EFFECTS OF RAT EPIDERMAL CHALONE ON UPTAKE OF THYMIDINE, URIDINE, SORBITOL AND WATER BY RAT EPIDERMIS

A Thesis

Presented to

the Faculty of the College of Pharmacy

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Christopher Galileo Russo

May 1974

ACKNOWLEDGMENTS

It is my pleasure to express my sincere thanks and deepest gratitude to my Thesis Committee Chairman and good friend, Dr. Michael B. Cramer and to my committee members for their help and guidance with this project.

I am, additionally, grateful to Mr. Clarence H. Cook, Jr. for his encouragement and cooperation throughout this study.

A special thanks is extended to the Neurochemistry and Addiction Section of the Veterans Administration Hospital, Houston, Texas, and especially to Dr. Virginia E. Davis and Mrs. Barbara McLaughlin for their advice and assistance with the scintillation counting.

Also, thanks are extended to Dr. Lindley A. Cates, University of Houston, College of Pharmacy, for allowing me to use certain instrumentation from his laboratory.

Finally, and most importantly, I am especially grateful for the support and encouragement given by my wife, Jean.

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ABSTRACT

A water soluble, endogenous substance (chalone) has been isolated from animal tissues in the past decade which has been shown to have mitotic depressant properties. This substance is further claimed to be tissue specific but not species specific. The present investigation was conducted to determine the effect of chalone on epidermal uptake of tritiated thymidine and tritiated uridine as an indirect index of DNA and RNA synthesis respectively. Additionally, studies were conducted to determine the effect of chalone, if any, on the extracellular fluid volume and cell membrane permeability using sorbitol ^{14}C and tritiated water respectively.

A chalone solution was prepared from rat epidermis by homogenization in aqueous buffer, pH 7.0. A portion of this solution was heated in an attempt to inactivate the chalone. Slices of epidermis taken by dermatome sectioning were incubated with either active chalone extract, heated chalone extract, or Bullough's Solution. In the thymidine and uridine uptake studies, epidermal sections were also treated with known inhibitors of DNA and RNA synthesis, cytosine arabinoside and actinomycin D respectively. These sections so treated were incubated for 15, 30, 45, 60, 90 and 120 minutes in each of the studies.

After incubation, the epidermal slices were solubilized for counting in a liquid scintillation counter, and results were expressed both as disintegrations per minute per milligram of wet tissue weight and disintegrations per minute per milligram of protein.

The results showed that chalone treated epidermis had less tritiated thymidine and uridine uptake at all incubation time periods than did epidermis treated with the Bullough's Solution, suggesting that chalone reduced mitosis by way of depressed nucleic acid synthesis. The chalone appeared to have no effect on the extracellular space or cell membrane permeability as there were no significant differences in sorbitol ^{14}C or tritiated water uptake at any of the incubation time periods.

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Chapter 1

INTRODUCTION

In the past few years a substance has been extracted from certain tissues which is claimed to have mitotic-depressant properties. The substance, named chalone, has been found to probably be tissue specific (1) but not species specific (2); thus, a chalone extracted from the epidermis of one vertebrate can be shown to depress mitosis in the same tissue of some other vertebrate, but not in other tissues. Mitotic inhibitors with the same general characteristics have been described for the lung (3), kidney (4), granulocytes (5), erythrocytes (6), lymphocytes (7), sebaceous glands (7) and liver (7).

A negative feedback system is the most popular explanation for the mechanism of chalone action. It has been proposed that the presence of chalone within the cell acts to restrict mitosis and only through its loss is cell proliferation facilitated (8).

With specific regard to the epidermal chalone, the factor has been found to be water soluble, thermolabile and non-dialyzable (9). Chemically, the active component has been shown to have a molecular weight of about 30,000-40,000 and to be a protein or glycoprotein (10). Additionally, it is speculated that hormonal cofactors, i.e., epinephrine and glucocorticoids, appear to be associated with chalone activity (1, 11, 12).

The primary purpose of this study was to compare the effect of epidermal chalone on the uptake of tritiated thymidine and of tritiated uridine by epidermal tissue as indicators of DNA and RNA synthesis respectively. Studies were also conducted to determine whether chalone might affect uptake of nucleotides by altering cell permeability or extracellular space. It is hoped that the results of this research will provide a base upon which to conduct further studies on the role of chalone in chemical carcinogenesis.

Chapter 2

LITERATURE SURVEY

The question as to what initiates the stimulus for cell proliferation has long been a fertile field of study. Recent thinking has changed from the concept of a stimulatory substance to one of natural restraints which block mitotic activity. It has also been proposed that three types of cells exist; those that are incapable of division, those that are stable and undergo mitosis only under suitable circumstances and those that are labile and continuously divide. Another concept is that of contact inhibition. With this view, cellular proliferation proceeds until cellular margins come into contact, then growth stops, and differentiation begins (13).

However, this study follows the proposal of Weiss who contends that a repressor substance exists within cells which when lost, as through injury, permits mitosis (13). As early as 1937, there were reports of suspected endogenous repressor substances. Simms and Stillman (14), working with trypsin extracts of sheep, dog and chicken aortas, found that the growth of fresh aorta tissue was depressed when treated with these thermolabile, non-dialyzable extracts. In 1956 it was found that liver and kidney macerates inhibit mitosis in rats following partial hepatectomy and nephrectomy (15). These macerates were also thermolabile and non-dialyzable; however, freezing followed by thawing and lyophylization did not inactivate the inhibitor.

Bullough (16), in an extensive treatise, referred to the repressor substance as "chalone". He took the position that cells prepare for mitosis unless diverted to differentiation by the mitotic inhibitor. Mitosis, he proposed, could be stimulated by decreased chalone concentration or chalone inactivation by hormones such as estrogens and androgens. However, certain hormones such as glucocorticoids and epinephrine are said, by Bullough, to act synergistically with the chalone. Further, he speculated that the substance was tissue specific and was produced by the cells upon which it acted. Evidence cited for his hypothesis was based on studies from wound healing, tissue regeneration and tissue extracts (both stimulatory and inhibitory). As confirmation of this concept, Iversen et al. (17) reproduced Bullough's *in vivo* work.

Other substances with activities similar to chalone have been isolated in the granulocytes (5), lung (3), erythrocytes (6), lymphocytes (7), sebaceous glands (7), kidney (4), and liver (7).

HORMONAL INFLUENCES

There have been two different influences recognized which may be imposed upon the action of chalone. Estrogens and androgens act as mitotic stimulating hormones, and thus, may operate to neutralize the effect of chalones (18). Conversely, epinephrine and hydrocortisone appear to favorably influence the mitotic inhibitory effects of chalone. Bullough and Laurence proposed that epinephrine augments the action of chalone by forming a chalone-epinephrine complex. Test tissues seem to

possess enough endogenous epinephrine for the first five hours of incubation. After that period, epinephrine washes are required to maintain the inhibitory activity of the chalone. It has also been suggested that there normally exists a chalone antagonist and that epinephrine inhibits this compound rather than assisting the chalone itself (10). It is proposed that hydrocortisone prevents the loss of epinephrine from the epidermal tissue by affecting cell permeability (18). It has also been suggested that hydrocortisone suppresses anti-chalone production (20).

On the other hand, it has been demonstrated that adrenalectomized animals respond as well to chalone as normal animals (18). Also, recent work indicates that epinephrine and other catecholamines stimulate the formation of cyclic adenosine-3',5'-monophosphate (cyclic AMP) which is followed by decreased mitotic rate with increased concentration, suggesting that epinephrine may exert mitotic inhibiting activity itself (18).

MECHANISM OF ACTION

The most popular explanation for the chalone mechanism of action has been that a negative feedback system is involved. Thus, the presence of chalone within the cell acts to restrict mitosis, but its loss, as through injury, facilitates cell proliferation (8). It has been further proposed that the negative feedback mechanism is exerted on the basal cell population (16). The research of Wiley et al. (21) supports the concept of a negative feedback system. They found that the

transient inhibition was followed by a recovery phase with augmented DNA synthesis. The recovery phase with increased activity was suggestive of negative feedback systems.

Most all mechanism of action proposals have associated the chalone activity with DNA synthesis. The question as to whether RNA synthesis is affected by chalone has not been totally avoided. Studies which compared chalone influence on DNA against RNA found DNA synthesis to be more strongly inhibited. Kiger and co-workers (22) compared DNA, RNA and protein synthesis in human lymphocyte tissue cultures using spleen chalone. Earlier work revealed that the spleen, a peripheral lymphoid organ, contained the lymphocytic chalone. The results, they claimed, indicated that DNA synthesis (measured by incorporation of tritiated thymidine) was inhibited to a greater extent than RNA synthesis (measured by tritiated uridine uptake) or protein synthesis (measured by the incorporation of radioactively labeled amino acids).

CELL CYCLE

The cell completes four phases in the production of its non-gamete daughter cells. The first three phases are preparatory to the actual division and are described as: (a) G_1 gap phase in which RNA's and proteins are synthesized in preparation for the onset of DNA synthesis, (b) S phase in which DNA replication occurs, and (c) G_2 gap phase in which RNA's and proteins are made in preparation for mitosis (18). These

are then followed by division, that is mitosis, which is further divided into prophase, metaphase, anaphase and telophase.[1]

It is in the preparatory phases that chalone is thought to exert its influence. Two chalone factors have been described (23): the M factor with epinephrine which inhibits mitosis at the G_2 gap phase, and the S factor which inhibits DNA snythesis in the late G_1 gap phase. Utilizing gel chromatographic techniques, Marks (23) speculated that the molecular weight of the M factor was 30,000-40,000 Dalton while that of the S factor was estimated at 100,000 Dalton. He also found the M factor to be destroyed by heat and proteolytic digestion, whereas, the S factor appeared resistent to neutralization by these methods.

Elgjo and Edgehill (24) found that by separating the basal cell layer from that of the differentiating cells they were also able to produce the two factor effect. The G_2 gap phase effect was found in the basal cell layer, whereas, the G_1 gap phase was associated with the differentiating cells.

Baden and Sviokla (25), however, were unable to elicit the G_1 gap phase effect. Utilizing whole tissue extract with epinephrine against a control of buffer solution, they tagged the samples with tritiated thymidine but failed to remove the isotope unassociated with the nucleic acid. This may have led to an erroneous conclusion, "that DNA synthesis is not the site of mitotic inhibition".

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[1]For a schematic of the cell cycle, see Appendix 3.

SPECIFICITY OF ACTIVITY

As previously stated, the chalone seems to be tissue specific but not species specific (16). Thus, the extract from the epidermis of one vertebrate can be shown to inhibit mitosis in the epidermis of some other vertebrate. Regarding tissue specificity, the granulocyte, erythrocyte, and lymphocyte chalones seem to be completely tissue specific (26). The epidermal chalone is probably tissue specific; however, there is evidence that it may inhibit other squamous epithelia, such as corneal epithelium (26). As for its lack of species specificity, the epidermal, granulocylic, erythrocytic and lymphocytic chalones all exhibit this property (2, 26). It has been shown that epidermal extracts of pigs, codfish, bats and man inhibited the mitotic rate in mouse epidermis when tested *in vivo* and *in vitro* (2).

BIOCHEMISTRY

Biochemical studies have produced some limited information regarding the nature of chalone. Attempts at further characterization have been largely impeded by the inability to isolate a purified chalone compound. The substance was found by Bullough (9), to be soluble both in distilled water and in saline and one unit of chalone was arbitrarily defined as "the activity contained in an aqueous extract of one milligram dry weight of mouse epidermis (including stratum corneum)". At the same time, he found the substance to be non-dialyzable (using a cellophane membrane) and heat labile. At 100 degrees centigrade for ten minutes,

one unit of chalone was found to completely neutralized. However, as mentioned earlier, the heat lability may pertain solely to the G_2 gap phase inhibiting factor. Alcohol precipitation revealed that the 70-80 per cent fraction produced the greatest depression (44%); second only to the whole water extract which produced 46 per cent depression.

The work of Marrs and Voorhees (27) in 1971 confirmed the above mentioned findings of Bullough. In addition, they found the chalone extract to be inactivated by trypsin. Prior to trypsinization the extract produced a 77 per cent depression in mitosis. Following the treatment only 16 per cent depression could be elicited.

