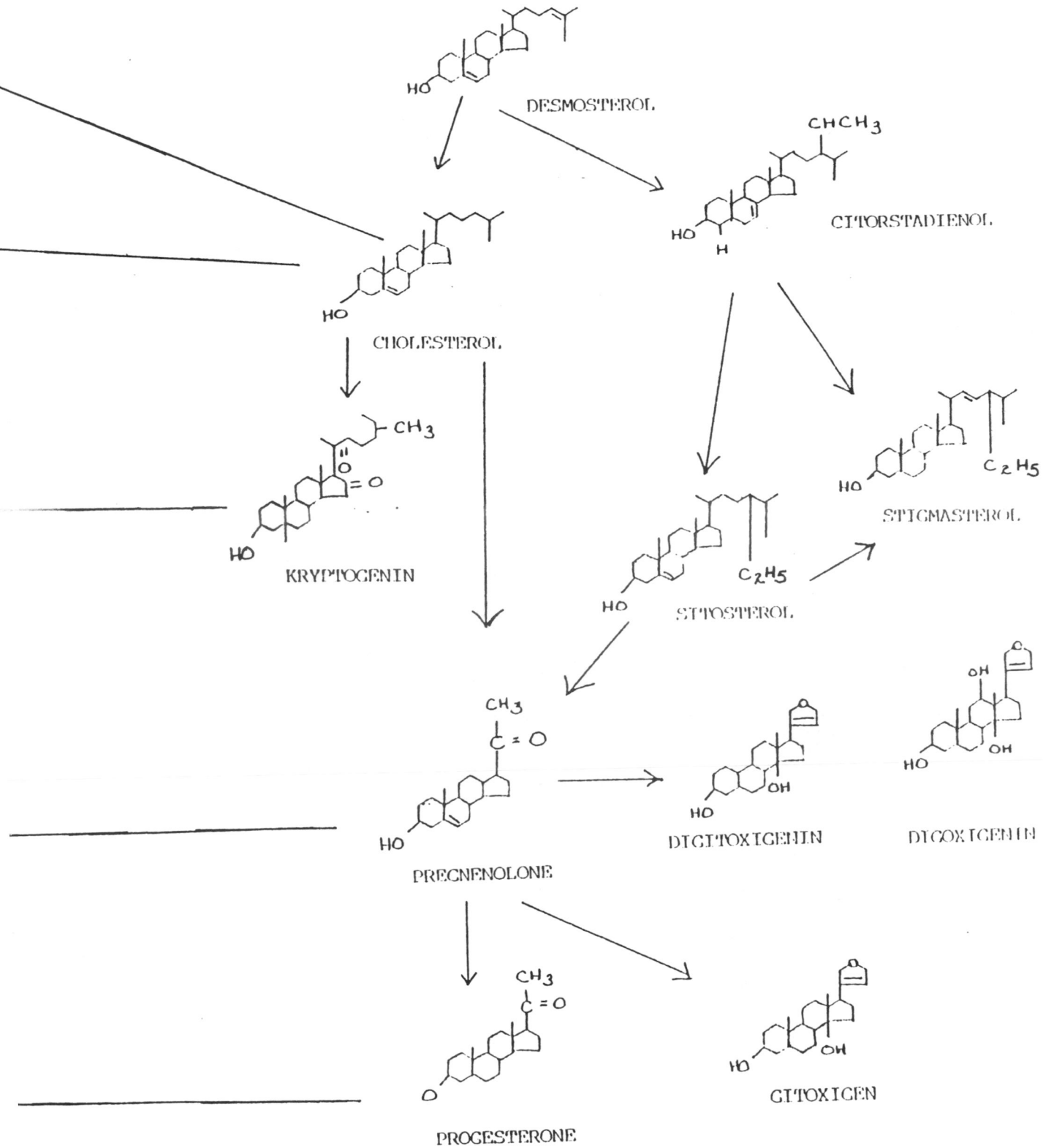
ESTRIOL



ESTRONE

FIGURE 4

PLANT STEROID METABOLISM



GA as was observed. Following this observation a search of the literature indicated that CCC would block the conversion of acetate to mevalonate and this would have an affect on sterol metabolism (27). If blocking sterol metabolism blocks the action of exogenous GA, then GA may function through sterol metabolism.

