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REGULATION OF MELANOGENESIS AT THE SUB-CELLULAR LEVEL IN CLOUDMAN MURINE MELANOMA CELLS

A Thesis Submitted to the Yale School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

Kenneth E. Rosenzweig

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ABSTRACT

REGULATION OF MELANOGENESIS AT THE SUB-CELLULAR LEVEL IN CLOUDMAN MURINE MELANOMA CELLS. Kenneth E. Rosenzweig and Jean L. Bolognia. Department of Dermatology, Yale University, School of Medicine, New Haven, CT.

The decarboxylation of dopachrome to 5,6-dihydroxyindole (DHI) appears to be a major control point in the biosynthesis of melanin, in particular the conversion of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA). The recent discovery of a factor, DHICA stablin, that stabilizes DHICA and inhibits its conversion to DHI has added insight into the regulation of this intermediary compound.

This study has shown that DHICA stablin activity is present in the melanosomal fraction of Cloudman murine melanoma cells and that this activity was observed by a new method using two complementary decarboxylase assays. When three known decarboxylase stabilizing cofactors (biotin, pyridoxal phosphate, and pyruvate) were added to melanosomal extracts, DHICA decarboxylase activity was enhanced but these factors did not decrease the lability of the decarboxylase enzyme.

Protein kinases have been shown to mediate an adenylate cyclase system that is involved in the regulation of morphology and proliferation of Cloudman murine melanoma cells. It is also known that coated vesicles are involved in the transfer of protein kinases and melanin precursors between internal cell components, which raises the possibility that coated vesicles are involved in the initiation of melanin synthesis and the formation of melanosomes. In order to further characterize this process, coated vesicles were isolated and the activity of their associated protein kinases was examined in the presence of calcium, magnesium, EGTA (a known calcium

chelator) and calmodulin (a ubiquitous intracellular receptor for calcium that mediates most calcium regulated processes).

Protein kinase activity associated with coated vesicles isolated from Cloudman murine melanoma cells was shown to be stimulated by pre-incubation with melanocyte stimulating hormone. This protein kinase appeared to be magnesium dependent and inhibited by calcium. These results were demonstrated by an assay of protein kinase activity as well as autoradiography. Together, these studies reveal new insights into the sub-cellular regulation of melanogenesis, and they underscore the high degree of complexity involved.

INTRODUCTION

There is a great diversity of color in the integument of vertebrates resulting largely from the type and distribution of melanin. Melanin is the principal pigment responsible for the five basic colors in human skin and hair: black, brown, yellow, red, and white (absence of pigment). The nature of the pigment is determined by the genetic background of the organism, and the biological functions of such pigments include camouflage, heat absorption, protection from ultraviolet light, and sexual recognition within species.[1]

The melanocyte is the site of pigment production within the skin. Melanin is formed within melanosomes, membrane-bound organelles located in the cytoplasm of the melanocyte. Melanocytes are found predominantly in the basal layer of the epidermis as well as in the matrix of the hair follicle. In addition, pigment cells reside in the dermis, the mucous membranes[2-4] , the leptomeninges[5], the retina and the uveal tract of the eye, and the cochlea and vestibular labyrinth of the ear.[6]

Pigment cells are primarily derived from precursor cells in the embryonic neural crest.[7] During embryogenesis, the immature melanoblasts arise in the neural crest, migrate ventrally and make their way to the epidermis and dermis where they complete the terminal stages of differentiation into functional mature melanocytes.

The epidermal melanin unit is composed of one melanocyte and the surrounding keratinocytes to which it supplies melanin.[8,9] Pigment transfer requires the keratinocyte to phagocytize the melanosome-laden dendritic tips of the melanocyte. The pigmentation of the skin is then determined primarily by the amount of melanin transferred to the keratinocytes via the melanosomes.

The melanosome is comprised of several proteins including structural proteins and the enzyme tyrosinase. The structural proteins of the

melanosome are derived from blebs of endoplasmic reticulum while the enzyme tyrosinase is synthesized on ribosomes and then accumulates in the Golgi-associated smooth endoplasmic reticulum (GERL) before it is incorporated into coated vesicles.[10,11] The coated vesicles then fuse with the structural proteins to form melanosomes. These structural proteins form a lamellar matrix within the melanosome upon which melanin is deposited. Lastly, there are proteins of unknown structure and function in the melanosome.

In addition to the transport of tyrosinase from the GERL to the melanosome, coated vesicles are involved in the endocytosis of receptors and macromolecules as well as the specific transfer of membrane components between internal cell components.[12] In pigment cells, coated vesicles also contain melanin precursors such as 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA).[13] It is therefore thought that coated vesicles play an important role in the formation of melanosomes and the regulation of pigment production.

In the Mason-Raper pathway of melanin synthesis, tyrosinase, a copper-containing enzyme, converts tyrosinase to dopa and oxidizes dopa to dopa quinone. The subsequent reactions that eventually lead to the formation of melanin are dopa quinone → leucodopachrome → dopachrome → DHI → indole-5,6-quinone → melanochrome → melanin (Fig. 1).[14,15] These reactions were originally believed to proceed spontaneously via auto-oxidation. However, it was subsequently shown that tyrosinase was involved in the conversion of DHI → melanin,[16] and dopachrome tautomerase (DT, also known as dopachrome conversion factor, dopachrome isomerase, and dopachrome oxidoreductase) was identified as a regulatory factor in the conversion of dopachrome to DHICA.[17,18]

DHICA is considered to be an intermediate compound in the dopachrome --> DHI reaction. It was not included in the original Mason-Raper

scheme because it was considered to be non-existent or short-lived. However, it has been found that at physiologic pH and temperature, DHICA is stable and does not readily lose its carboxyl group to form DHI.[19-21] On the other hand, DHICA does rapidly decarboxylate to DHI in the presence of mushroom tyrosinase, and a black insoluble melanin precipitate is formed.[22] Therefore, in the classic Mason-Raper pathway, a branch point after dopachrome indicating the conversion of dopachrome to either DHI or DHICA should be included (Fig. 2).[23]

Melanins from various zoological sources have been shown to contain unexpectedly high contents of carboxyl groups. The carboxyl groups have been shown to be derived from the incorporation of DHICA monomers into melanin.[24] These "DHICA-melanins" are highly soluble at a neutral pH and are a rich golden-brown in color. This is in contrast to the insoluble and black "DHI-melanin" which is a polymer formed through the auto-oxidation of dopachrome or through tyrosinase-catalyzed reactions. For example, it has been observed that melanins from the hair of brown mice can be readily solubilized into a brown solution with a Tris/urea extraction, whereas the melanins from the hair of black mice remain insoluble under the extraction conditions.[23] This has led to the speculation that the brown melanins in nature may be predominantly DHICA-rich melanins, whereas the black melanins may be predominantly DHI-rich melanins.[23]

Stablins are factors present in the extracts of mouse melanoma cells that stabilize indoles such as DHI and DHICA, and thus prevent the polymerization process.[25] They apparently act through continual binding rather than through enzymatic processes. The activity of stablin is non-dialyzeable and does not adhere to wheat germ agglutinin. Because stablin activity has also been found in other tissue sources, including keratinocytes, the biological role for stablins is unknown, but it has been theorized that they are modulators of melanogenesis.[25]

Melanotropins (also known as melanocyte-stimulating hormone [MSH]) are small peptide hormones ranging in size form 12 to 18 amino acids. In vertebrates, they are known products of the intermediate lobe of the pituitary gland.[26] The three forms of MSH, α,β, and γ, are cleavage products of a common precursor peptide, pro-opiomelanocortin (POMC).[27] Other peptide hormones that are derived from the POMC precursor include corticotropin (ACTH), corticotropin-like intermediate lobe peptide, β-lipotropin, and β-endorphin. The ability of MSH to cause an increase in skin pigmentation has been demonstrated in several animal models as well as humans.[28,29]

Cloudman mouse melanoma cells have been used extensively in studies on the mechanism of action of MSH.[1,30] Evidence has been obtained to support the following conclusions: (1) MSH binds to specific high-affinity receptors on the cell's surface, predominantly during the G2 phase of the cell cycle;[31-33] (2) the formation of the hormone receptor complex is followed by a stimulation of the adenylate cyclase system and a net increase in intracellular levels of cyclic adenosine monophosphate (cAMP);[34,35] (3) the increased levels of cAMP result in increased tyrosinase activity and melanin deposition, as well as in changes in morphology and rates of proliferation;[36] (4) these processes are mediated, at least in part, by cAMP-dependent protein kinases;[37,38] and (5) there appears to be a internal receptor for MSH that is an important criterion for cellular responsiveness to MSH.[39,40] In Cloudman mouse melanoma cells, an increase in pigment production can be seen following the addition of anyone of the several agents that raise intracellular levels of cAMP. These compounds include MSH, isobutylmethylxanthine (MIX or IBMX; a compound that potentiates the effects of MSH), cholera toxin, forskolin, and dibutryl cAMP.[41,42]