In 1968, the chemical nature of the chalone was elaborated utilizing purified samples obtained by water extraction and ethanol fractionation followed by column electrophoresis. At that time, Hondius-Bolding and Laurence (10) found the epidermal chalone to be a glycoprotein with a molecular weight of approximately 30,000-40,000 Daltons containing 44.48 per cent carbon, 29.85 per cent oxygen, 13.22 per cent nitrogen, 6.8 per cent hydrogen, and 1.91 per cent ash. Sixty-three per cent of the compound consisted of amino acids. The remaining 37 per cent was accounted for as carbohydrate and water; 15 per cent being glucose and to a lesser extent galactose, fucose and galactosamine. Water made up the remaining 20 per cent.

Regarding the question of stability, the aqueous solution was found to be stable for several days at zero degrees centigrade and several weeks at minus twenty degrees centigrade (9). It was later added by Elgjo (28) that extracts rapidly lose their inhibiting

capacity at thirty-seven degrees centigrade. Extracts at this temperature for thirty minutes had no effect when tested in vivo. Chalone was also found to be most stable at a pH between three and six and inactivated in alkaline solutions (10).

THERAPEUTIC IMPLICATIONS

CANCER:

A possible explanation for the overgrowth of tissue and increased post-mitotic aging may be a reduction in intracellular chalone. This reduction may be absolute as in the case of inadequate chalone synthesis or increased cellular loss as a result of membrane permeability. The reduction may also be functional if the tumor cells are unable to respond to the chalone (7, 29).

As support for depressed response to chalone by tumor cells, Bullough and co-workers (29) produced a 62 per cent reduction in the Vx2 epidermal tumor by using normal tissue chalone in concentrations, up to 5000 units (one unit equals the amount dissolved in four milliliters of saline necessary to depress mitosis 50 per cent). This reaction seemed to depend also on the presence of epinephrine and hydrocortisone since results similar (53 per cent depression) to the above in vivo response could only be elicited in vitro after the addition of the hormones. In vivo, the stress of cancer may produce high concentrations of these hormones (29).

The proposal that there is inadequate chalone synthesis is refuted by an experiment in which extracts made from tumor cells

depressed mitosis in normal epidermis by 47 per cent (29). This was three per cent more than the depression produced by the normal tissue extracts.

As support for the increased membrane permeability proposal, Rytomaa and Kiviniemi (5) found that chloroleukemic cells contain less than one-tenth the chalone of normal granulocytes with an increased amount in the blood. Related to this, Bullough and Laurence (29) believe that if the afflicted organism survives long enough, the blood chalone level will increase to the point of inhibiting the tumor. This is indirectly supported by Schatten (39), perhaps idealistically, who claims that the removal of one tumor, a source of chalone, stimulated the proliferation of others.

Other work lending optimistic results has been conducted on epithelioma and melanoma. Work done on a keratizing hamster epithelioma produced a 34 per cent difference between an epidermal extract group and a liver extract group (30). Mohr et al. (31), working with Harding-Possey transplanted melanoma, found in 275 cases, all tumors treated with chalone regressed completely. There were no spontaneous regressions in the controls.

Some are not so optimistic about the usefulness of chalones in cancer. Iversen (32), in his review on the subject, commences by saying that transplantable tumors, as those in the instances above, probably do not regress as a result of the chalone but rather through some mechanism mediated through the vasculature. "Tumors," he continues,

"are not always characterized by a rapid rate of cell proliferation; in fact, many tumor cell populations have a lower rate of proliferation than that of the tissue of origin." Thus, a growth inhibitor such as chalone would inhibit the normal population before the malignant cells.

Iversen (17) does not, however, rule out the idea of chalone being effective in cancer therapy. He believes this can be determined only after chalone is purified and satisfactorily characterized. Bullough (7), in a similar vein, proposes a secondary role for chalones in the treatment of cancer. In this way, chalone would arrest cancer growth, however, eradication would be brought about by some other mechanism such as the immune system.

PSORIASIS:

Studies done with psoriatic tissue have clouded the situation even more. Chopra and Flaxman (33) found that normal extracts inhibited psoriatic tissue from 44-66 per cent depending on concentration. However, they introduced the concept of a balance between the inhibitor and a stimulator. Unpublished evidence for this is based on experiments with serum obtained from psoriatic patients said to contain the stimulator. With the serum, they were able to both stimulate mitosis and block the effect of chalone treated tissue.

It has also been found that psoriatic tissue extracts possess mitotic inhibiting properties based on depressed tritiated thymidine uptake (34). A one per cent concentration was found to allow only 4 to 27 per cent isotope uptake; whereas, a one per cent concentration

of normal tissue extract allowed 34 to 61 per cent uptake. This increased activity by the psoriatic tissue extracts may be explained by the increased number of malpighian cells which, according to Bullough et al. (2), are said to produce the chalone.

Chapter 3

METHODOLOGY

GENERAL PROCEDURES:

ANIMALS:

All animals used for this study were male, Sprague-Dawley rats weighing 150-175 grams at the beginning of the study. The animals were maintained in air conditioned quarters at the University of Houston College of Pharmacy, Houston, Texas, on a diet of Purina Laboratory Chow and water to which they had free access.

CHEMICALS:

Data on the radiolabelled chemicals used are summarized in Table 1. The following chemicals were purchased from Sigma Chemical Company, St. Louis, Mo. 63178: Cytosine arabinoside (Lot: 61C-0560), actinomycin D (Lot: 42C-0691-9), and bovine serum albumin (Lot: 33C-8160). NCS Tissue Solubilizer (Lot: A-425) was purchased from Amersham/Searle Corporation, Arlington Heights, Ill. 60005. The Phenol Reagent (Lot: 731397) used in protein determinations was purchased from Fisher Scientific Corporation, Fair Lawn, New Jersey 07410. The depilatory, Nair^{R} (Carter Products, a division of Carter-Wallace, Inc., Cranbury, New Jersey 08512), was obtained at a retail outlet. The scintillation cocktail[1] was donated by the Veterans Administration Hospital, Houston,

[1]For composition of the Scintillation Cocktail, see Appendix 1.

Table 1

Specific Activities and Sources of Labelled Chemicals

Chemical	Specific Activity	Lot	Source
Thymidine (Methyl-^{3}H)[a]	24 C/m mole	YR2573	Schwarz/Mann, Orangburg, N.Y. 10962
Uridine-5-^{3}H[b]	26.1 C/m mole	78F-04F	New England Nuclear, Boston, Mass. 02118
D-Sorbitol-^{14}C-UL[c]	185 mC/m mole	628971	International Chemical and Nuclear Corp.,Irvine, California 92664
$^{3}H_2O$ Biological Grade[d]	100 mC/ml	584364	International Chemical and Nuclear Corp.,Irvine, California 92664

[a]Referred to in the text as tritiated thymidine.
[b]Referred to in the text as tritiated uridine.
[c]Referred to in the text as sorbitol ^{14}C.
[d]Referred to in the text as tritiated water.

Texas. All other chemicals used in the study were reagent grade, available from the College of Pharmacy stockroom.

CHALONE EXTRACTION:

Fourteen animals were anesthesized with ether. Their trunks were shaved and depilated with Nair^{R} for approximately three minutes. After rinsing in distilled water to remove the depilatory and hair, skin from each animal was rapidly excised and placed in cold normal saline. The animals were then sacrificed by ether inhalation. A scraping procedure was performed with razor blades on the underside of the skins to remove the dermis. The remaining epidermis (approximately 150 grams) was minced with scissors and placed in Bulloughs Solution (5 ml/Gm of tissue)[1]. This preparation was then homogenized using a Tekmar Homogenizer, Model SDT (Tekmar Co., P.O. Box 37202, Cincinnati, Ohio 45222) for twenty minutes. Homogenization rendered a pink suspension which was centrifuged using an International Equipment Company Refrigerated Centrifuge, Model B-20 (International Equipment Company, 300 Second Ave., Needham Heights, Mass. 02194) at 12,000 rpm (20,000g's) and minus four degrees centigrade for fifty minutes. The pink supernate, hereafter referred to as the chalone extract, was then filtered and frozen at minus ten degrees centigrade.

EFFECTS OF CHALONE ON UPTAKE OF TRITIATED THYMIDINE BY EPIDERMIS:

Three animals were anesthesized with ether. Their dorsal sides were shaved and depilated for ten minutes with Nair^{R}. The treated areas

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[1]For composition of Bullough's Solution, see Appendix 1.

of skin were removed and spread with the corneum side up on a padded board. The animals were sacrificed by ether inhalation. Slices of epidermis, approximately 400 microns thick, were taken using a Davol/ Seimon dermatome Model 3291 (Davol Inc., Providence, R.I. 02901). The first section, taken from the exterior surface of the skin and containing primarily stratum corneum, was discarded. The second section was used to prepare the test samples. To assess the effects of chalone on DNA synthesis, these sections were stored in cold Bullough's Solution until added to incubation flasks. Four groups of flasks were prepared, each containing a 15-30 mg portion of skin. One group contained, as the incubation medium, 2.5 ml chalone extract previously prepared. The second group of flasks contained 2.5 ml of chalone extract which had been heated at 100 degrees centigrade for thirty minutes. The third group of flasks contained 2.5 ml Bullough's Solution and the fourth group of flasks contained cytosine arabinoside, an inhibitor of DNA synthesis (35) at 25 mcg per ml in 2.5 ml Bullough's Solution. After addition to each flask of five micro C thymidine-methyl^{3}H in 0.1 ml of Bullough's Solution, skin samples from each of three rats were incubated separately in each medium at 37 degrees centigrade under 95 per cent oxygen and five per cent carbon dioxide for 15, 30, 45, 60, 90, or 120 minutes.

After incubation, the samples were immediately removed from the solution and blotted dry. Each sample, including a blank (a portion of skin not incubated with radioactivity), was then placed in a test tube with three ml of cold, six per cent perchloric acid,

shaken, and left overnight at zero degrees centigrade. On the following day, the samples were rinsed in three ml of fresh, cold, six per cent perchloric acid for a period of one hour. This was done twice. The purpose of the perchloric acid rinses was to remove low molecular weight tritiated substances, insuring virtually all the remaining activity was in DNA. Following this, each sample was rinsed once in normal saline, blotted dry and placed in a counting vial with one ml of NCS Tissue Solubilizer until dissolved. The dissolution required approximately 14 hours when conducted in a water bath at 50 degrees centigrade.

Fifteen ml of a scintillation cocktail was added to each sample, which was then stored in the dark at four to five degrees centigrade for 48 hours. After this storage, the samples were counted for ten minutes in a Packard Tri-Carb liquid scintillation spectrometer (Model 3390), Packard Instrument Co., Inc., Downers Grove, Ill. 60515, set for tritium and whose efficiency had been determined by external standardization.

EFFECT OF CHALONE ON UPTAKE OF TRITIATED URIDINE BY EPIDERMIS:

To determine the effects of chalone on RNA synthesis, the same procedure was followed as in the previous experiment, with the following exceptions: (a) uridine-5^{3}H (five micro C per flask) was used in place of thymidine-methyl^{3}H and (b) actinomycin D, an inhibitor of DNA-directed RNA synthesis (36), was used at five mcg/ml in place of cytosine arabinoside.

EFFECT OF CHALONE ON UPTAKE OF SORBITOL-^{14}C BY EPIDERMIS:

To determine whether or not differences in nucleotide uptake could be attributed to alteration in extracellular fluid, three animals were prepared as in the two previous experiments and the same incubation was followed. However, in this study only three incubation media were employed: chalone extract, heated chalone extract and Bullough's Solution. All samples were incubated with D-Sorbitol-^{14}C-UL, 0.5 micro C per flask. The previous procedure was followed except that the perchloric acid rinses were eliminated. Nine additional samples (three of active chalone, three of heated chalone and three of Bullough's Solution) were incubated with tritiated thymidine as before for 90 minutes as a control for chalone stability over time.

EFFECT OF CHALONE ON UPTAKE OF TRITIATED WATER BY EPIDERMIS:

This experiment was conducted in the same manner as was the sorbitol ^{14}C study except that tritiated water (two micro C/ml) was added to each of the flasks instead of sorbitol ^{14}C. Nine thymidine controls were again carried through the 90 minute incubation.

PROTEIN DETERMINATION:

A procedure similar to that outlined by Lowry and co-workers (40) was followed to determine the epidermal protein content in the twelve animals used in the previously mentioned experiments.