The auxin indoleacetic acid (IAA) causes elongation in coleoptiles. Applications of CCC will retard elongation by decreasing the amount of diffusable auxin. When IAA is added the inhibition is overcome. Inhibition by CCC of coleoptile growth is overcome by GA. Both exogenous IAA and GA give an increase in diffusable auxin (23). Gibberellic acid will overcome the CCC inhibition by stimulating production of IAA oxidase (13). Indoleacetic acid oxidase catalyzes the conversion of IAA into methoxyindole. Methoxyindole is active in assays for IAA (25).

An interesting association of GA in animal metabolism is pertinent to the research reported here. In the adult honeybee, Apis mellifera L., pollen is the natural diet. When raised in caged colonies development of larval stages required natural pollen. Replacement of the natural pollen with GA allowed the larvae to develop (26). The need for the GA additions to the diet corresponds to the development of the prothoracic glands which produce ecdysone, a product also produced by plants (Fig. 4) from cholesterol.

The actions of the sex steroids in animals are fairly well documented. The androgens cause a masculinizing of the female and the estrogens effect an effeminization of the male. Gibberellic acid when applied to female flowers will cause the formation of male flower parts and no effect is noticed on the male flower of Cannabis sativia, a dioecious plant (24).

Treatment of a monoecious plant, Fagopyrum esculentum Moench, cv Silver Hull, with GA caused the production of functional males by a decrease in the gynoecium, the female reproductive structure (11). Treatment of Proserpinaca with GA caused the abortion of anthers and the production of short peduncles on sessile flowers (16).

As interesting as similarities between GA-steroid effects on plants and animals may be, it remains to be seen whether or not the basic biochemical mechanisms are related. Before any definitive work on the possibility that gibberellins function in steroid metabolism, there must be evidence that gibberellins do affect steroid metabolism. The purpose of the work presented here is to study the effect of GA on changes in serum cholesterol levels in the albino rat.

METHODS AND MATERIALS

Experimental subjects for the investigation were male albino rats (Ratus ratus L.) of the Holtzman Strain. They were retired breeders of approximately one year of age. Upon arrival from Holtzman, the rats were allowed to equilibrate at least 48 hours in the vivarium. Environmental conditions within the vivarium were maintained at constant levels during the equilibration and testing period: 20 to 25°C, average 22°C, photoperiod of 14 hours of light, humidity varied with external conditions. (Humidity in the vivarium was not monitored.) Food and water were supplied ad libitum. Retired breeders were chosen for their size due to the fact that repeated collections of blood from smaller animals left them anemic. Males were selected since there would be no observable effects of sex steroids.

Each experiment consisted of 3 phases:

1. Injections of treatment solutions (buffered GA, buffer, buffer components, and physiological saline)
2. Collection of serum
3. Assay for serum cholesterol

INJECTIONS: All treatments were given by intraperitoneal (IP) injection. Treatments were given on a concentration-to-weight ratio, 0.2 0.4, and 0.6 mg GA/100 g body weight. There were 3 series of treatments:

1. Gibberellic acid from a stock solution of 5.0 mg/ml buffer at pH 6.9 (McIlvaines citric acid and phosphate buffer, 0.1 M citric acid and 0.2 M disodium phosphate; the potassium salt of GA_3 was used.)

2. Sham injections of McIlvaines buffer at pH 6.9. The sham injections were given on the same concentration to weight basis as the GA injections, the rats received an equivalent amount of buffer as if the treatment were from the GA stock solution.
3. Buffer components, citrate and phosphate each injected separately, were injected at 4 levels: 0.2 M; 0.1 M; 0.05M; and 0.01 M; at pH 6.9 from stock solutions. The buffer components were injected at the volumes required to equal the volumes given from the concentration to weight ratio of the 0.4 mgGA/100 body weight.

Treatment solutions came from stock solutions so the volumes of the injections varied as to the weight of the rat and the concentration of the treatment. High concentration stock solutions were used and volumes given were under 0.5 ml.

Variability due to circadian rhythms was reduced by scheduling injections and sampling at the same time intervals to reduce the possibility of steroidal circadian rhythm variations. Injections were given at 10 a.m. and sampling took place after the correct time intervals.