The aim of this project was to examine the regulation of melanin synthesis in Cloudman murine melanoma cells. The regulation of the phosphorylation of proteins involved in melanin synthesis was examined by

isolating coated vesicles that contained the associated protein kinases. These protein kinases were also characterized by examining their activity in the presence of various ions (i.e., calcium, magnesium). In addition, the effects of EGTA, a known calcium chelator,[43] and calmodulin, a ubiquitous intracellular receptor for calcium that mediates most calcium-regulated processes,[44] were tested. To further characterize DHICA stabilization, the effect of melanosomal extracts on the decarboxylation of DHICA was studied. The activity of this decarboxylation reaction was also examined in the presence of the cofactors pyridoxal 5'-phosphate, pyruvate and biotin, all of which have been shown to be essential in the function of various carboxylase enzymes.[45]

Cell culture

Cells were cultured in monolayer in Ham's F10 medium supplemented with 10% horse serum.[46] The Cloudman melanoma cell line used for these studies is amelanotic unless MSH or other agents which raise cyclic AMP are added to the culture medium. Cells in the phosphorylation experiments were either grown as above or were incubated for 24 hours with 5% horse serum and 2×10^{-7} M MSH. In order to potentiate the effects of MSH, 1×10^{-4} M MIX was also added. The cells used in the decarboxylase experiments were incubated with MSH plus MIX in the above concentrations for approximately 48 - 60 hours prior to harvesting.

Isolation of melanosomes

Approximately 3×10^{8} cells were suspended in 0.1 M sodium phosphate buffer pH 6.8 and lysed by five passages each through a 21g and 30g needle and homogenization. Multiple centrifugations of the supernatant (1000g and 20,000g) resulted in a pellet which was resuspended in 0.1 M sodium phosphate buffer pH 6.8 and layered on a discontinuous sucrose gradient (1.0 M - 2.0 M) which was spun at 100,000g for 60 minutes. The black band at 1.4 M was collected and resuspended in 0.1 M sodium phosphate buffer pH 6.8 and spun at 100,000g for 60 minutes. The pellet was then resuspended in lysis buffer and used in subsequent experimentation as the melanosomal fraction (Fig. 3). When the melanosomal extract was examined in the presence of the cofactors biotin (1×10^{-7} M, Sigma), pyridoxal 5'-phosphate (1×10^{-7} M, Sigma) and pyruvate (1×10^{-7} M, Sigma), the extract was placed in a buffer to which protease inhibitors had been added.

A portion of the melanosomal extract was passed through wheat germ agglutinin column (lectin from Triticum Vulgaris, Sigma). Two major fractions

were collected. The first represented eluant that initially passed through the column and the second the eluant that was released when N-acetyl-glucosamine (NAGA) was added to the column. The first eluant was called "wheat germ flow-through" and the second, "NAGA-eluant."

Synthesis of ^{14}C-carboxy-labelled DHICA

Five microcuries of DL-3,4-dihydroxyphenylalanine-1-^{14}C (48.5 mCi/mmole, New England Nuclear Corp.) in 150 µl 0.1 M sodium phosphate, pH 6.8 was mixed vigorously (1 min, 20°) with 1 mg of silver oxide to form dopachrome-1-^{14}C as described by Körner and Pawelek.[17] Silver oxide was removed by filtration (0.22 micron Acrodisc Filters) and the orange-red ^{14}C-dopachrome was then incubated with partially purified dopachrome tautomerase (10 µg) in a total volume of 200 µl. The dopachrome tautomerase preparation was free of stablin and dopachrome decarboxylase activities. After 20 minutes (20°), the orange-red dopachrome was enzymatically converted to colorless ^{14}C-DHICA. The material was either used as is, with no further purification, or the ^{14}C-DHICA was purified free of dopachrome tautomerase by HPLC separatory procedures. The results were the same in either case.

Measurement of decarboxylase activity

$^{14}CO_2$ release from ^{14}C-carboxy-labelled DHICA was monitored by two techniques.[17] The ^{14}C-DHICA was incubated with buffer and cell extracts in a total volume of 250 µl. At various timepoints, the reaction mixture was vortexed and aliquots (10 µl) were removed and counted in a scintillation counter. A disappearance of counts was assumed to be due to the evolution of $^{14}CO_2$. The alternative method involved the incubation of buffer or extracts in tubes that were sealed with natural corks soaked in NaOH (0.1 M). At various timepoints, the corks were removed and counted in a scintillation counter. ^{14}C

counts from the corks were assumed to be due to $^{14}CO_2$ which had been trapped in the form of $Na_2{}^{14}CO_3$.

Isolation of coated vesicles

Coated vesicles were isolated from Cloudman S91 murine melanoma cells by a modification of the method of Pearse.[47] Approximately 2 x 10^8 cells that were grown in culture were suspended in 0.1 M 2[N-Morpholino]-ethane-sulfonic acid (MES) buffer pH 6.8 and broken by homogenization. Multiple centrifugations of the supernatant (700g, 20,000g and 100,000g) resulted in a pellet which was resuspended and spun twice on a discontinuous sucrose gradient (5-60%) for 1-2 hr at 55,000g (Fig. 4). The following fractions were collected: 10%/40% interface (first spin) and the 5%/30% interface (second spin). The final pellet was examined by electron microscopy to confirm the presence of coated vesicles. The fractions were then added to a buffer containing calcium, magnesium, EGTA, and/or calmodulin.

Measurement of protein kinase activity

Protein kinase activity was assessed by measuring the incorporation of ^{32}P from γ-labelled adenosine triphosphate (ATP) into proteins; the latter were precipitated from the coated vesicles with trichloroacetic acid. These phosphoproteins were then analyzed by SDS-PAGE and autoradiography. Due to the relatively low efficiency of the procedure, it was necessary to expose the x-ray film for 6 weeks before developing.

All of the above experiments were done completely by the author. The only exceptions are the synthesis of ^{14}C-carboxy-labeled DHICA which was done by Dr. John Pawelek and the electron microscopy of the coated vesicles which was done by Elizabeth Kuklinska.

I. Studies on the Metabolism of DHICA

The assay for DHICA decarboxylase activity involved incubating fractionated melanoma homogenates with ^{14}C-DHICA and measuring (a) the disappearance of radioactivity in the form of $^{14}CO_2$ from the incubation mixture; and (b) the appearance of radioactivity in the form of $Na_2{}^{14}CO_3$ on NaOH-saturated corks used to seal the reaction tubes. In the first set of experiments (Figs. 6 and 7), it was found that indeed, an increase in radioactivity in the corks and the disappearance of radioactivity from the reaction mixture occurred in a time-dependent fashion. However, depending on the concentration of homogenate used, the increase in $^{14}CO_2$ in the corks was often significantly diminished from that seen in control tubes containing buffer only ($p < .05$). This result was seen in the complementary assay: the reaction mixture with the higher concentration of melanosomal extract did not lose any $^{14}CO_2$. These results are consistent with the expression of DHICA stablin rather than decarboxylase activity.

A second set of experiments was done to examine the possibility of the presence of an antagonist of decarboxylation such as DHICA stablin. In these experiments, the concentration of extract varied from 0-16% (Figs. 8 and 9); the greatest ^{14}C activity in the form of $Na_2{}^{14}CO_3$ was seen in the middle range of extract concentrations (2% and 4%) and again, there was significantly less activity at higher concentrations ($p < .01$). Similar results were observed in the complementary assay where the largest decrease in $^{14}CO_2$ counts from the reaction mixture was exhibited by these same concentrations. This result was also consistent with the expression of DHICA stablin in the extract.

Since stablin had been shown to not adhere to a wheat germ agglutinin column,[25] melanosomal extract was passed through such a column to see if this "DHICA stablin-like" activity we observed behaved in a similar fashion.

Various concentrations of: (1) extract alone; (2) extract passed through a wheat germ column; and (3) eluant released from the wheat germ column by NAGA were tested. In the extract alone (Fig. 10), the highest level of ^{14}C activity in the form of $Na_2{}^{14}CO_3$ was observed in the control (no extract) sample ($p < .005$). The latter contained 2.4 X the activity at 3 hours as compared to the samples that contained melanosomal extract. The wheat germ flow-through showed a similar pattern of activity (Fig. 11) - the control (Triton X 0.5% buffer passed through a separate control column) had the highest activity by a factor of 3.1 at 3 hours compared to the samples that contained wheat germ flow-through extract ($p < .01$). The NAGA-eluant also had the greatest activity in the control sample (NAGA passed through the separate control column) by a factor of 1.8 at 3 hours as compared to the samples that contained NAGA-eluant extract ($p < .05$; Fig. 12). The complementary assay showed similar results, with the greatest decrease in activity occurring in the control samples (Figs. 13 and 14). However, it was necessary to adjust for the protein concentration of the extracts.