Three samples of epidermis were taken from each animal's skin, accurately weighed and placed in a test tube with five ml of 0.5 N potassium hydroxide. Three standards of bovine serum albumin in five ml of 0.5 N potassium hydroxide (0.5, 1.0, 1.5 mg/ml) and a blank of 0.5 N potassium hydroxide were prepared and run through the entire procedure. These samples were then dissolved in a heated water bath. A 200 micro l aliquot was taken from each sample solution and transferred to a test tube with 0.5 ml of 0.5 N potassium hydroxide. Five ml of Reagent A[1] was added to each. The solutions were well mixed and allowed to stand for twenty minutes at room temperature. Then 0.5 ml of Reagent B[2] was added to each with rapid stirring. Samples were allowed to stand at room temperature for forty minutes to insure complete color development. Each sample was then centrifuged to remove a white precipitate that resulted. The samples were then read against a blank at 500 nm in a Beckman DB-GT grating spectrophotometer, Beckman Instruments, Inc., Fillerton, Calif. 92634, (Tungsten lamp-slit program controls).

All data were initially analyzed using the Balanova Analysis of Variance[3] to compare the treatments at each incubation time period. For each time point for which the results of this test indicated the existence of a significant ($p<.05$) difference, each pair of means was compared using Student's t-Test. Any pair of means for which $p<.05$ was taken as statistically significant.

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[1]For composition of Reagent A, see Appendix 1.

[2]For composition of Reagent B, see Appendix 1.

[3]For explanation of Balanova Analysis of Variance, see Appendix 2.

Chapter 4

RESULTS

UPTAKE OF TRITIATED THYMIDINE:

Samples were incubated with tritiated thymidine and incorporation into non-diffusible perchloric acid-insoluble substances was used as an index of DNA synthesis. The results expressed as disintegrations per minute per milligram of wet tissue weight (Figure 1) indicated that at all incubation times the epidermis incubated with the chalone extract had a reduced tritiated thymidine uptake when compared to controls. A decrease in uptake was also observed when active chalone extract was compared with heated chalone extract, one exception being at the 30 minute incubation time period. Only at the 15 minute incubation time did the epidermis incubated with chalone extract have less uptake than did the epidermis incubated with a known inhibitor of DNA synthesis, cytosine arabinoside. Statistically significant differences ($p<.05$) were observed in the 45, 60, 90 and 120 minute incubation time periods.

At the 45 minute incubation period, a statistically significant difference was found between the control group and the cytosine arabinoside group. At the 60 minute period, there was a significant difference between epidermis incubated with active chalone and control (Bullough's Solution) and between epidermis incubated with cytosine arabinoside and control. At the 120 minute incubation period, there was a significant difference between active chalone and control and between cytosine arabinoside and control preparations.

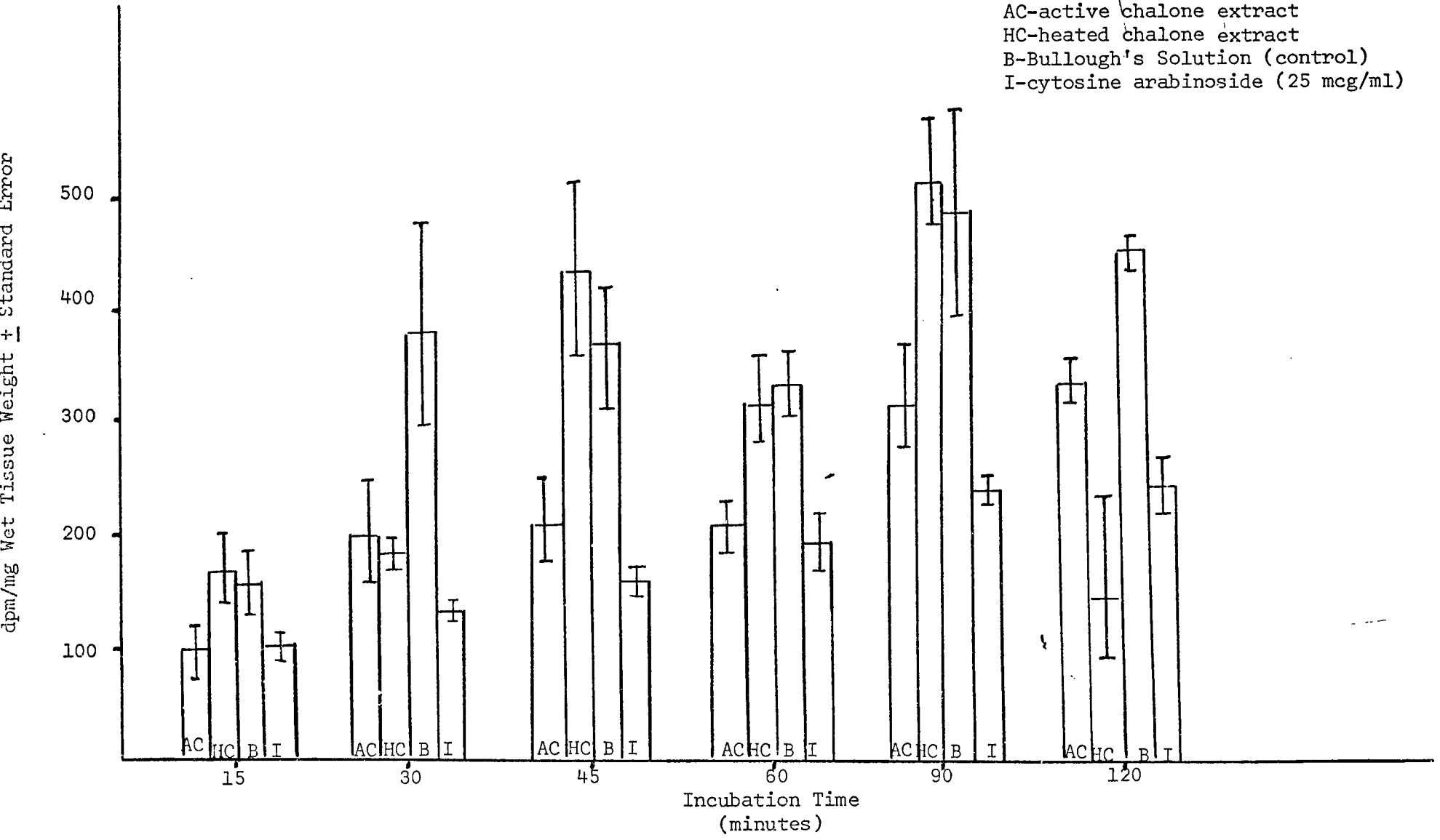

Figure 1. Effect of Chalone on Uptake of Tritiated Thymidine by Rat Epidermal Slices (mean dpm/mg of wet tissue weight ± standard error)[a]

[a]Each mean is based upon epidermal sections from three animals.

Figure 2 graphically represents the per cent depression of thymidine uptake comparing chalone to control and cytosine arabinoside to control.

When the results were expressed as disintegrations per minute per milligram of tissue protein (Figure 3), all epidermal samples incubated with active chalone extract again had depressed tritiated thymidine uptake compared to those incubated with either control or heated chalone extract. In two instances, at the 15 and 60 minute incubation time periods, the active chalone preparation had less uptake than did epidermis treated with cytosine arabinoside; however, these comparisons were not statistically significant. The four groups were found to have statistically significant differences at the 90 and 120 minute incubation time periods. At the 90 minute incubation period, there was a significant difference between cytosine arabinoside and control. At the 120 minute period, there was a significant difference between active chalone and heated chalone, and between cytosine arabinoside and control.

A graphic representation of the per cent depression when thymidine uptake of chalone was compared to control and when cytosine arabinoside was compared to control is depicted in Figure 4.

UPTAKE OF TRITIATED URIDINE:

To determine the effects of chalone on RNA synthesis, samples were incubated with tritiated uridine. The results expressed as

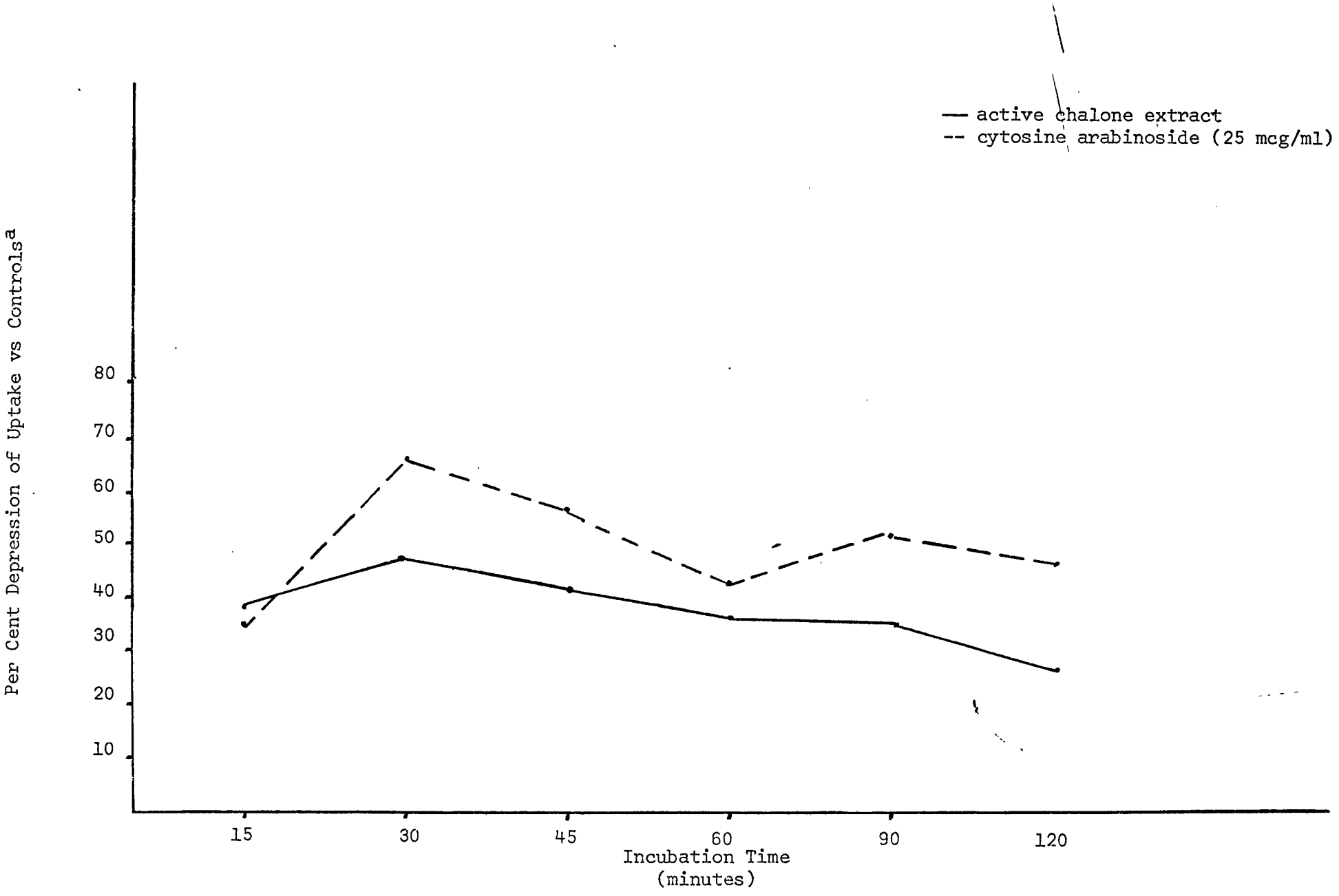

Figure 2. Depression of Tritiated Thymidine Uptake in Rat Epidermal Slices by Chalone and Cytosine Arabinoside (based on dpm/mg of wet tissue weight)

[a]Controls represented zero depression.