SERUM COLLECTION: Blood samples were taken from the subjects by means of clipped tails. An internal control for all the times, levels, and types of treatment was obtained by taking the first sample prior to the treatment injection, designated as 0 hours. Samples were collected from the same subject at 0, 2, and 4 hours and from a second subject at 0, 6, and 8 hours for each level and type of treatment. The sampling process was completed in approximately one month.

Sampling was accomplished by anesthetizing with ether, heating the tail under water to increase blood flow, clipping 1 cm off the tail tip and allowing the subject to bleed into a heparinized centrifuge tube. The clipping was done with a microtome blade which did not traumatize the cut and allowed the blood to flow freely. After each sample was collected the clipped tail was tied off to stop the bleeding.

Sample volume was approximately 1.5 to 2.0 ml and samples were stored on ice during the sampling period and in a refrigerator until all could be centrifuged. Plasma was separated from the cells by spinning the samples in a refrigerated centrifuge for 10 minutes at 8,000xG. The isolated plasma was deproteinated by pipetting 0.5 ml plasma into 5.0 ml ethylacetate: ethanol (1:1, V:V) mixture. Serum was separated from the protein precipitate by refrigerated centrifugation for 10 minutes at 8,000xG. The serum was then used for assaying for cholesterol levels.

ASSAY: Collected serum was assayed for cholesterol content by the method described in Gradwhal's Clinical Laboratory Methods and Diagnosis (17). In this procedure serum was mixed with ferric chloride, with color development from the addition of concentrated sulfuric acid. The measurement of cholesterol was accomplished spectrophotometrically. This assay procedure did not detect GA_3 nor did GA_3 interfere with cholesterol detection (Appendix 1).

Quantification of cholesterol was the same for each series by using the optical density (OD) values from the triplicate standards for that series. The standards followed Beers Law; the triplicate standards (OD

values) were used to calculate a regression coefficient. The OD values from duplicate samples were converted to mg cholesterol/100 ml blood (mg%) by using the calculated regression coefficient in a linear regression (Appendix 2).

RESULTS

The action of GA upon cholesterol levels in the white rat is summarized in Tables 2 and 3 and graphically represented by Figure 5.

Figure 5 shows the relation of serum cholesterol levels to the different treatments and different times. All of the points on the graphs are the average mg% cholesterol value from 3 rats. The times shown, 2, 4, 6, and 8 hours are the intervals between injection of treatment solutions and bleeding.

The normal values are from animals receiving physiological saline. The normal cholesterol level is between 50 and 90 mg% cholesterol with 70 mg% cholesterol level as the average. Normal values are placed on the graph as a reference point for the buffer treatment.

The effects of the GA can be readily seen by comparing the cholesterol levels of the buffer and the GA. The GA gives a consistent decrease in cholesterol levels, with the greatest decrease at a concentration of 0.4 mg GA after 2 hours.

EFFECT OF BUFFER: Injections of buffer caused an increase in cholesterol after 2 hours at all volumes. After 4 hours the buffer with the least volume (same volume as used for 0.2 mg GA) showed a small decrease below normal while the other 2 volumes showed an increase. After 6 hours the only volume of buffer that showed an increase in cholesterol was the largest, corresponding to 0.6 mg GA. The 8 hour assay had the middle volume being elevated and the smallest and largest volumes depressed (Table 1).

TABLE 1

SERUM CHOLESTEROL VALUES AFTER TREATMENT OF MALE RATS WITH GIBBERELLIC ACID AND BUFFER*

TIME	TREATMENTS						
HOUR AFTER TREATMENT	NORMAL	BUFFER (SHAM)			GIBBERELLIC ACID IN BUFFER		
		0.2 mg/100 g	0.4 mg/100g	0.6 mg/100g	0.2 mg/100g	0.4 mg/100g	0.6 mg/100g
0	67.47	70.82	73.68	76.71	58.89	62.27	60.12
2	56.17	63.24	79.99	77.04	52.87	45.75	52.99
4	64.74	60.49	82.47	79.97	48.90	56.17	48.50
6	71.66	62.73	72.11	80.18	60.86	57.31	54.67
8	67.33	56.79	79.02	53.95	49.64	50.29	59.17

*The values shown are the mean value in mg%/3 rats except the 0 hour values which are the mean value from 6 rats.

EFFECT OF BUFFERED GA: Injections of the GA caused a decrease from normal serum cholesterol levels. With the buffer-only treatment causing elevated levels, the decrease in cholesterol is even more pronounced (Table 1).