When the ^{14}C activity in the form of $Na_2{}^{14}CO_3$ was adjusted for the protein concentration of the extract, the wheat germ flow-through had 2.3 times as much ^{14}C activity as the melanosomal extract alone (Fig. 15), suggesting that the inhibition of decarboxylation was possibly due to DHICA stablin because the "stablin-like" activity had not adhered to the wheat germ agglutinin column (DHICA stablin does not adhere to wheat germ agglutinin[25]). The NAGA-released eluant had a protein concentration that was too low to be measured accurately and was not used for comparison.

A variety of cofactors (biotin, pyridoxal 5'-phosphate, and pyruvate) that are known stabilizers of decarboxylase activity[45] were added to the melanosomal extracts to examine their effect on DHICA decarboxylase activity. The results (Figs. 16-19) demonstrated that 10% melanosomal extract in the presence of all cofactors had the highest decarboxylase activity in

comparison to samples with no cofactors or individual cofactors ($p < .05$). When 50% melanosomal extract was used, the samples with all of the cofactors had significantly increased decarboxylase activity as compared to each cofactor alone or the control ($p < .05$). When the assay was repeated 24 hours later using extracts which had been refrigerated overnight at 4° (figures not shown), the previous results could not be repeated - all of the samples had the same activity.

II. Studies on MSH-Sensitive Protein Kinase Activity in Coated Vesicles

When protein kinase activity (measured by the incorporation into proteins of ^{32}P from γ-labelled ^{32}ATP) was examined in isolated coated vesicles, protein kinase activity was significantly elevated when the cells had been pre-incubated in MSH and MIX ($p < 0.05$; Figs. 20 and 21). In addition, protein kinase activity was significantly increased in the presence of magnesium ($p < 0.05$; Fig. 20) and greatest in the presence of EGTA (a preferential calcium chelator) plus magnesium ($p < 0.01$; Fig. 21). In comparison, less protein kinase activity was observed when EGTA, magnesium, and calcium were combined, which suggested that calcium interfered with protein kinase activity. However, the latter combination had a higher protein kinase activity than in coated vesicles incubated in the presence of magnesium plus calcium (Fig. 21), which suggested that EGTA enhanced the protein kinase, most likely by chelating free calcium which was interfering with kinase activity. Figure 22 shows lower protein kinase activity in the extract incubated with calcium or calcium plus calmodulin as compared to EGTA plus magnesium, which is in agreement with the previous results.

Coated vesicle phosphoproteins, analyzed by SDS-PAGE and autoradiography (Fig 23), showed increased phosphorylation in cells that were incubated in MSH as compared to the control. The greatest radioactivity was observed when the proteins had been incubated in the presence of EGTA

plus magnesium and there was decreased activity in the presence of calcium. This was in agreement with the results from the protein kinase assays which also showed the most activity in the presence of magnesium plus EGTA and inhibition by calcium. There was generally increased phosphorylation in two bands (denoted by arrows) that seem to show specific stimulation by MSH.

I. Studies on the Metabolism of DHICA

Evidence has accumulated to support the idea that the decarboxylation of dopachrome to DHI via DHICA represents an important control point in the biosynthesis of melanin. The conversion of dopachrome to DHICA by DT (Figs.1,2) has been well studied,[17,23] but there are still details of this conversion that need to be examined. The discovery of a factor, DHICA stablin, that stabilizes DHICA and inhibits its conversion to DHI[25] has added insight into the regulation of this intermediary compound.

In the set of experiments described in this paper, attempts were made to further study the regulation of DHICA by measuring DHICA decarboxylase activity in melanosomal extracts. Decarboxylation was measured by two methods: (a) the disappearance of radioactivity in the form of $^{14}CO_2$ from the incubation mixture; and (b) the appearance of radioactivity in the form of $Na_2{}^{14}CO_3$ on NaOH-saturated corks used to seal the reaction tubes. In the majority of experiments performed, the two methods were complementary, that is if the production of $Na_2{}^{14}CO_3$ increased, then the amount of $^{14}CO_2$ decreased. In the initial set of experiments (Figs. 6-9), as the concentration of melanosomal extract was increased in the reaction mixture, decarboxylase activity in the assay decreased, i.e. there was less $Na_2{}^{14}CO_3$ activity in the corks and more $^{14}CO_2$ activity remaining in the reaction mixture. The results from both methods indicated that the melanosomal extract was preventing the decarboxylation reaction from occurring.

The source of ^{14}C within the reaction mixture was ^{14}C-carboxy-labelled-DHICA. The decarboxylation reaction occurring was assumed to be ^{14}C-DHICA → DHI with the release of ^{14}C-labelled CO_2. If the melanosomal extract was preventing this reaction from occurring, then it was behaving similarly to stablin, which has been shown to stabilize DHICA.[25]

An interesting possibility was that these two complementary assays represented a new method of assessing DHICA stablin activity. Previously, the presence of DHICA stablin had been measured by observing the absence of pigmentation in a reaction mixture of DHICA plus extract as compared to DHICA alone (which would polymerize to a black pigment); the drawback of this latter assay was that it required an incubation time of 24-48 hours. However, before the melanin assay could be replaced by the $^{14}CO_2$ assays, it was necessary to determine if the "stablin-like" activity observed had the same properties as DHICA stablin. To accomplish this, the melanosomal extract was first passed through a wheat germ agglutinin column. This was done because it had been previously shown that stablin activity did not adhere to wheat germ agglutinin.[25] Therefore, there should be more stablin-like activity (less decarboxylation) in the extract passed through the column (flow-through) as opposed the melanosomal extract alone or eluant released by passing N-acetyl-glucosamine (NAGA) through the column (NAGA-eluant).

This proved to be the case when the assays were run (Figs. 10-12). In all three extracts (melanosomal extract alone, wheat germ flow-through, and NAGA-eluant), the control (no extract) had the highest decarboxylation activity ($p < 0.05$), indicative of low stablin activity. The presence of extract decreased decarboxylation activity and the decrease was independent of the concentration of cell extract, i.e., both 12% and 40% decreased activity to the same extent (Figs. 10-12). The wheat germ flow-through did decrease decarboxylase activity to the greatest extent, that is it had the most stablin activity. This was demonstrated by the high ratio (3.1:1) of activity in the blank sample as compared to the samples containing extract. In contrast, the NAGA-eluant had the lowest ratio of blank to extract activity (1.8:1).

It was already known that the melanosomal extract contained DHICA stablin activity. However, it was important to determine if the "stablin-like" activity observed had similar properties to DHICA stablin. The fact that there

was greater activity in the wheat germ flow-through, as compared to the initial cell extract or NAGA-eluant was evidence in favor of the "stablin-like" activity being secondary to stablin. This is based on the previous observations that stablin passes through the column and the flow-through has the highest stablin activity.[25] It was also necessary to adjust all of these samples for protein concentration. When this was done (Fig. 15), the extract that passed through the wheat germ column had 2.3 X as much activity as the melanosomal extract alone demonstrating that the increased activity was not just due to increased protein in the flow-through.

Previous experiments with DHICA decarboxylase in our laboratory had shown it to be quite labile; its activity often lasted less than twelve hours. In an effort to stabilize the enzyme, cofactors were added that have been previously shown to stabilize the activity of various decarboxylase enzymes. These cofactors are biotin, pyridoxal 5'-phosphate, and pyruvate.[45] When these factors were added to the reaction mixture (Figs. 16-19), each alone had no effect, but in the presence of all three cofactors, decarboxylase activity was enhanced. However, there was no evidence for stabilization of the decarboxylase enzyme when the extract was tested the next day.

II. Studies on MSH-Sensitive Protein Kinase Activity in Coated Vesicles

Protein kinases have been shown to mediate an adenylate cyclase system that is involved in the regulation of morphology and proliferation of Cloudman murine melanoma cells.[37,38] It is also known that coated vesicles from these melanoma cell lines are involved in the transfer of protein kinases and melanin precursors between internal cell components,[47] which raises the possibility that coated vesicles are involved in the initiation of melanin synthesis and the formation of melanosomes. In order to further characterize this process, coated vesicles were isolated and the activity of their associated protein kinases was examined in the presence of calcium, magnesium, EGTA

(a known calcium chelator[43]) and calmodulin (a ubiquitous intracellular receptor for calcium that mediates most calcium regulated processes[44]).

Magnesium alone was shown to increase protein kinase levels in coated vesicles (Fig. 20). In contrast, the presence of calcium alone and calcium with calmodulin consistently lowered protein kinase activity (Figs. 21 and 22). The kinase activity was also shown to be the greatest when incubated in the presence of magnesium and EGTA (Fig. 21). EGTA, when added in conjunction with calcium increased protein kinase activity, assumedly because it chelated free calcium. These results have led to the conclusions that the kinase is magnesium-dependent and the kinase is inhibited by calcium and calcium plus calmodulin. An autoradiogram of the phosphoproteins (Fig. 23) confirms these results with the darkest lane (Lanes 11 and 12) occurring in the presence of magnesium and EGTA.