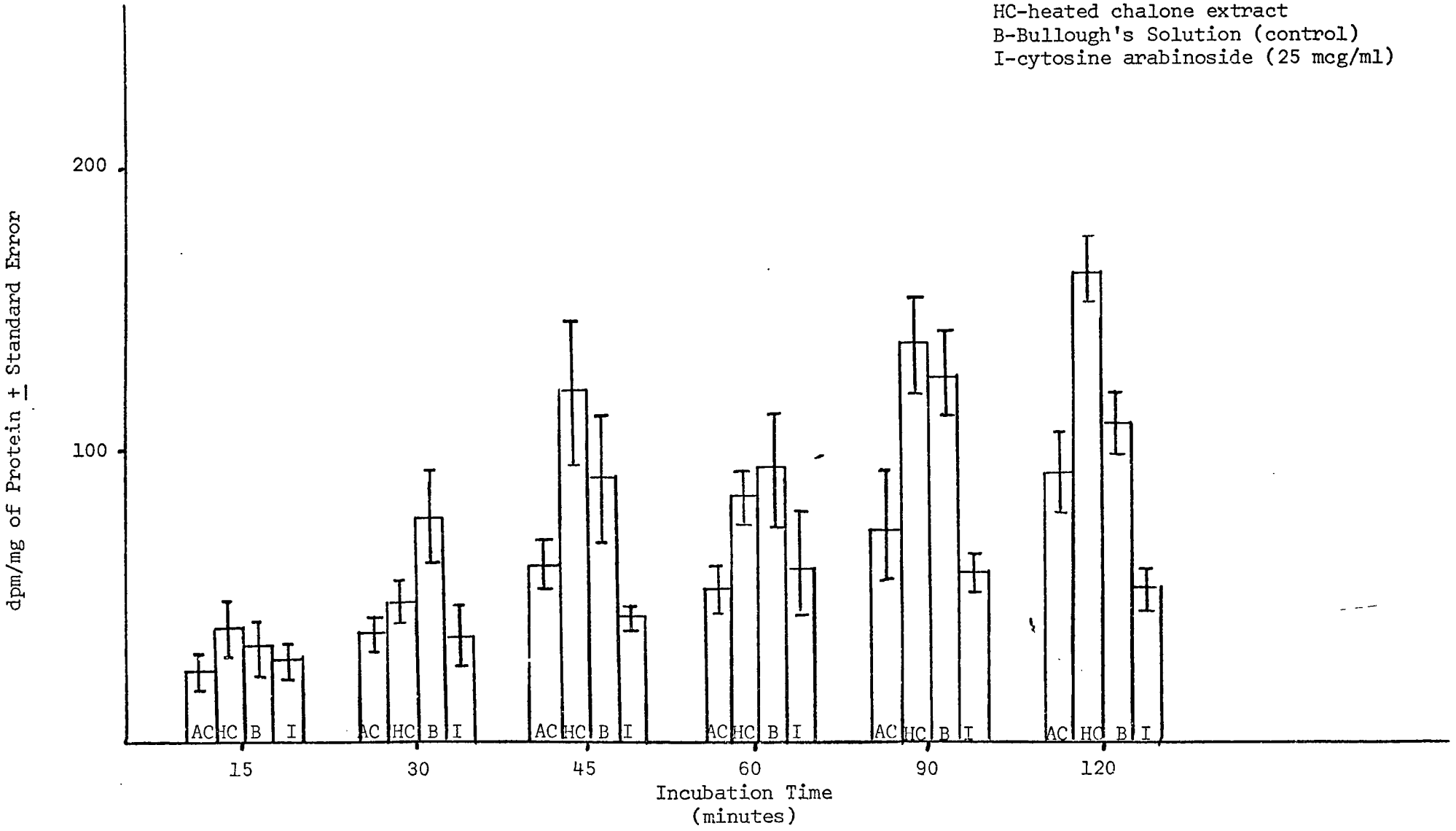

Figure 3. Effect of Chalone on Uptake of Tritiated Thymidine by Rat Epidermal Slices (mean dpm/mg of epidermal protein ± standard error)[a]

[a]Each mean is based upon epidermal sections from three animals.

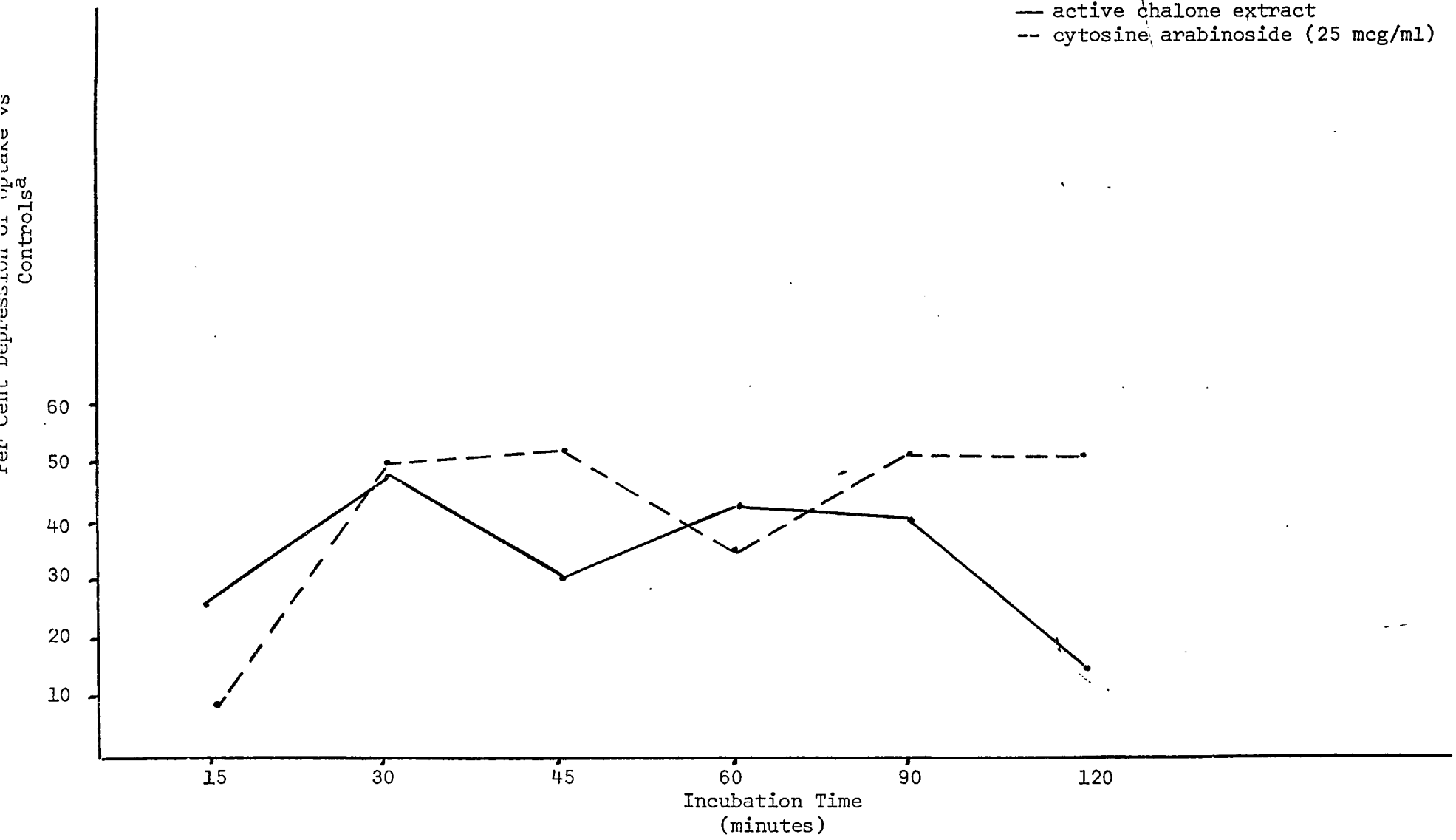

Figure 4. Depression of Tritiated Thymidine Uptake in Rat Epidermal Slices by Chalone and Cytosine Arabinoside (based on dpm/mg of epidermal protein)

[a]Controls represented zero depression.

disintegrations per minute per milligram of wet tissue weight (Figure 5) indicated that incubation with chalone resulted in less uptake of the radioactive nucleotide than incubation with either Bullough's Solution (control) or a known RNA synthesis inhibitor, actinomycin D. The Balanova Analysis of Variance revealed a statistical significance between the four treatment groups at the 45, 60, 90 and 120 minute incubation time periods. At the 45 minute incubation period, there was a significant difference between active chalone and control and between heated chalone and control. At the 60 minute period, there was a significant difference between active chalone and control and between heated chalone and control. At the 90 minute incubation there was a significant difference between the results of the following treatments: active chalone and control, heated chalone and control, and actinomycin D and control. At the 120 minute incubation period, there was a significant difference between active chalone and control and between heated chalone and control.

A graphic representation of the per cent depression in uridine uptake of chalone compared to control and actinomycin D compared to control is depicted in Figure 6.

When the results were expressed as disintegrations per minute per milligram of protein (Figure 7), the epidermis incubated with active chalone again had less tritiated uridine uptake than either epidermis incubated with control or actinomycin D. A Balanova Analysis of Variance indicated statistically significant results among the four treatment groups at the 45, 60 and 120 minute incubation periods. At

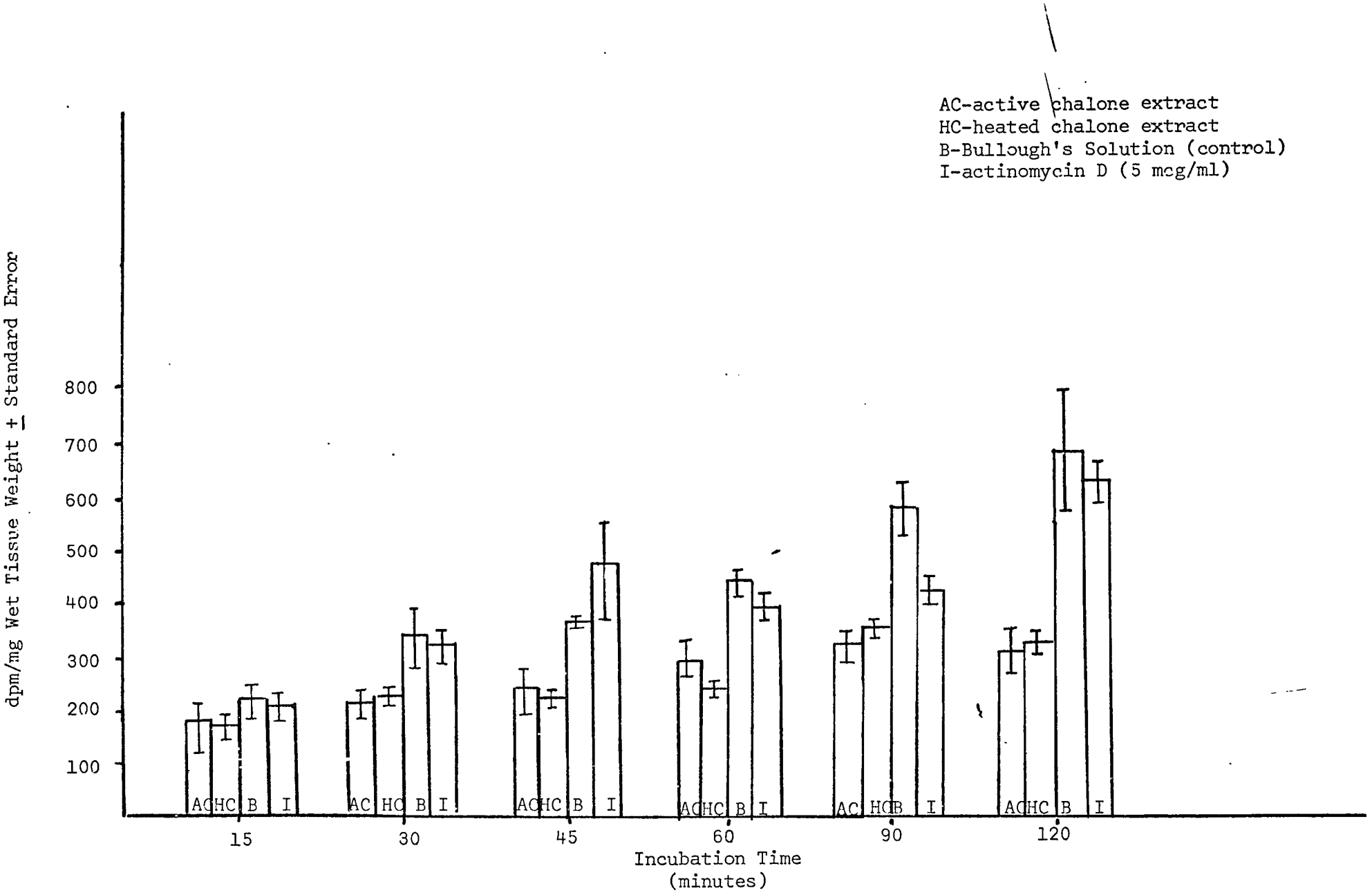

Figure 5. Effect of Chalone on Uptake of Tritiated Uridine by Rat Epidermal Slices (mean dpm/mg of wet tissue weight ± standard error)[a]

[a]Each mean is based upon epidermal sections from three animals.