The experimental design was such that a partitioned analysis of variance was used to determine statistical significance and where the significance would be located (Table 2).

Table 2

Values for Analysis of Variance

Source	df	SS	MS	F
Time	28	5288.55	188.88	NS
Injections	6	6767.41	1127.90	2.412*
Normal=Buffer	1	277.64	277.90	NS
Normal=GA	1	1330.40	1330.40	2.845*
GA 0.2=Buffer 0.2+ Normal	1	1261.72	1261.72	NS
GA 0.4=Buffer 0.4+ Normal	1	1997.41	1997.41	4.27*
GA 0.6=Buffer 0.6+ Normal	1	2163.85	2163.85	4.63**
Error	70	32734.77	467.64	

Changes due to time were not significant while changes in serum cholesterol were significant at the 0.05 level. Partitioning show where the significant changes were.

The elevation of serum cholesterol level due to buffer was not sufficient to be significant but the depression of serum cholesterol due to GA was significant to the 0.1 level.

The different concentrations of GA all caused a decrease in serum cholesterol. The decrease from GA at 0.2 mg/100 g body weight were significant at the 0.05 level. (See Appendix 2 for sample calculations.)

EFFECT OF BUFFER CONSTITUENTS: Serum cholesterol levels remained almost normal with the various injections of citrate and phosphate (Table 3). The lack of change from either citrate or phosphate is in agreement with the statistical analysis indicating that changes in serum cholesterol due to buffer were not significant.

TABLE 3

SERUM CHOLESTEROL VALUES AFTER TREATMENT OF MALE RATS WITH CITRATE AND PHOSPHATE*

TREATMENT	TIME		CHANGE
	0 HOUR	2 HOURS	
Normal	88.98	84.54	-4.44
Physiological Saline	87.50	86.43	-1.07
Citrate 0.2 M	83.58	84.03	+0.45
Citrate 0.1 M	78.52	78.98	+.46
Citrate 0.05 M	70.88	73.02	+3.86
Citrate 0.01 M	77.74	73.03	-4.71
Phosphate 0.2 M	98.94	96.65	-1.29
Phosphate 0.1 M	65.12	64.42	-0.70
Phosphate 0.05 M	74.53	77.88	+3.35
Phosphate 0.01 M	96.04	84.11	-11.93

*The values shown are mean values in mg% for 4 rats/treatment.