Cells that had been incubated in the presence of MSH consistently showed protein kinase activity that was 2-3 times greater than the activity seen in the control cells (Figs. 20-22). The autoradiogram (Fig. 23) also shows increased activity in the lanes representing coated vesicles from cells that had been grown in the presence of MSH (Lanes 9-16) as compared to coated vesicles from control cells (Lanes 1-8). This is the first time that an increase in protein kinase activity shown in coated vesicles from cells incubated with MSH has been demonstrated. There also appears to be disproportionate increases in activity in specific protein kinases in certain bands (denoted by arrows) as well as generalized increased activity.

In conclusion, this study has shown that DHICA stablin activity is present in the melanosomal fraction of Cloudman murine melanoma cells and that this activity was observed by a new method using two complementary decarboxylase assays. When three known decarboxylase stabilizing cofactors (biotin, pyridoxal phosphate, and pyruvate) were added to melanosomal extracts, DHICA decarboxylase activity was enhanced, however these factors

did not decrease the lability of the decarboxylase enzyme. In addition, the protein kinase activity associated with coated vesicles isolated from Cloudman murine melanoma cells was shown to be stimulated by pre-incubation with MSH . This protein kinase appeared to be magnesium-dependent and inhibited by calcium. These results were demonstrated by an assay of protein kinase activity as well as autoradiography.

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REFERENCES

1. Pawelek JM and Körner AM. The biosynthesis of mammalian melanin. *American Scientist* 1982; **70**: 136-145.

2. Laidlaw GF, Cahn LR. Melanoblasts in the gum. *J. Dent. Res.* 1932; **12**: 534-537.

3. Zak FG, Lawson W. The presence of melanocytes in the nasal cavity. *Ann. Otol. Rhinol. Laryngol.* 1974; **83**: 515-519.

4. Nigogosyan G, De La Pava S, Pickren JW. Melanoblasts in the vaginal mucosa. *Cancer* 1964; **17**: 912-913.

5. Goldgeier MH, Klein LE, Klein-Angerer S, Moellmann G, Nordlund JJ. The distribution of melanocytes in the leptomeninges of the human brain. *J. Invest. Dermatol* 1984; **82**: 235-8.

6. Savin C. The blood vessels and pigmentary cells of the inner ear. *Ann. Otol. Rhinol. Laryngol.* 1965; **74**: 611-622.

7. Weston JA. The migration and differentiation of neural crest cells. In: Abercrombie M, Brachet J, King T, eds. Advances in morphogenesis, New York: Academic Press, 1970; 8: 41-114.

8. Fitzpatrick TB, Miyamoto M, Ishikawa K. The evolution of concepts of melanin biology. *Arch. Dermatol.* 1967; **96**: 305-323

9. Jimbow K, Quevedo WC, Fitzpatrick TB, Szabo G. Some aspects of melanin biology: 1950-1970. *J. Invest. Dermatol.* 1976; **67**: 72-89.

10. Maul GG. Golgi-melanosome relationship in human melanoma *in vitro*. *J. Ultrastruct. Res.* 1969; **26**: 163-176.

11. Novikoff AB, Albala A, Biempica L. Ultrastructural and cytochemical observations on B-16 and Harding Passey mouse melanomas. *J Histochem Cytochem* 1968; **16**: 299-319.

12. Pearse BMF, Crowther RA. Structure and assembly of coated vesicles. *Ann. Rev. Biophys. Biophys. Chem.* 1987; **16**: 49-68.

13. Hatta A, Mishima Y, Ichihashi M, Ito S. Melanin monomers within coated vesicles and premelanosomes in melanin synthesizing cells. *J. Invest. Dermatol.* 1988; **91**: 181-184.

14. Raper HS. The aerobic oxidases. *Physiol. Rev.* 1928; **8**: 245-82.

15. Mason HS. The chemistry of melanin. III. Mechanism of the oxidation of dihydroxyphenylalanine by tyrosinase. *J. Biol. Chem.* 1948; **172**: 83-99.

16. Körner AM, Pawelek JM. Mammalian tyrosinase activity catalyzes three reactions in the biosynthesis of melanin. *Science* 1982; **217**: 1163-1165.

17. Körner AM, Pawelek JM. Dopachrome conversion: a possible control point in melanin biosynthesis. *J. Invest. Dermatol.* 1980; **75**: 192-195.

18. Pawelek JM, Körner AM, Bergstrom A, Bolognia J. New regulators of melanin biosynthesis and the autodestruction of melanin cells. *Nature* 1980; **286**: 617-619.

19. Körner AM, Gettins P. Synthesis *in vitro* of 5,6-dihydroxyindole-2-carboxylic acid by dopachrome conversion factor from Cloudman S91 melanoma cells. *J. Invest. Dermatol.* 1985; **85**: 229-231.

20. Napolitano A, Chioccara F, Prota G. A reexamination of the zinc catalyzed rearrangemant of dopachrome using immobilized tyrosinase. *Gazz. Chim. Ital.* 1985; **115**: 357-359.

21. Palumbo A, d'Ischia M, Misuraca G, Prota G, Schultz TM. Structural modicication in biosynthetic melanins induced by metal ions. *Biochim. Biophys. Acta*. 1988; **964**: 193-199.

22. Pavlovich M. Chakraborty A, Pawelek JM. Pathways of melanogenesis differ between mushrooms and mammals. (In press, *J. Invest. Dermatol.*)

23. Pawelek JM. After dopachrome? *Pigment Cell Research* 1991; **4**: 53-62.

24. Ito S. Reexamination of the structure of eumelanin. *Biochim. Biophys. Acta* 1986; **883**: 155-161.

25. Pawelek JM, Chakraborty A, Pavlovich M, Osber M, Grove K, Min K, Myerson N, Orlow S, Bolognia J. Stablins: ubiquitous indole stabilizing factors. (Submitted for publication)

26. Li CH. The chemistry of melanotropins. In: Li CH, ed. Hormonal proteins and peptides. New York: Academic Press, 1978; 5: 1-33.

27. Nakanishi S, Inoue A, Kita T, et. al. Nucleotide sequence of cloned cDNA for bovine corticotropin-beta-lipotropin precursor. *Nature* 1979; **278**: 423-427.

28. Lerner AB, Shizune K, Fitzpatrick TB, Mason HS. MSH: the melanocyte stimulating hormone. *Arch. Dermatol. Syph.* 1954; **70**: 669-674.

29. Lerner AB, McGuire JS. Effect of alpha- and beta-melanocyte stimulating hormones on the skin color of man. *Nature* 1961; **189**: 176-179.

30. Pawelek JM. Factors regulating growth and pigmentation of melanoma cells. *J. Invest. Dermatol.* 1976; **66**: 201-209.

31. Wong G, Pawelek JM, Sansone M, Morowitz J. Response of mouse melanoma cells to melanocyte-stimulating hormone. *Nature* 1974; **248**: 351-354.

32. Varga JM, DiPasquale J, Pawelek JM, McGuire JS, Lerner AB. Regulation of melanocyte stimulating hormone action at the receptor level: discontinuous binding of hormone to synchronized mouse melanoma cells during the cell cycle. *Proc. Natl. Acad. Sci. USA* 1974; **71**: 1590-1593.

33. Lambert DT, Lerner AB. Optimization of melanotropin-reeceptor binding assay by reversed-phase high performance liquid chromatography. *J. Chromatography* 1983; **266**: 567-576.

34. Kreiner PW, Gold CJ, Keirns JJ, Brock WA, Bitensky MW. Hormonal control of melanocytes. MSH-sensitive adenyl cyclase in Cloudman melanoma. *Yale J. Biol. Med.* 1973; **46**: 583-591.

35. Pawelek JM, Wong G, Sansone M, Morowitz J. Molecular controls in mammalian pigmentation. *Yale J. Biol. Med.* 1973; **46**: 430-443.

36 Wong G, Pawelek JM. Control of phenotypic expression of cultured melanoma cells by melanocyte stimulating hormones. *Nature* [New Biol] 1973; **241**: 213-215.

37. Körner AM, Pawelek JM. Activation of melanoma tyrosinase by a cyclic AMP-dependent protein kinase in a cell-free system. *Nature* 1977; **267**: 444-447.

38. Pawelek JM.Evidence suggesting that a cyclic AMP-depencdent protein kinase is a positive regulator of proliferation in Cloudman S91 melanoma cells. *J. Cell. Physiol.* 1979; **98**: 619-625.

39. Orlow SJ, Hotchkiss S, Pawelek JM. Internal binding sites for MSH: analyses in wild-type and variant Cloudman melanoma cells. *J. Cell. Physiol.* 1990; **142**: 129-136.

40. Chakraborty AK, Orlow SJ, Bolognia JL, Pawelek JM. Structural/functional relationships between internal and external MSH receptors: modulation of expression in Cloudman melanoma cells by UVB radiation. *J. Cell. Physiol.* 1991; **147**: 1-6.