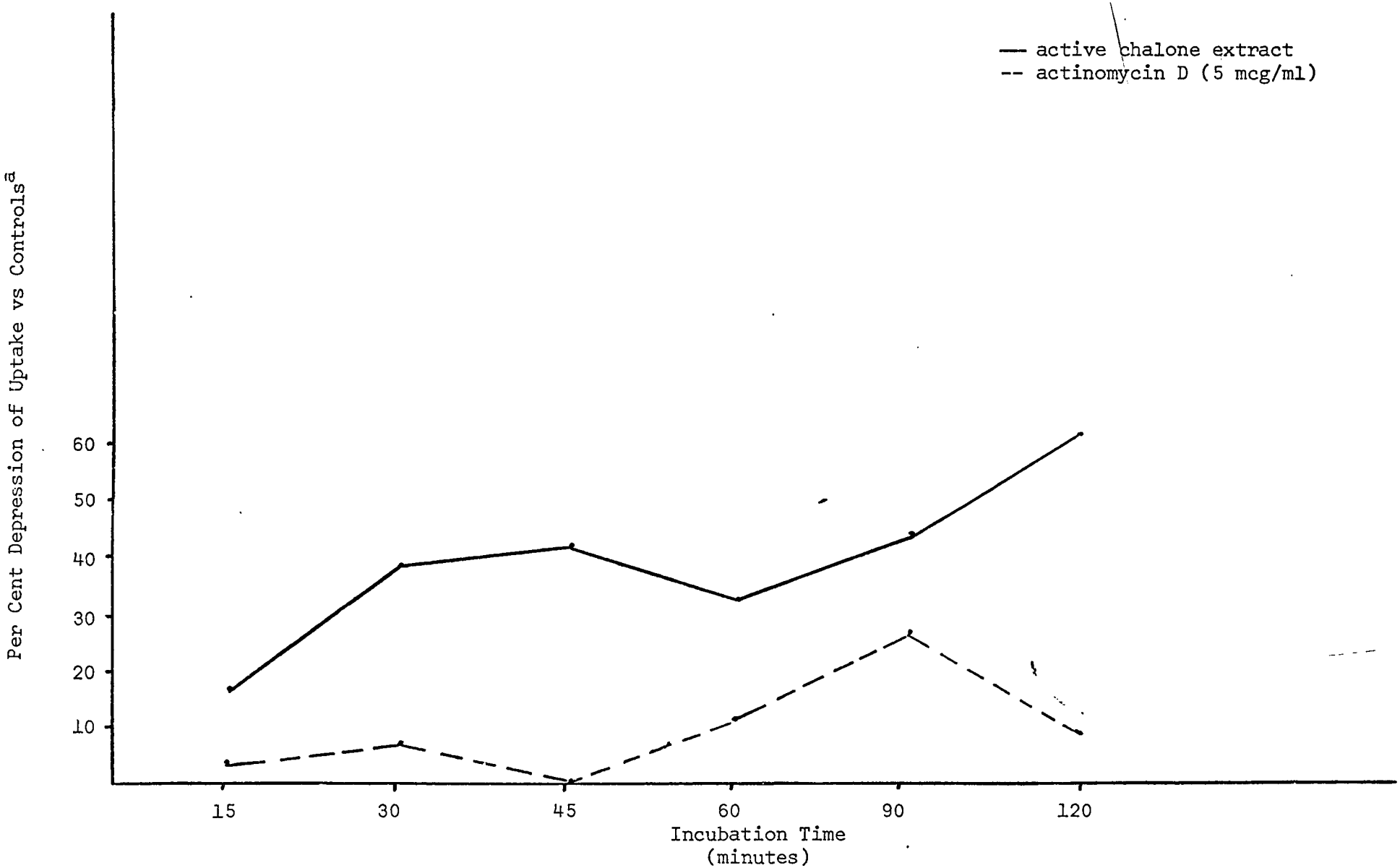

Figure 6. Depression of Tritiated Uridine Uptake in Rat Epidermal Slices by Chalone and Actinomycin D (based on dpm/mg of wet tissue weight)

[a]Controls represented zero depression.

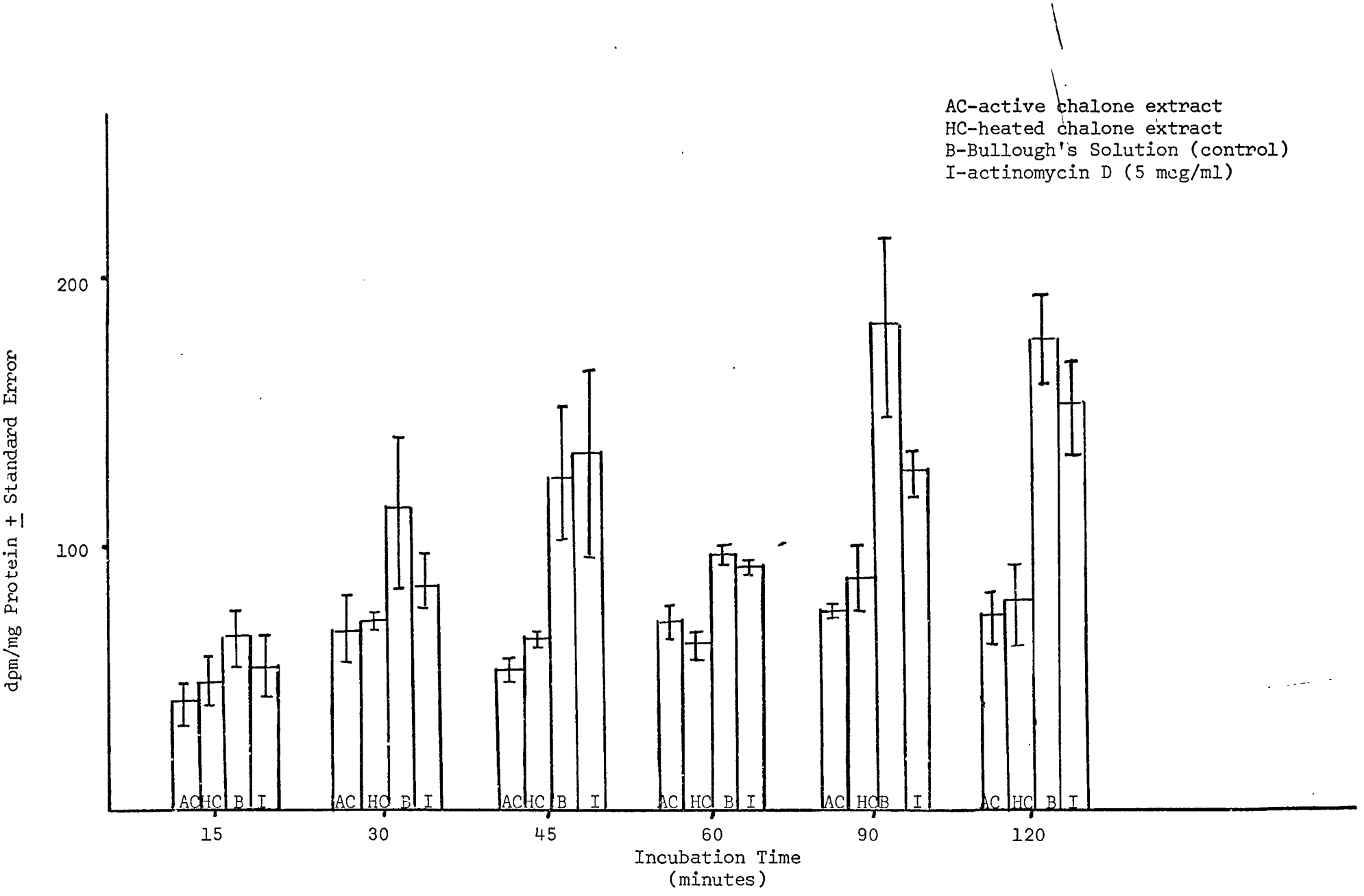

Figure 7. Effect of Chalone on Uptake of Tritiated Uridine by Epidermal Slices (mean dpm/mg of epidermal protein ± standard error)[a]

[a]Each mean is based upon epidermal sections from three animals.

the 45 minute time period, there was a significant difference between active chalone and heated chalone. At the 60 minute period there was a significant difference between active chalone and control. At the 120 minute incubation period a significant difference was noted between active chalone and control groups.

Figure 8 is a graphic representation of the per cent depression in uridine uptake when chalone was compared to control and when actinomycin D was compared to control.

UPTAKE OF SORBITOL ^{14}C:

Sorbitol ^{14}C was used to determine the effect of the chalone on the extracellular space. Whether the results were expressed as disintegrations per minute per milligram of wet tissue weight (Figure 9) or disintegrations per minute per milligram of protein (Figure 10) there appeared to be no definite pattern in the radioactive uptake by the three treatments. The Balanova Analysis of Variance supported this since at no incubation time period was there a statistical significance among the treatment groups.

UPTAKE OF TRITIATED WATER:

Tritiated water was used to determine the effect of chalone on cell permeability. The results expressed as disintegrations per minute per milligram of wet tissue weight (Figure 11) or disintegrations per minute per milligram of protein (Figure 12) revealed no definite pattern

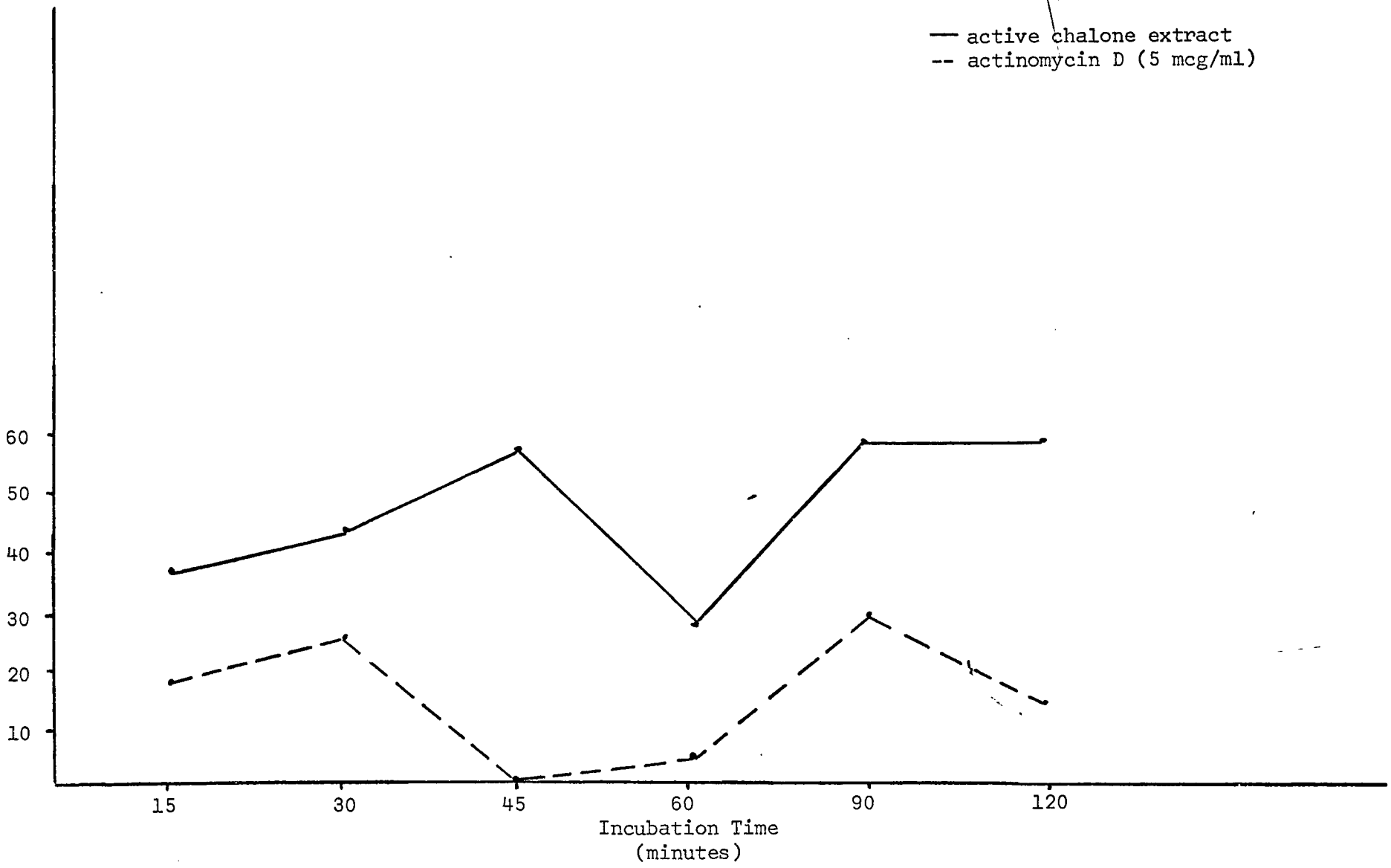

Figure 8. Depression of Tritiated Uridine Uptake in Rat Epidermal Slices by Chalone and Actinomycin D (based on dpm/mg of epidermal protein)

[a] Controls represented zero depression.