Fig. 5

Changes in Serum Cholesterol Levels in Relation

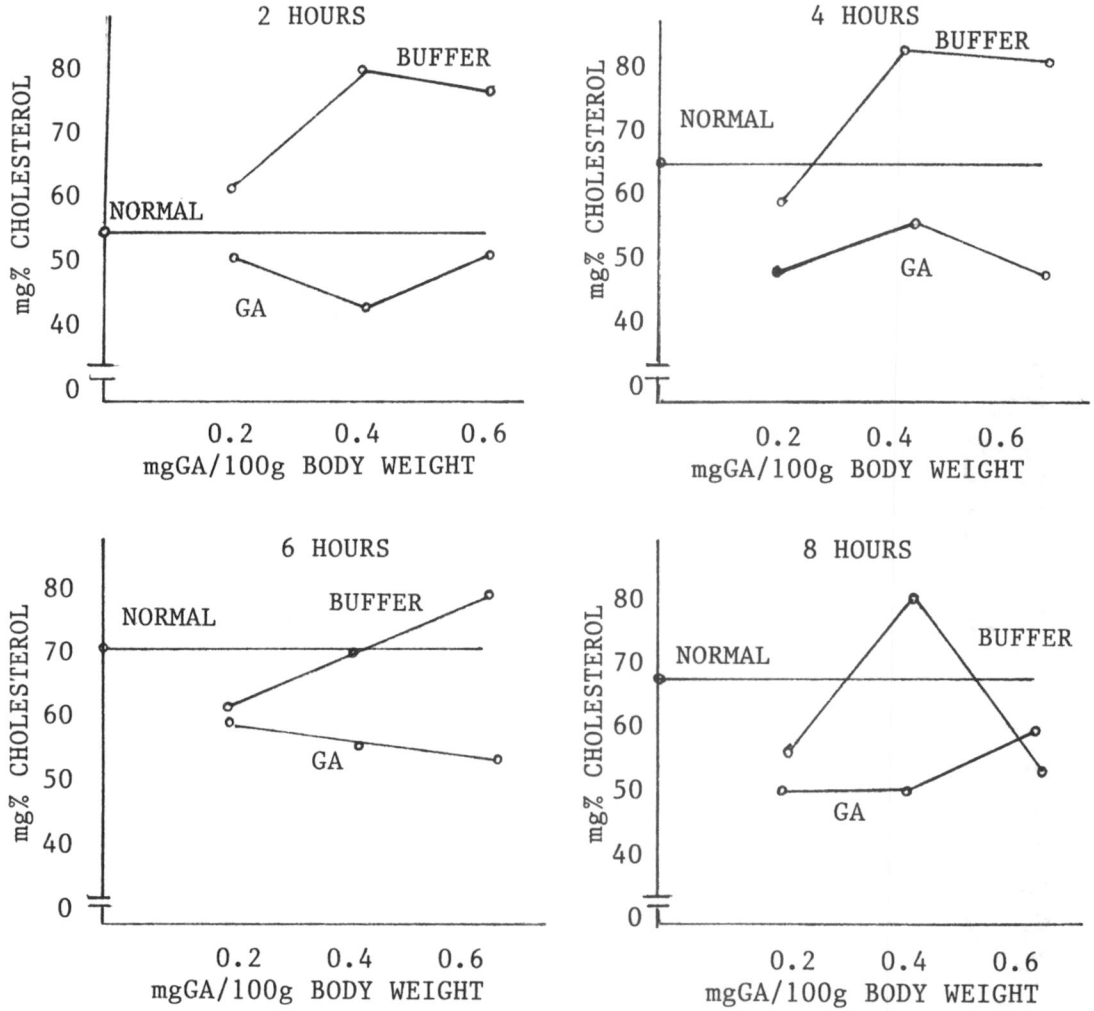
To

Treatment and Time

Fig. 5 shows the relation of serum cholesterol levels to the different treatments and different times. All of the points on the graphs are the average mg% cholesterol value from 3 rats. The times shown 2, 4, 6, and 8 hours are the intervals between injection of treatment solutions and bleeding.

FIGURE 5

CHANGES IN CHOLESTEROL LEVELS IN RELATION TO TREATMENT AND TIME



DISCUSSION

Since its discovery in the 1920's, GA has been studied extensively for its action on plant growth responses. Recent research emphasis has been on isolation, structural determinations and classifying the various gibberellins. Gibberellic acid is classed with auxins and kinins as a plant hormone; it produces its effect with small concentrations and affects certain areas, i.e. growth and development.

Another area of research with plant growth has been with retardants. These plant growth regulators will retard plant growth, and applications of plant hormones will generally reverse the effect of the retardant (33, 2). The traditional approach to research with plant growth regulators has been to treat plant material with the regulators and determine the growth response. The material is then treated with different hormones to determine which would reverse the action of the regulator.

Research I conducted with the semiaquatic angiosperm, P. palustris, while following the above protocol led me to consider the possibility that GA could be involved in sterol metabolism.

Plant and animal steroid biosynthesis are shown in Figures 2, 3, and 4. In both plants and animals biosynthesis of steroids is from acetate (Fig. 2). The main pathway (29) for steroid biosynthesis is through terpene additions and subsequent cyclization.

The common pathway is from acetate to mevalonate to the monoterpenes, isopentenyl and dimethylallyl. The two monoterpenes add head to tail to form the diterpene, geranyl. Geranyl adds head to tail with isopentenyl

to form the triterpene, farnesyl. Two farnesyl units add to form squalene which will cyclize into the standard steroid ring structure. From lanosterol, there are small changes as the different steroids are formed (Figs. 3 and 4).

Branches from the main pathway are for plant products. Squalene can cyclize in different manners and produce Beta-amyrin, euphol, or hydroxyhopanone. Farnesyl will add to isopentenyl to form geranylgeranyl which undergoes ring closure to form (-) kaurene. Kaurene can form either steviol or gibberellic acid.

The animal steroid pathway is a combination of androgens, estrogens, and corticosteroids (Fig. 3). Cholesterol is at the top, being the precursor for all 3 classes of steroids shown. The estrogen pathway represented is pregnenolone, progesterone, testosterone, estrone, and estriol. The androgen pathway represented is pregnenolone, hydroxypregnenolone, dehydroepiandosterone, androsterone, and testosterone. The pathway for the corticosteroids is pregnenolone, progesterone, deoxycorticosterone, corticosterone, aldosterone, hydroxyprogestrone, deoxycortisol, and cortisol.