41. Wikswo MH. Action of cyclic AMP on pigment donation between mammalian melanocytes and keratinocytes. *Yale J. Biol. Med.* 1973; **46**: 592-601.

42. Kiang JG, Wu YY, Lin MC. Heat treatment induces an increase in intracellular cAMP content in human epidermoid A-431 cells. *Biochem J.* **276**: 683-9.

43. Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. Molecular Biology of the Cell, 2 ed., New York: Garland, 1989, p.872.

44. Cheung WY. Calmodulin. *Sci. Am. 1982;* 246 (6): 48-56.

45. Snell EE. Vitamin B_6 and decarboxylation of histidine. *Annals NY Acad. Sci.* 1990; **585**: 1-12.

46. Pawelek JM: Melanoma Cells, Methods in Enzymology, Vol. LVII. Edited by W Jacoby, I Pastan. 1979, pp. 564-70.

47. Pearse BMF. Coated vesicles from pig brain: purification and biochemical characterization. *J. Mol. Biol.* 1975; **97**: 93-8.

Figure 1

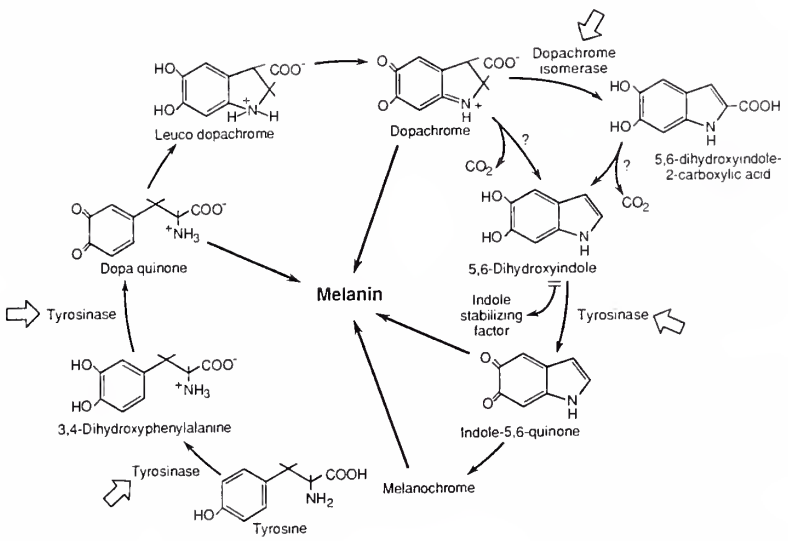

Leuco dopachrome
Dopachrome
Dopachrome isomerase
5,6-dihydroxyindole-2-carboxylic acid
5,6-Dihydroxyindole
Dopa quinone
Melanin
Indole stabilizing factor
Tyrosinase
Tyrosinase
3,4-Dihydroxyphenylalanine
Indole-5,6-quinone
Tyrosinase
Melanochrome
Tyrosine

Figure 2

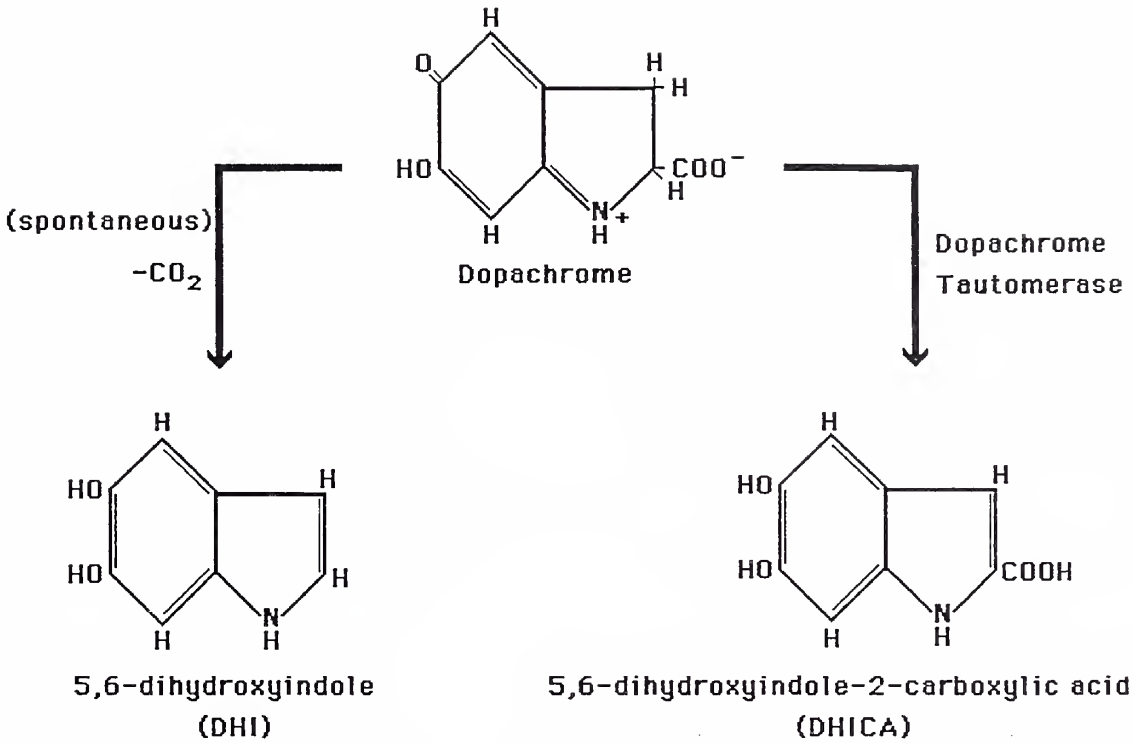

(spontaneous)
-CO2
Dopachrome
Dopachrome
Tautomerase
5,6-dihydroxyindole
(DHI)
5,6-dihydroxyindole-2-carboxylic acid
(DHICA)

Figure 3

ISOLATION OF MELANOSOMES

Start with 3-4 X 10^8 cells

(1) Suspend cells in 10 ml sodium phosphate buffer
Pass through 21g needle x 5, then 30g needle x 5
Homogenize

(2) Centrifuge (1000g, 5 min)
Save supernatant
Repeat steps #2 and #3 on pellet

(3) Centrifuge supernatant (20,000g, 20 min)
Resuspend pellet in 3 ml sodium phosphate buffer

(4) Layer resuspended pellet on sucrose gradient
[1.0M/1.2M/1.4M/1.6M/1.7M/2.0M]

(5) Centrifuge (100,000g, 60 minutes)

(6) Isolate band at 1.4M
Resuspend in 8 ml sodium phosphate buufer

(7) Centrifuge (100,000g, 60 minutes)

Figure 4

ISOLATION OF VESICLES

3-4 x 10^8 Cells

(1) Rinse with 0.1 M MES, 0.5 mM $MgCl_2$ (pH 6.8)
Centrifuge (1000g, 10 min, 4 C)

(2) Resuspend pellet in 5 ml MES buffer
Pass through 21g needle x 5, then 26g neddle x 5

(3) Centrifuge (700g, 5 min)
Save supernatant
Repeat steps #2 and #3 on pellet

(4) Centrifuge supernatant (20,000g, 30 min)
Discard pellet

(5) Centrifuge supernatant (100,000g, 60 min)
Resuspend pellet in 1-1.5 ml MES buffer

(6) Layer resuspended pellet on sucrose gradient
[60/50/40/10/5]
Centrifuge (55,000g, 120 min)

(7) Isolate bands at 10%/40% and 40%/50% interfaces
Resuspend in MES buffer
Centrifuge (100,000g, 60 minutes)

(8) Layer resuspended pellet on sucrose gradient
[60/30/5]
Centrifuge (55,000g, 45 min)

(9) Isolate bands at 5%/30%
Resuspend in MES buffer
Centrifuge (100,000g, 60 min)