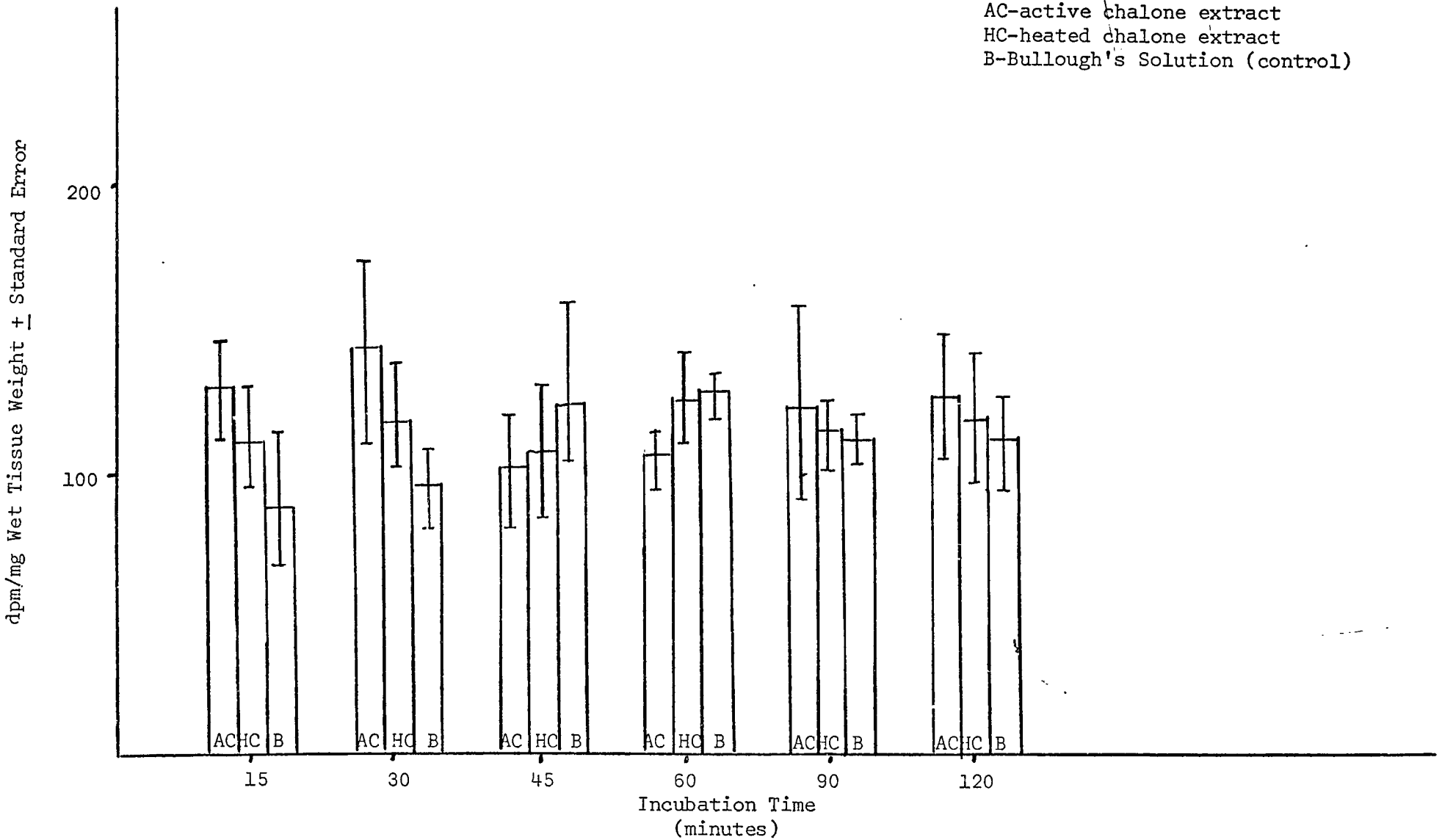

Figure 9. Effect of Chalone on Uptake of Sorbitol ^{14}C by Epidermal Slices (mean dpm/mg of wet tissue weight ± standard error)[a]

[a]Each mean is based upon epidermal sections from three animals.

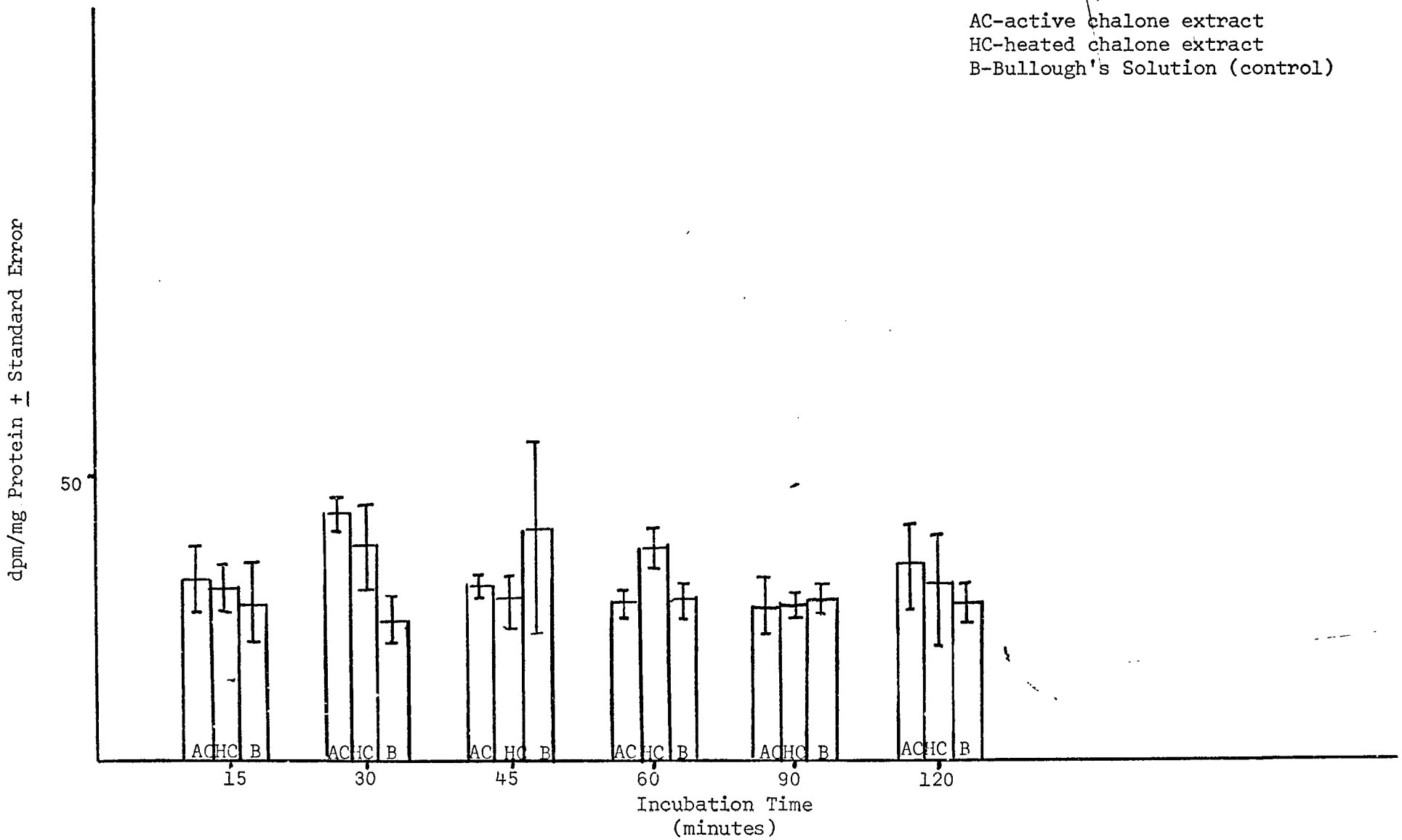

Figure 10. Effect of Chalone on Uptake of Sorbitol ^{14}C by Epidermal Slices (mean dpm/mg of epidermal protein ± standard error)[a]

[a]Each mean is based upon epidermal sections from three animals.

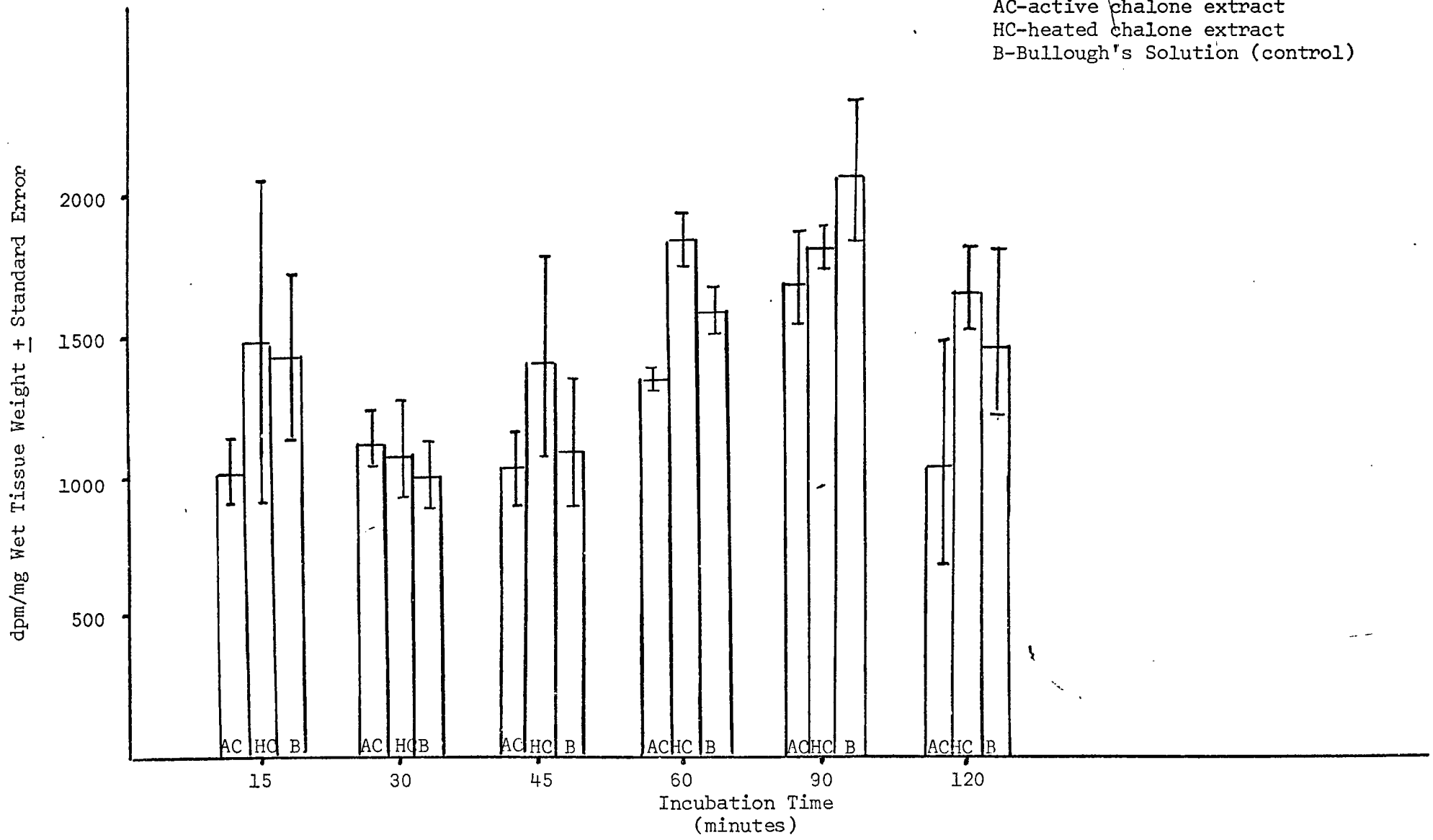

Figure 11. Effect of Chalone on Uptake of Tritiated Water by Epidermal Slices (mean dpm/mg of wet tissue weight ± standard error)[a]

[a]Each mean is based upon epidermal sections from three animals.