The estrogens are the female sex hormones, the androgens are the male sex hormones, and the corticosteroids are the hormones produced by the adrenal cortex.

The plant steroid pathway (Fig. 4) shows that cholesterol is not the ultimate precursor for steroids (3, 4, 5, 6, 7, 18, 19, 21). In the plant, there is a split from desmosterol to form cholesterol and citrostadienol. From citrostadienol, the other branch from desmosterol, are found the traditional plant steroids, stigmasterol and sitosterol. Both stigmasterol and sitosterol lead to the production of pregnenolone.

From pregnenolone two different classes of steroids are produced, the cardenolides and the alkaloids. A third class of compounds, the sapogenins, are produced from cholesterol. The cardenolides are composed of digitoxigenin, digoxigenin, gitoxigenin; the alkaloids are represented by holaphyllamine and hollaphylline; the sapogenins shown are kryptogenin and diosgenin.

Also produced from cholesterol are the sterols ergosterol and ecdysterone. Ergosterol is converted to vitamin D_2 by UV radiation. Ecdysterone is a steroidal hormone that is active in insect moulting and is produced from cholesterol by insects.

It is also noticeable that there are no androgens or corticosteroids in the plant pathway. In reviewing the literature to construct the plant steroid pathway, I found no mention of any attempts to isolate either androgens or corticosteroids. (Many plant physiology textbooks cite the 1945 experiment of Love and Love with sex change in plants upon applications of animal estrogens and androgens.) The most noticeable difference between plant and animal steroid pathways is the split from desmosterol to cholesterol and citrostadienol in the plant system. The split was interpreted from the literature and with more research may be found to be a branch from cholesterol.

In the aminal system GA may mimic cholesterol, after some structural changes, and act as a feedback inhibitor. If GA replaced cholesterol, there would be a build up of precursors which would halt production and create a decrease in cholesterol. This possibility is supported by findings that ingestion of plant sterols will reduce the production of sterols in bees.

With all known enzymes being proteins, it is highly improbable that GA will be the exception. Gibberellic acid may function as part of a conjugated enzyme or as a cofactor. The possibility of GA acting in a position of a cofactor is supported in that the SK&F 7997 (Tris (2-dimethylaminoethyl) phosphate) reverses the effects of exogenous GA in seedlings and floral initiation prior to the photoinductive cycle (9). Both are suggestive of an induced enzyme system. Gibberellic acid also could serve as a cofactor to an enzyme that is rate limiting. By increasing the reaction rate of sterol metabolism, there would be a decrease in the level of cholesterol as the equilibrium values shifted.

Gibberellic acid has been shown to act as an androgen in its action on sex reversal. Gibberellic acid could function as an androgen precursor or an androgen-like hormone. In animals hormones are carried by blood proteins throughout the system. In plants, GA is carried through the vascular system bound to water soluble proteins (22).

SUMMARY

To test an hypothesis of GA function in steroid metabolism, the action of GA on serum cholesterol levels in white rats was studied. Cholesterol is the primary sterol in formation of estrogens, androgens and corticosteroids (Fig. 3). Changes in sterol metabolism should be reflected by changes in serum cholesterol levels.

Data gathered from this work support the hypothesis that GA can function through sterol metabolism. Serum cholesterol levels decreased upon injection of GA. The research was on an animal system and possible GA effects on plant steroid metabolism can only be speculated upon until research is completed on plant systems. Likewise, with the information available, we can only speculate how GA can cause a decrease in cholesterol.

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APPENDIX 1

ASSAY PROCEDURE FOR SERUM CHOLESTEROL

Determination of serum cholesterol levels were according to Gradwhals Clinical Laboratory Methods and Diagnosis, 7th edition.