Figure 5

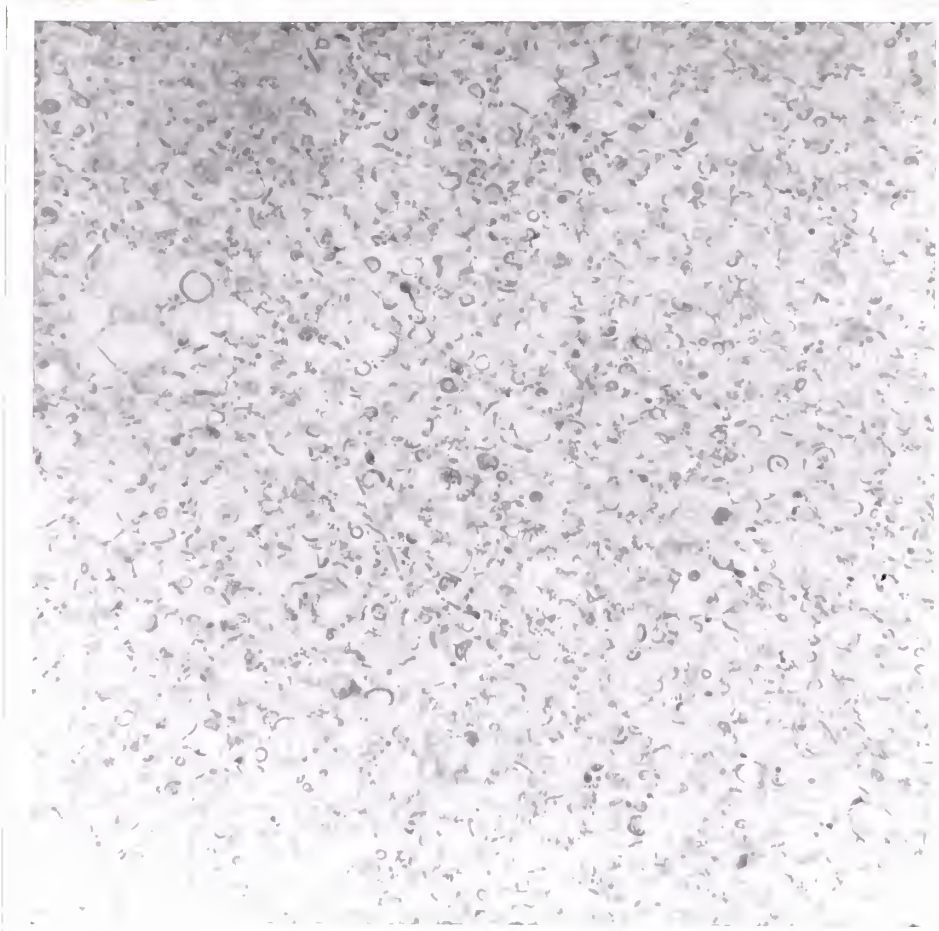


Figure 6. Decarboxylase Assay
Activity Accumulated in Corks

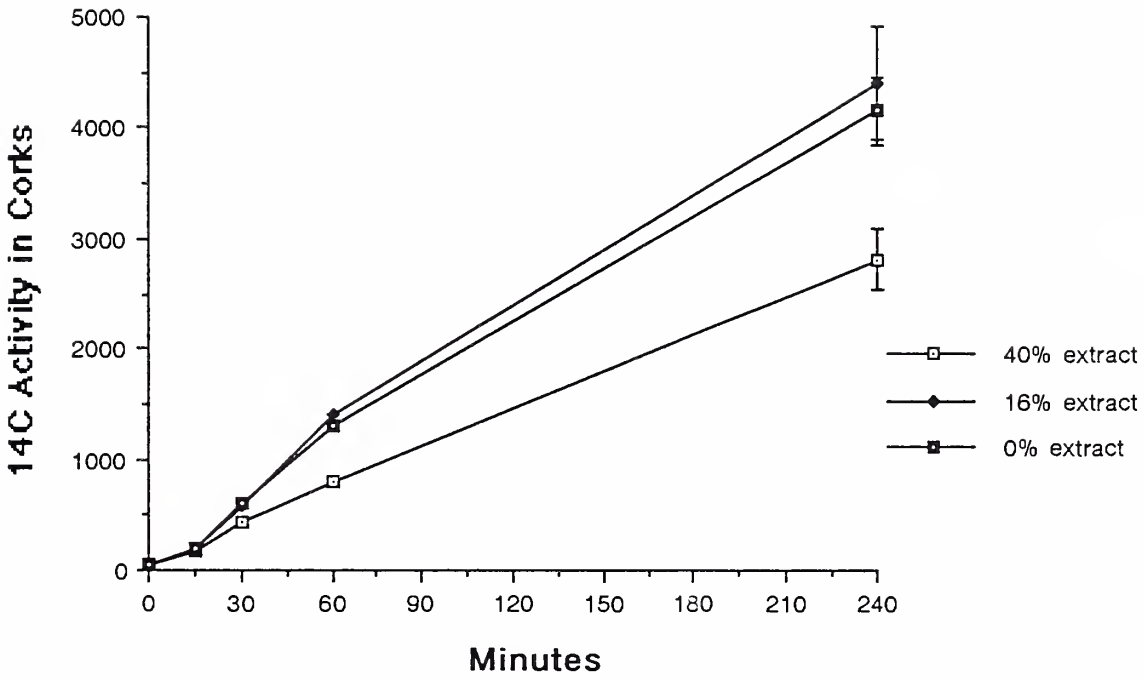


Figure 7. Decarboxylation Assay
Activity Remaining in Reaction Mixture

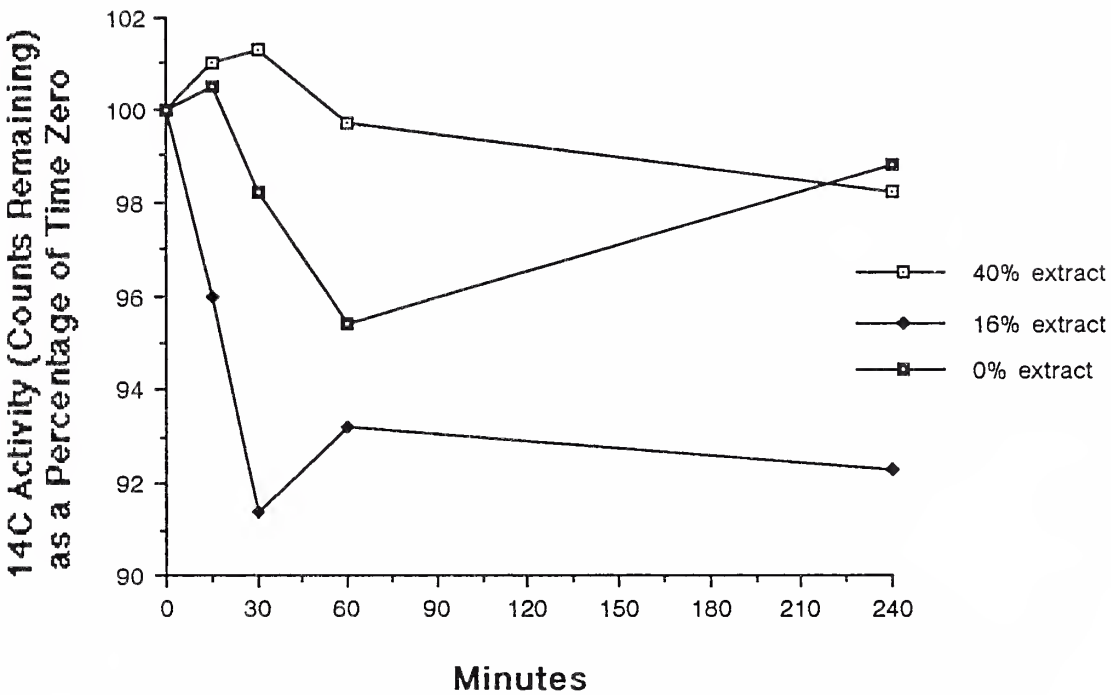


Figure 8. Decarboxylation Assay
Actiivty Accumulated in Corks