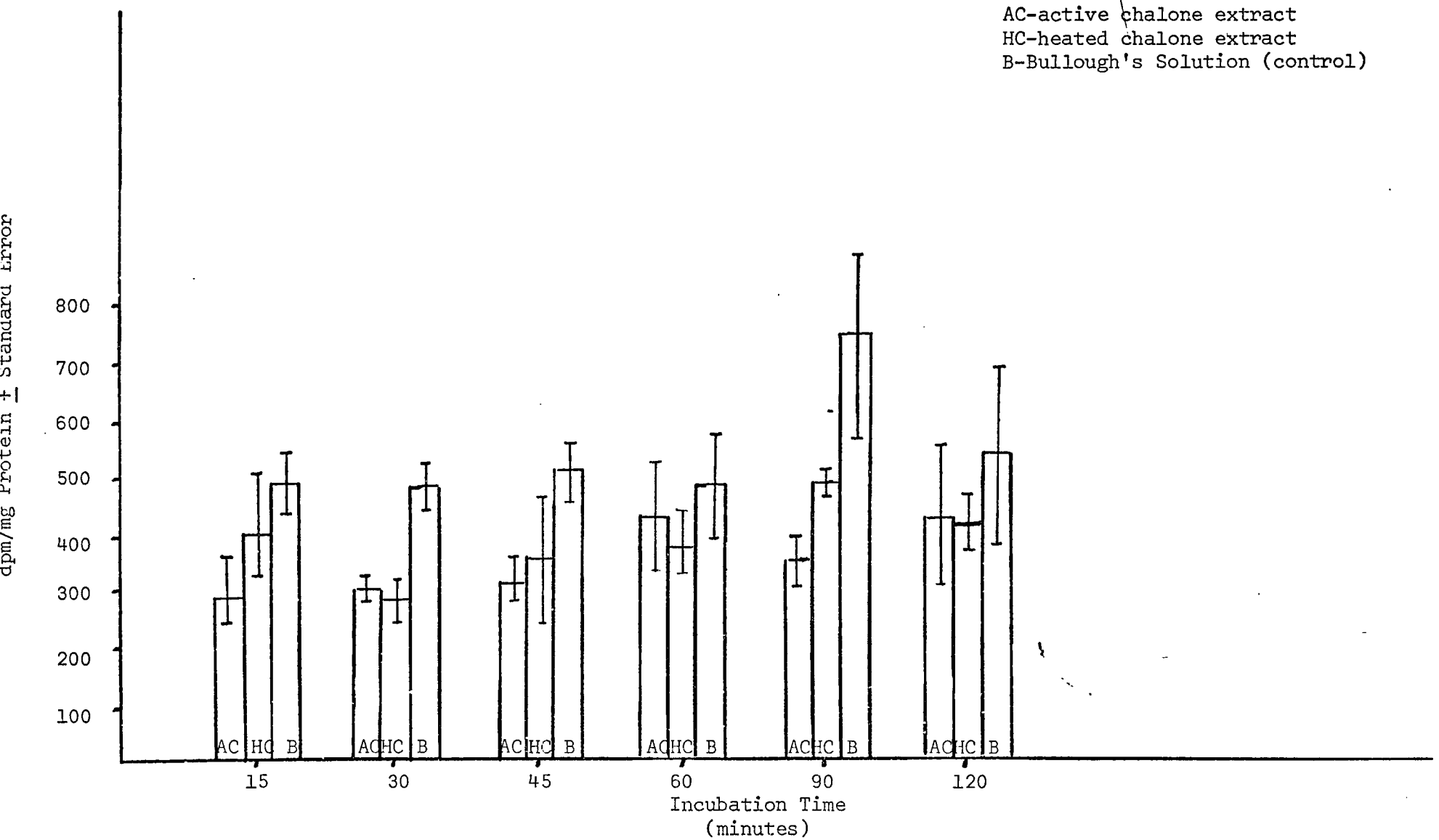

Figure 12. Effect of Chalone on Uptake of Tritiated Water by Epidermal Slices (mean dpm/mg of epidermal protein ± standard error)[a]

[a]Each mean is based upon epidermal sections from three animals.

in uptake by the three epidermal treatments. Balanova Analysis of Variance showed no statistical significant differences among these treatments.

Normally, the means were calculated from the results of three samples. However, in two instances samples were lost. This occurred in the thymidine control group at the 120 minute incubation time period and in the uridine heated chalone group at the 45 minute incubation time period. The means in these instances were calculated by first finding the average disintegrations per minute of the two available samples. That figure was then used as a substitute for the missing sample's data when calculating the mean.

Chapter 5

DISCUSSION

Chalone, a tissue specific endogenous substance, is said to possess mitotic depressing properties. Most studies have measured and associated this activity with depressed DNA synthesis (23, 24, 37). Kiger and co-workers (22) using human lymphocyte tissue compared DNA, RNA and protein synthesis using spleen chalone. They found that DNA synthesis was the most markedly influenced by the chalone activity.

The purpose of this research was threefold: (a) to compare the effect of chalone on epidermal DNA and RNA synthesis by considering the uptake of radioactive precursors as an indirect index of the synthesizing activity; (b) to determine the influence of chalone on the extracellular space using a substance (sorbitol) known to be unable to penetrate cell membranes; and (c) to determine the effect of chalone on cell permeability using a radioactive substance which readily diffuses through the cell membrane.

The results of these experiments were expressed as disintegrations per minute per milligram of wet tissue weight and as disintegrations per minute per milligram of protein. The latter was calculated since there would presumably be less variability in epidermal protein content then in wet tissue weight. However, an analysis of the variability revealed that the results were approximately the same regardless of the method of expression. It is probable that a more realistic basis would be per unit of the appropriate nucleic acid.

Three basic treatments were employed in all experiments. These were chalone, chalone heated at 100 degrees centigrade for 30 minutes in an attempt to inactivate it and a control. In the tritiated thymidine and uridine uptake studies, known DNA and RNA synthesis inhibitors were also utilized as additional controls for the system. These inhibitors were cytosine arabinoside and actinomycin D respectively.

The results of the thymidine uptake study (Figures 1, 2, 3 and 4) indicated a depression in DNA synthesis brought about by the active chalone extract at all incubation time periods when compared to the control. However, these results were only significant ($p<.05$) at the 60 and 120 minute periods for the wet weight analysis. There were no significant differences between active chalone extract and control when the results were based on protein weight.

The results of the tritiated uridine study (Figures 5, 6, 7 and 8) also indicated a depression in RNA synthesis brought about by active chalone extract at all incubation time periods when compared to control. When the results were based on wet tissue weight there was a significant difference between active chalone and control results at the 45, 60, 90 and 120 minute incubation time periods. When expressed on the basis of protein weight the results were significant at the 60 and 120 minute periods, only. This apparently resulted because of less variability associated with the protein analysis.

The results of both the thymidine and uridine uptake studies suggest that chalone does depress the synthesis of DNA and RNA respectively.

The last two experiments, effects of extracellular space and cell permeability, showed that chalone does not appear to effect either of these using the methods employed herein. The uptake of the radioactive substances was not significantly different at any incubation time when active chalone extract was compared to control. Thus, chalone apparently does not produce cellular swelling which would reduce the extracellular space and inhibit diffusion of radioactivity. In the case of inhibited diffusion, the centrally located cells of a sample would have little or no radioactive substance associated with them and the count rates would, therefore, be low. Also, chalone does not apparently reduce membrane permeability which would also inhibit the radioactive diffusion into the cell and result in low count rates.

The results of these tests lend further evidence to the supposition that depressed radioactive uptake produced by chalone is related to nucleic acid synthesis rather than nonspecific effects on the cell or the extracellular space.

This study did not produce as great a depression in DNA synthesis as has been reported by other investigators (24, 29). A possible explanation for this includes nonuniform tissue sections. Production of a uniform tissue section proved to be a difficult task with the dermatome employed. Because of this, it was impossible to be certain that all sections contained comparable quantities of the basal cell layer which is the epidermal area of mitosis. It might be possible to rectify this problem by either using a tissue which does not have a

critical zone of mitosis as does epidermis or by using a technique in which the results could be expressed as disintegrations per minute per weight of nucleic acid.

The method employed to inactivate the chalone proved to be somewhat unsatisfactory in that irregular effects were noted. This was particularly evident in the tritiated uridine studies as the uptake of the heated chalone group in several instances was not comparable to that of the control. The technique used, heating in a boiling water bath for 30 minutes, is an accepted procedure for inactivating many enzyme proteins, however, it has been reported that chalone can be broken down into two factors. One of these, the M factor, is heat labile; the other, the S factor, is resistant to this form of neutralization (23). It is, therefore, suggested that future attempts at inactivation be conducted using a proteolytic enzyme such as trypsin which Hondius-Bolding and Laurence (10) found to be an acceptable aid in the characterization of chalone as a protein. Additionally, Marrs and Voorhees (27) found trypsinized chalone to have an effect on mitosis comparable to that of a buffer control and concluded that trypsin would inactivate it.

The inability to depress RNA synthesis with actinomycin D is difficult to explain in the uridine uptake study. Hennings and co-workers (36) using mouse skin slices found that actinomycin D inhibited all DNA dependent RNA synthesis and according to Cantarow et al. (38) recent evidence indicated that all RNA is synthesized on a DNA template. Since the dose used in this project was twice that used by Hennings'

group, possible explanations might be inadequate length of incubation time or need to incubate with actinomycin D with the tissue for a period of time prior to the introduction of the uridine into the system.

Chapter 6

SUMMARY

Epidermal sections were treated in one of several ways to determine the effect of chalone on DNA synthesis (by way of tritiated thymidine uptake), RNA synthesis (by way of tritiated uridine uptake), extracellular space (by way of sorbitol ^{14}C uptake) and cell permeability (by way of tritiated water uptake).

Various controls were employed. Since chalone is thought to be a protein or glycoprotein, the chalone extract was heated in an attempt to inactivate the preparation and in that way control the possibility that some non-heat labile substance in the solution was bringing about the mitotic depressing activity. Bullough's Solution was also employed as a control since it is not considered to have mitotic depressing activity. Finally, in the studies for the determination of DNA and RNA synthesis, known inhibitors of these (cytosine arabinoside and actinomycin D respectively) were employed to assess the validity of the procedure.

It can be concluded, based upon the conditions of this study that:

1. Chalone possesses mitotic depressing activity when based on the uptake of labeled nucleotides.

2. Chalone does not affect the extracellular space.

3. Chalone does not affect cell permeability.

4. There appears to be, as has been reported, a heat labile fraction and non-heat labile fraction in the chalone structure. This was suggested by different effects of heated chalone preparation on thymidine uptake and uridine uptake.

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BIBLIOGRAPHY

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APPENDICES

APPENDIX 1

FORMULA FOR SCINTILLATION COCKTAIL

Triton X-100	300 ml
Toluene	667 ml
1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP)	0.2 g
2,5-diphenyloxazolyl (PPO)	4.0 g

FORMULA FOR BULLOUGH'S SOLUTION

NaCl (0.90%)	100
KCl (1.15%)	4
$CaCl_2$ (1.22%)	3
KH_2PO_4 (2.11%)	3
$MgSO_4$ 7·H_2O (3.82%)	1
$NaHCO_3$ (1.30%)	3

Use nine parts of the above and add one part of 0.1M phaophate buffer (pH 7.4) which is made by dissolving 14.1 g of Na_2HPO_4 in 20 ml of normal HCl diluted to one liter with distilled water.

FORMULA FOR REAGENT A

Cupric Sulfate (1%)	1 ml
Potassium Tartrate (2.7%)	1 ml
Sodium Carbonate (2%)	100 ml

FORMULA FOR REAGENT B

Folin phenol reagent diluted to 1N with distilled water immediately before use.