REAGENTS:

ethylacetate:ethanol 1:1 (V:V)

0.005 M Fe Cl_3 in glacial acetic acid

1 mg cholesterol/ml ethylacetate:ethanol

conc. H_2SO_4

heparin

EXPERIMENTAL ASSAY:

1. Collect 1.5-2 ml blood from clipped tail at the correct time into a heparinized centrifuge tube.
2. Spin the blood at 8,000xG for 10 minutes in a refrigerated centrifuge.
3. Pipet 0.5 ml of the supernatan into 5.0 ml ethylacetate:ethanol.
4. Spin the ppt. protein at 8,000xG for 10 minutes in a refrigerated centrifuge.
5. Pipet 0.3 ml of the supernatant into a test tube.
6. Pipet 1.0 ml Fe Cl_3 into the test tube and mix.
7. Pipet 1.0 ml conc. H_2SO_4 into the test tube and mix.
8. Allow the color to develop and determine the OD at 550 nm.
9. Calculate the mg% cholesterol using the experimental OD values and a regression coefficient calculated from cholesterol standards.

STANDARDS ASSAY:

1. Pipet 0.0, 0.5, 1.0, 1.5, 2.0, and 2.5 ml of cholesterol standard soln. into test tubes and dilute to 5.0 ml with ethylacetate:ethanol.
2. Pipet 0.5 ml physiological saline to each tube and mix well.
3. Pipet 0.3 ml of each above soln. into each of 3 tubes.
4. Pipet 1.0 ml Fe Cl_3 into each tube and mix well.
5. Pipet 1.0 ml conc. H_2SO_4 into each tube and mix well.
6. Allow the color to develop and determine the optical density at 550 nm.
7. From the optical density values at the known cholesterol concentrations a regression coefficient is calculated and used to calculate a linear regression for use with experimental optical density values.

NOTE: The viscosity of the conc. H_2SO_4 required the use of an automatic test tube shaker to mix the reagents.

APPENDIX 2

REGRESSION COEFFICIENT: The assay standards were linear, sample calculations followed Beers Law, in absorbance. The OD values from triplicate standards were used to calculate a regression coefficient from the formula:

$$b = \frac{\Sigma XY - \frac{\Sigma X \Sigma Y}{N}}{\Sigma X^2 - \frac{(\Sigma X)^2}{N}}$$

EXAMPLE:

Cholesterol Standards (mg%)	OD VALUES		
	1	2	3
100	0.305	0.305	0.314
200	0.658	0.619	0.662
300	0.90	0.90	1.00
400	1.19	1.19	1.20
500	1.21	1.40	1.43

$$b = \frac{4772.20 - 3984.9}{13.900 - 11.762} = 368{,}241$$

$N=15$ $\quad Y=4500$ $\quad \overline{Y}=300$ $\quad \overline{X}=0.886$

$\Sigma X=13.283$ $\quad \Sigma X^2=13.900$ $\quad \frac{(\Sigma X)^2}{N} = 11.762$ $\quad \Sigma XY=4772.20$

$\frac{\Sigma X \Sigma Y}{N}=3984.9$

LINEAR REGRESSION: Using the calculated regression coefficient, b, the mean from duplicate experimental OD values were used to convert from OD to mg%. The following formula was used:

$$Yp = \overline{Y} + b\ (X-\overline{X})$$

$$Yp = 300 + 368.241\ (X-0.886)$$

EXAMPLE:

EXPERIMENTAL OD		MEAN	mg% CHOLESTEROL
1	2		
0.180	0.181	0.180	40.02

$$Yp = 300 + 368.241\ (0.180-0.886) = 40.02$$

ANALYSIS OF VARIANCE: When the OD values had been converted to mg% they were placed in table form. (Table 2 is the table containing the calculated values, the values are given as the mean of triplicate experiments.) Cholesterol values from the table were used to construct an analysis of variance table. The analysis of variance table is found in the Results section.

EXAMPLE: Normal = Buffer (partitioned section)

$$\frac{(X_N)^2}{N_N} + \frac{(X_{B_1} + X_{B_2} + X_{B_3})^2}{N_N} - \frac{(X_N + X_{B_T})^2}{N_T} =$$

$$\left(\frac{(985.47)^2}{15}\right) + \left(\frac{961.89 + 1116.60 + 1101.47)^2}{45}\right) - \left(\frac{(985.47 + 961.89 + 1116.60 + 1101.47)^2}{6}\right) =$$

$$64743.41 + 224714.35 - 289180.12 = 277.64$$

SOURCE	df	SS	MS	F
Normal = Buffer	1	277.64	277.64	NS
ERROR	70	32734.77	467.64	