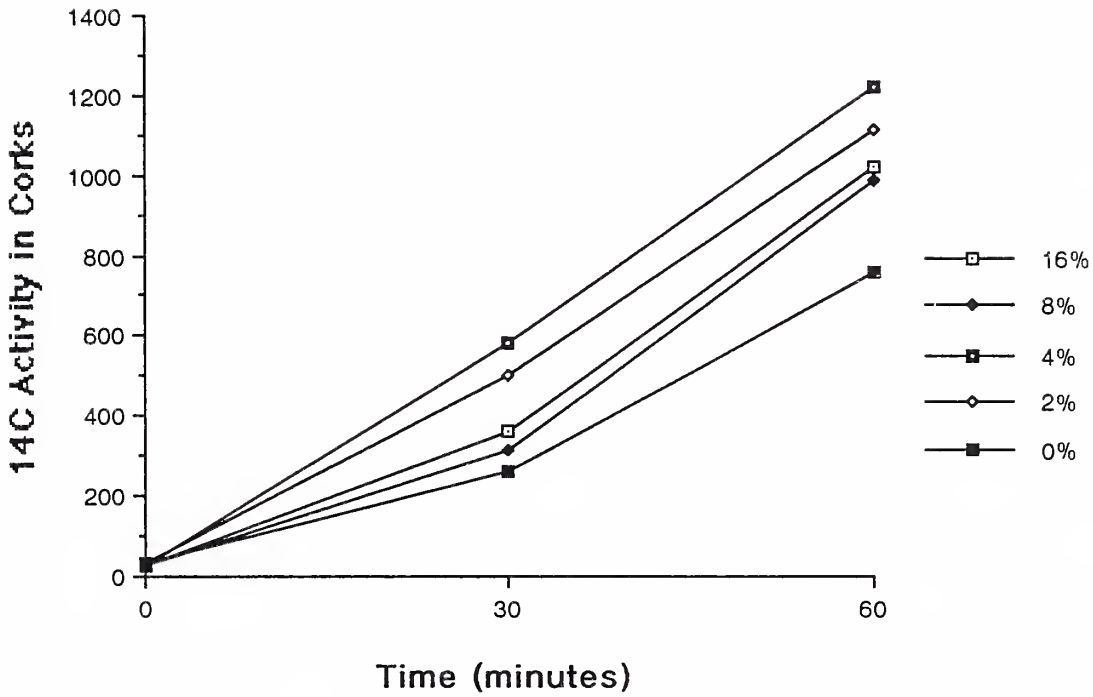


Figure 9. Decarboxylation Assay
Activity Remaining in Reaction Mixture

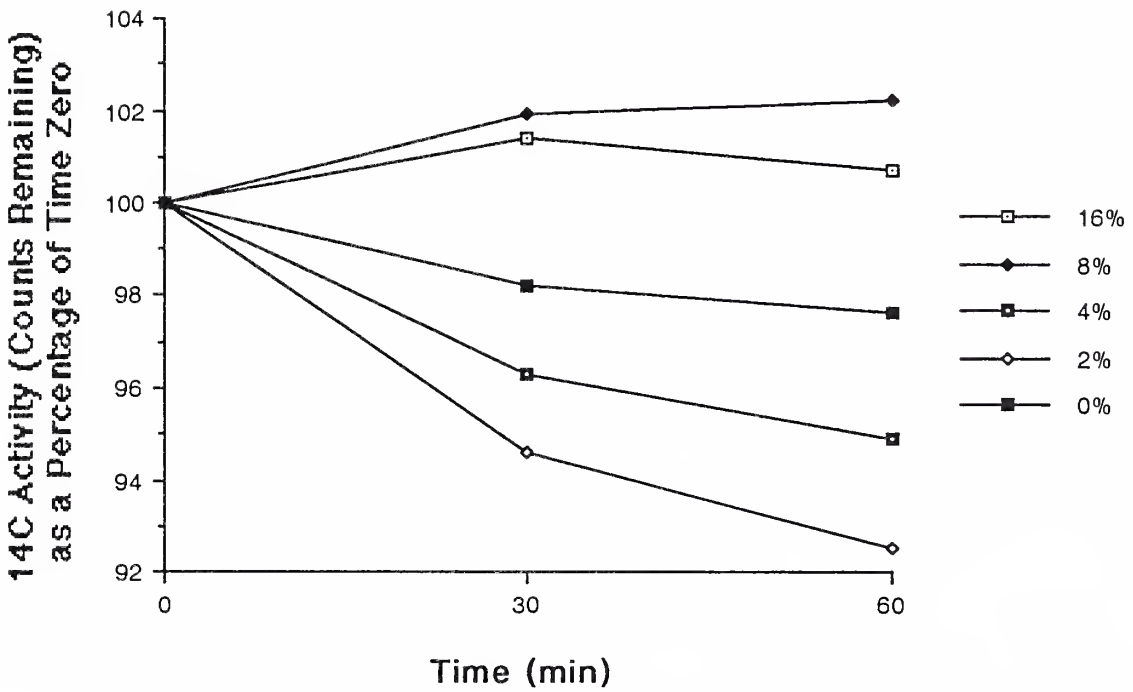


Figure 10. Decarboxylase Assay
Activity Accumulated in Corks

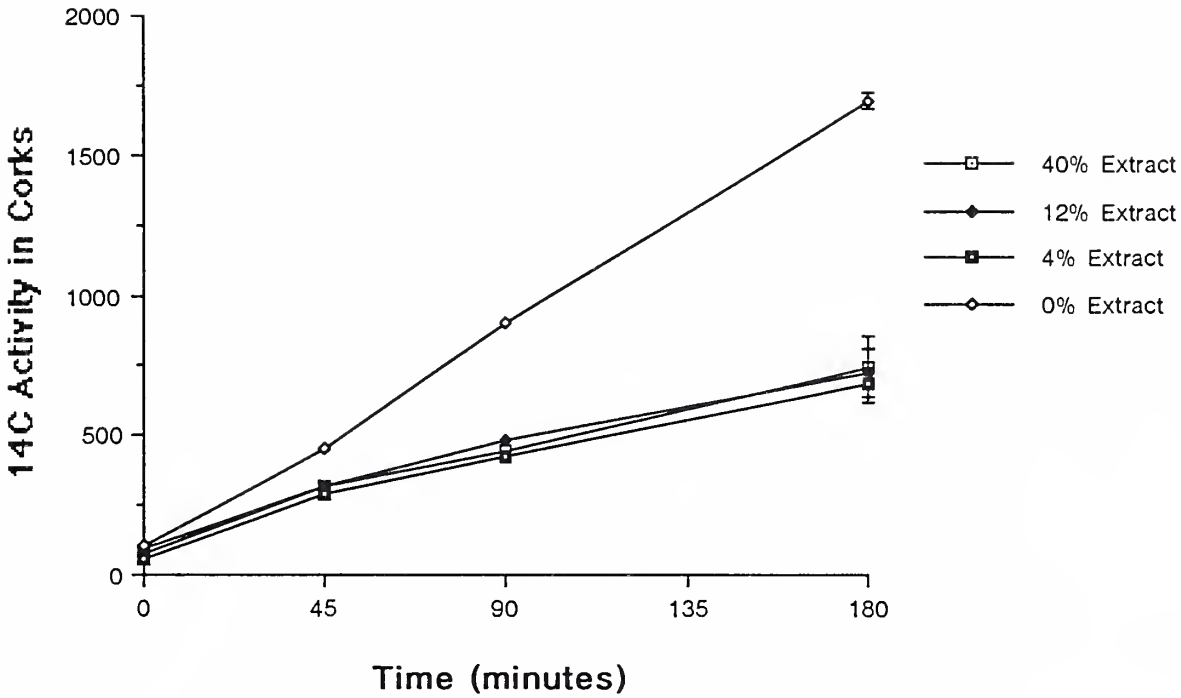


Figure 11. Decarboxylase Assay
Activity Accumulated in Corks
Wheat Germ Flow-Through