APPENDIX 2

BALANOVA ANALYSIS OF VARIANCE

Balanova is a general analysis of variance applicable to a wide range of experimental design. The basic program was written by Paul Herxberg at York University in Canada. Originally, it was adapted to Fortran IV by Roger Hall at the University of Houston, Subsequent adaption has been made by William Rowley, Director of the University of Houston Computer Center for the Univac[R] 1108, Sperry Rand Corp., Terrytown, New York.[1]

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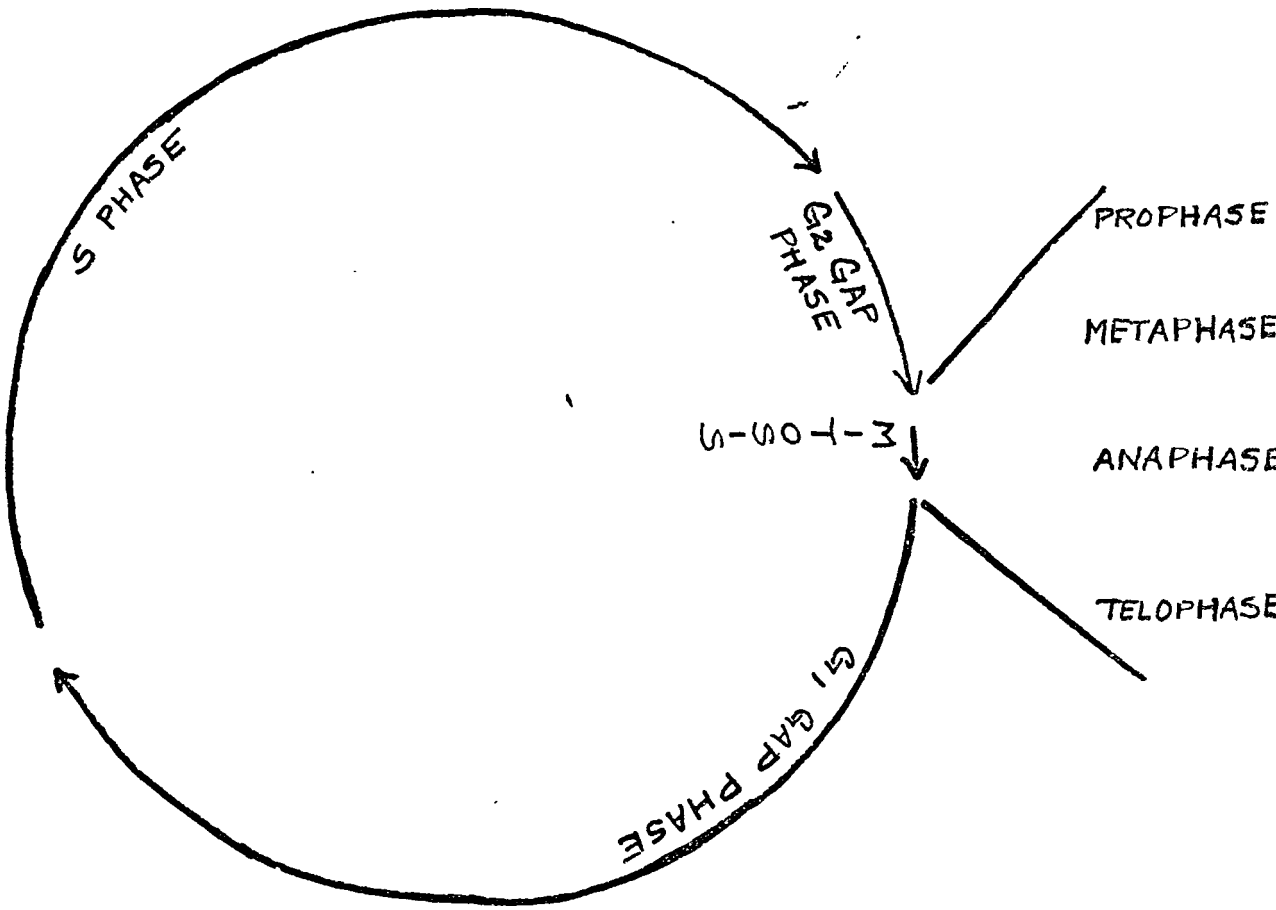
[1] Harberson, W., University of Houston Computer Center, personal communication.

APPENDIX 3

CELL CYCLE



APPENDIX 4

DESCRIPTIVE STATISTICS

TRITIATED THYMIDINE DATA EXPRESSED AS

MILLIGRAMS OF WET TISSUE WEIGHT

Variable	Incubation Time (Minutes)	Mean[a]	Standard Deviation
Active Chalone	15	96.25	29.35
Heated Chalone	15	169.73	60.42
Buffer	15	155.14	51.93
Cytosine Arabinoside	15	100.17	13.63
Active Chalone	30	198.16	68.81
Heated Chalone	30	188.71	15.85
Buffer	30	371.21	180.81
Cytosine Arabinoside	30	126.02	17.22
Active Chalone	45	218.77	78.41
Heated Chalone	45	435.37	147.68
Buffer	45	370.27	91.47
Cytosine Arabinoside	45	161.72	21.37
Active Chalone	60	211.87	32.41
Heated Chalone	60	314.61	75.16
Buffer	60	330.63	41.00
Cytosine Arabinoside	60	188.64	51.73
Active Chalone	90	317.06	97.87
Heated Chalone	90	511.19	108.62
Buffer	90	487.20	145.94
Cytosine Arabinoside	90	239.31	19.36
Active Chalone	120	328.37	28.17
Heated Chalone	120	602.15	141.16
Buffer	120	448.67	32.45
Cytosine Arabinoside	120	240.34	38.91

[a]Each mean is based upon epidermal sections from three animals.

TRITIATED THYMIDINE DATA EXPRESSED AS

MILLIGRAMS OF PROTEIN

Variable	Incubation Time (Minutes)	Mean[a]	Standard Deviation
Active Chalone	15	24.46	7.60
Heated Chalone	15	40.05	17.40
Buffer	15	34.06	14.55
Cytosine Arabinoside	15	31.06	10.59
Active Chalone	30	39.95	7.67
Heated Chalone	30	49.31	9.14
Buffer	30	79.13	28.64
Cytosine Arabinoside	30	38.32	18.25
Active Chalone	45	64.05	12.46
Heated Chalone	45	127.78	42.51
Buffer	45	94.73	34.53
Cytosine Arabinoside	45	43.19	4.13
Active Chalone	60	54.58	14.88
Heated Chalone	60	87.65	12.85
Buffer	60	96.63	35.13
Cytosine Arabinoside	60	61.45	33.45
Active Chalone	90	159.54	107.46
Heated Chalone	90	140.11	28.45
Buffer	90	129.01	25.41
Cytosine Arabinoside	90	60.11	10.35
Active Chalone	120	94.19	25.72
Heated Chalone	120	165.16	19.54
Buffer	120	111.14	19.04
Cytosine Arabinoside	120	53.73	9.43

[a]Each mean is based upon epidermal sections from three animals.

TRITIATED URIDINE DATA EXPRESSED AS

MILLIGRAMS OF WET TISSUE WEIGHT

Variable	Incubation Time (Minutes)	Mean[a]	Standard Deviation
Active Chalone	15	178.70	70.57
Heated Chalone	15	177.01	18.48
Buffer	15	215.64	40.00
Actinomycin D	15	206.26	36.08
Active Chalone	30	210.38	47.74
Heated Chalone	30	214.06	28.74
Buffer	30	343.32	90.31
Actinomycin D	30	320.91	50.18
Active Chalone	45	231.84	59.34
Heated Chalone	45	228.06	10.20
Buffer	45	406.20	1.68
Actinomycin D	45	466.17	149.06
Active Chalone	60	287.82	40.86
Heated Chalone	60	254.33	8.16
Buffer	60	431.35	33.96
Actinomycin D	60	382.82	52.69
Active Chalone	90	310.71	37.57
Heated Chalone	90	340.75	20.74
Buffer	90	558.52	69.73
Actinomycin D	90	406.02	27.71
Active Chalone	120	291.23	63.23
Heated Chalone	120	310.33	15.40
Buffer	120	674.91	179.14
Actinomycin D	120	616.06	57.64

[a]Each mean is based upon epidermal sections from three animals.

TRITIATED URIDINE DATA EXPRESSED AS

MILLIGRAMS OF PROTEIN

Variable	Incubation Time (Minutes)	Mean[a]	Standard Deviation
Active Chalone	15	41.93	12.30
Heated Chalone	15	48.91	8.57
Buffer	15	65.25	18.74
Actinomycin D	15	53.58	18.30
Active Chalone	30	68.02	21.22
Heated Chalone	30	71.22	2.53
Buffer	30	114.21	45.59
Actinomycin D	30	85.40	17.22
Active Chalone	45	52.27	3.80
Heated Chalone	45	65.84	1.27
Buffer	45	125.20	42.54
Actinomycin D	45	134.16	52.55
Active Chalone	60	70.81	10.14
Heated Chalone	60	62.83	10.90
Buffer	60	96.55	3.33
Actinomycin D	60	91.50	1.56
Active Chalone	90	74.22	2.00
Heated Chalone	90	87.53	23.38
Buffer	90	180.62	56.44
Actinomycin D	90	127.84	10.65
Active Chalone	120	71.85	18.14
Heated Chalone	120	78.45	25.11
Buffer	120	176.12	32.64
Actinomycin D	120	150.99	23.96

[a]Each mean is based upon epidermal sections from three animals.

SORBITOL ^{14}C DATA EXPRESSED AS

MILLIGRAMS OF WET TISSUE WEIGHT

Variable	Incubation Time (Minutes)	Mean[a]	Standard Deviation
Active Chalone	15	129.53	27.73
Heated Chalone	15	110.94	31.24
Buffer	15	87.72	48.78
Active Chalone	30	142.40	61.32
Heated Chalone	30	118.74	29.20
Buffer	30	95.55	26.80
Active Chalone	45	100.34	35.49
Heated Chalone	45	106.05	39.25
Buffer	45	123.38	63.74
Active Chalone	60	106.20	15.76
Heated Chalone	60	125.61	26.45
Buffer	60	127.63	9.17
Active Chalone	90	120.85	64.98
Heated Chalone	90	113.73	20.25
Buffer	90	110.60	70.00
Active Chalone	120	125.90	39.60
Heated Chalone	120	118.89	43.49
Buffer	120	121.16	25.46

[a] Each mean is based upon epidermal sections from three animals.

SORBITOL ^{14}C DATA EXPRESSED AS

MILLIGRAMS OF PROTEIN

Variable	Incubation Time (Minutes)	Mean[a]	Standard Deviation
Active Chalone	15	30.33	8.87
Heated Chalone	15	31.48	7.17
Buffer	15	28.59	13.33
Active Chalone	30	39.35	6.00
Heated Chalone	30	34.51	10.59
Buffer	30	25.25	6.12
Active Chalone	45	30.66	1.21
Heated Chalone	45	28.40	9.26
Buffer	45	41.11	29.89
Active Chalone	60	28.21	3.98
Heated Chalone	60	38.53	5.18
Buffer	60	29.20	4.52
Active Chalone	90	26.97	10.28
Heated Chalone	90	27.78	2.67
Buffer	90	27.91	2.24
Active Chalone	120	35.19	12.32
Heated Chalone	120	31.39	18.75
Buffer	120	29.18	4.35

[a]Each mean is based upon epidermal sections from three animals.

TRITIATED WATER DATA EXPRESSED AS

MILLIGRAMS OF WET TISSUE WEIGHT

Variable	Incubation Time (Minutes)	Mean[a]	Standard Deviation
Active Chalone	15	1114.60	190.33
Heated Chalone	15	1491.17	1024.60
Buffer	15	1429.99	536.80
Active Chalone	30	1137.48	171.18
Heated Chalone	30	1099.94	339.38
Buffer	30	1023.20	214.72
Active Chalone	45	1050.03	226.59
Heated Chalone	45	1408.41	687.24
Buffer	45	1101.81	454.18
Active Chalone	60	1358.92	44.94
Heated Chalone	60	1860.44	168.21
Buffer	60	1603.58	152.56
Active Chalone	90	1702.68	335.76
Heated Chalone	90	1812.79	156.50
Buffer	90	2082.70	425.80
Active Chalone	120	1043.67	784.39
Heated Chalone	120	1676.44	283.04
Buffer	120	1470.69	538.35

[a]Each mean is based upon epidermal sections from three animals.

TRITIATED WATER DATA EXPRESSED AS

MILLIGRAMS OF PROTEIN

Variable	Incubation Time (Minutes)	Mean[a]	Standard Deviation
Active Chalone	15	298.03	138.46
Heated Chalone	15	408.66	195.04
Buffer	15	496.95	103.89
Active Chalone	30	307.91	35.19
Heated Chalone	30	289.12	74.82
Buffer	30	493.00	97.73
Active Chalone	45	332.62	100.58
Heated Chalone	45	371.53	235.11
Buffer	45	530.52	102.11
Active Chalone	60	442.21	237.66
Heated Chalone	60	382.74	139.77
Buffer	60	493.36	170.62
Active Chalone	90	354.45	104.71
Heated Chalone	90	497.98	45.27
Buffer	90	757.38	307.64
Active Chalone	120	439.12	251.53
Heated Chalone	120	430.74	78.72
Buffer	120	548.60	297.99

[a]Each mean is based upon epidermal sections from three animals.