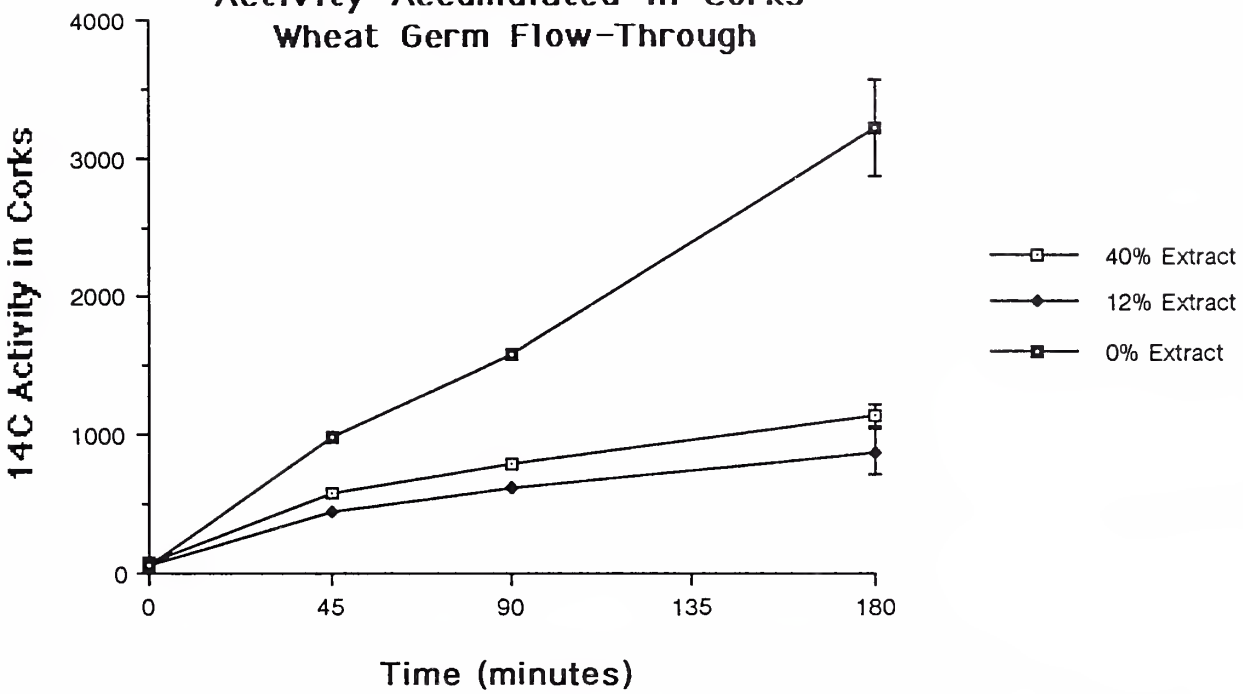


Figure 12. Decarboxylase Assay
Activity Accumulated in Corks
NAGA-Eluant

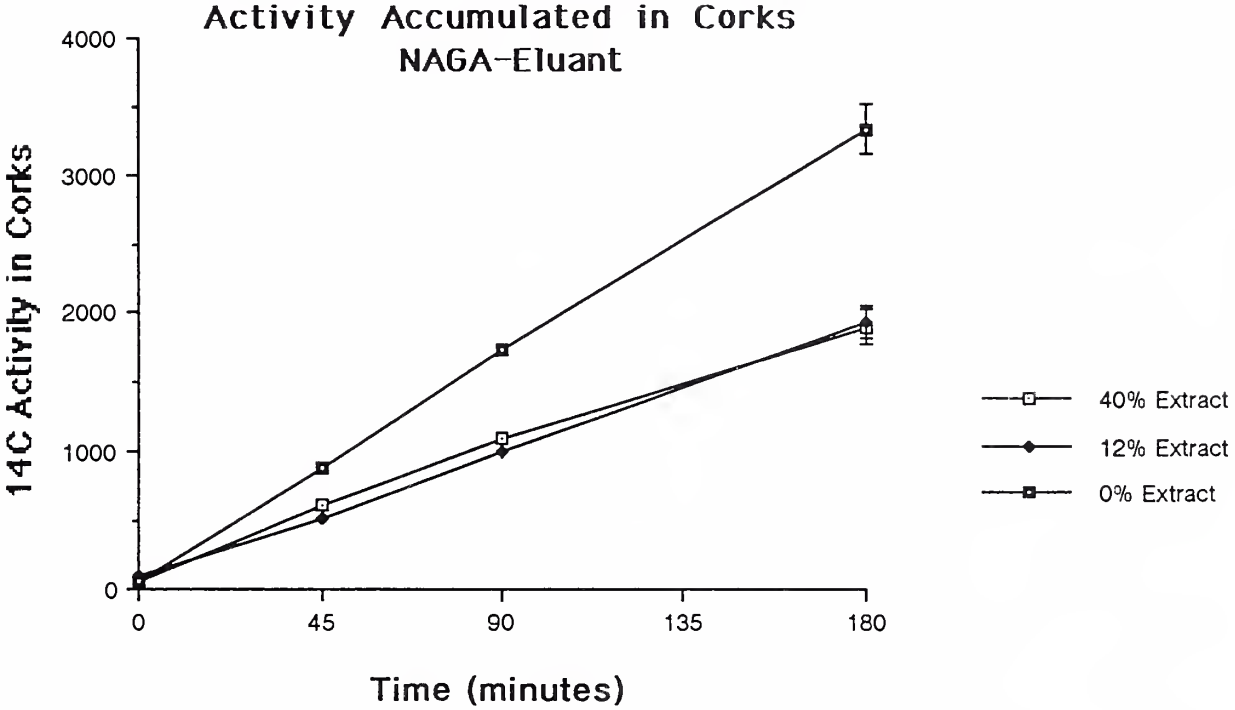


Figure 13. Decarboxylase Assay
Activity Remaining in Reaction Mixture

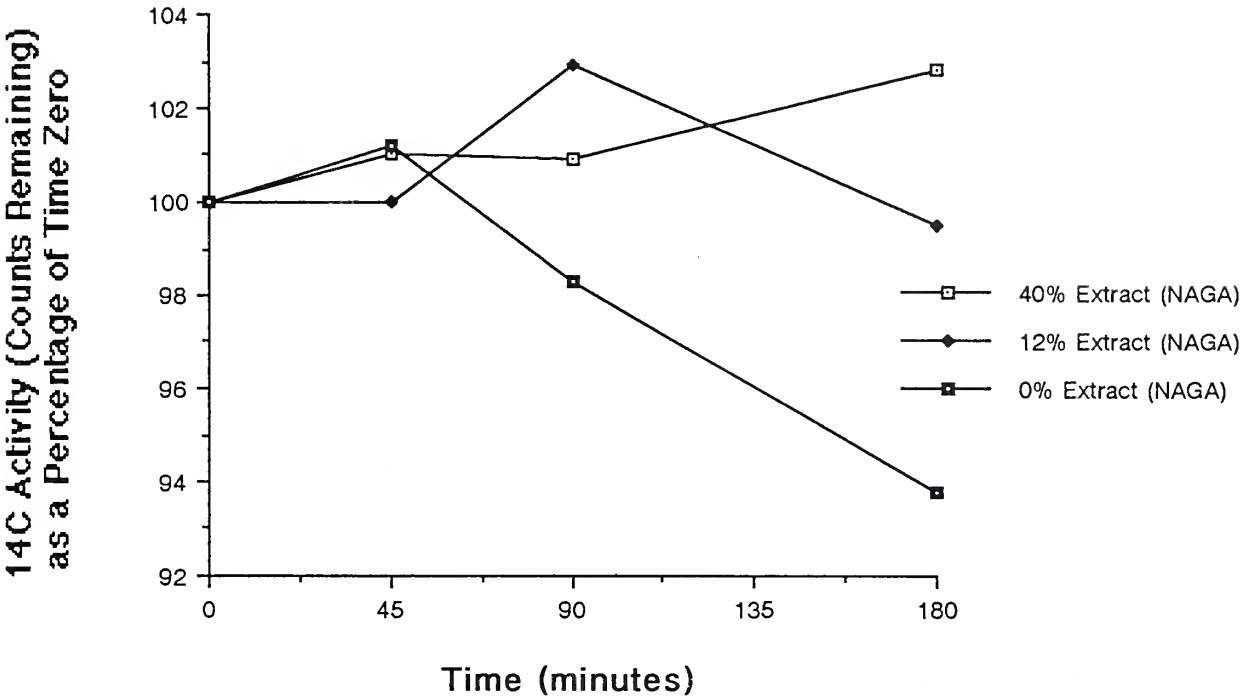


Figure 14. Decarboxylase Assay
Activity Remaining in Reaction Mixture

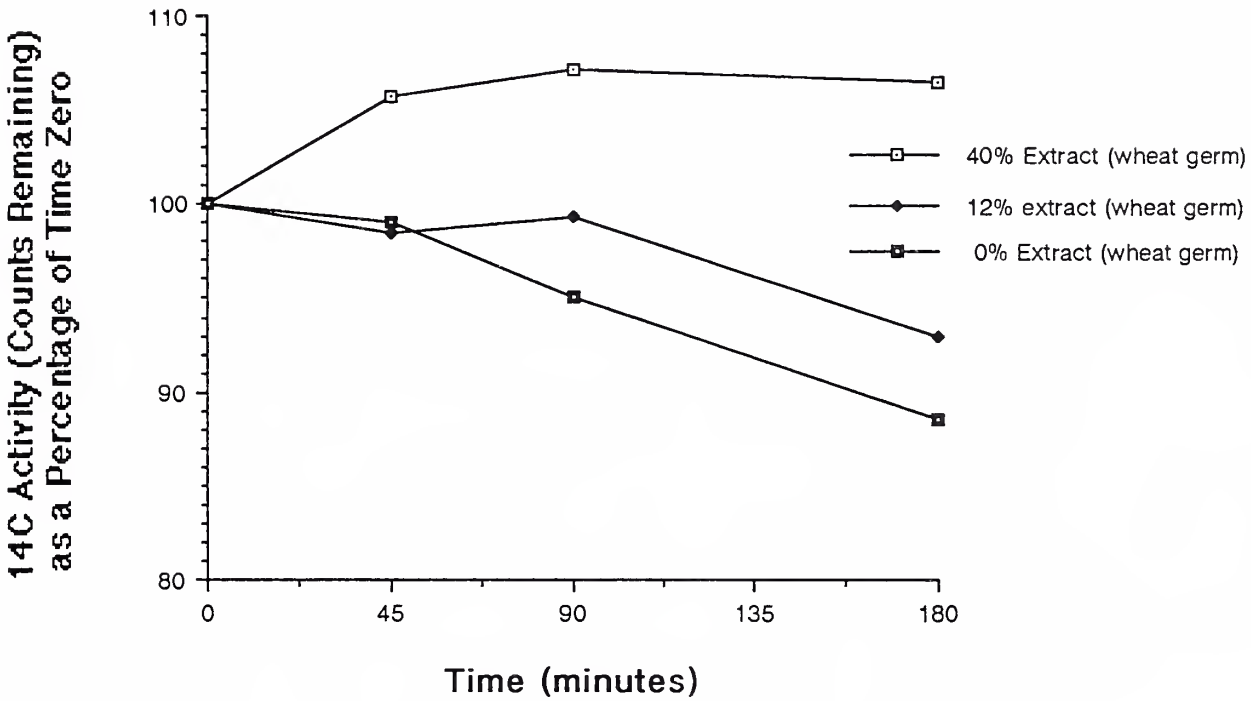


Figure 15. Decarboxylation Assay
Comparison of Extract and
Wheat Germ Eluant

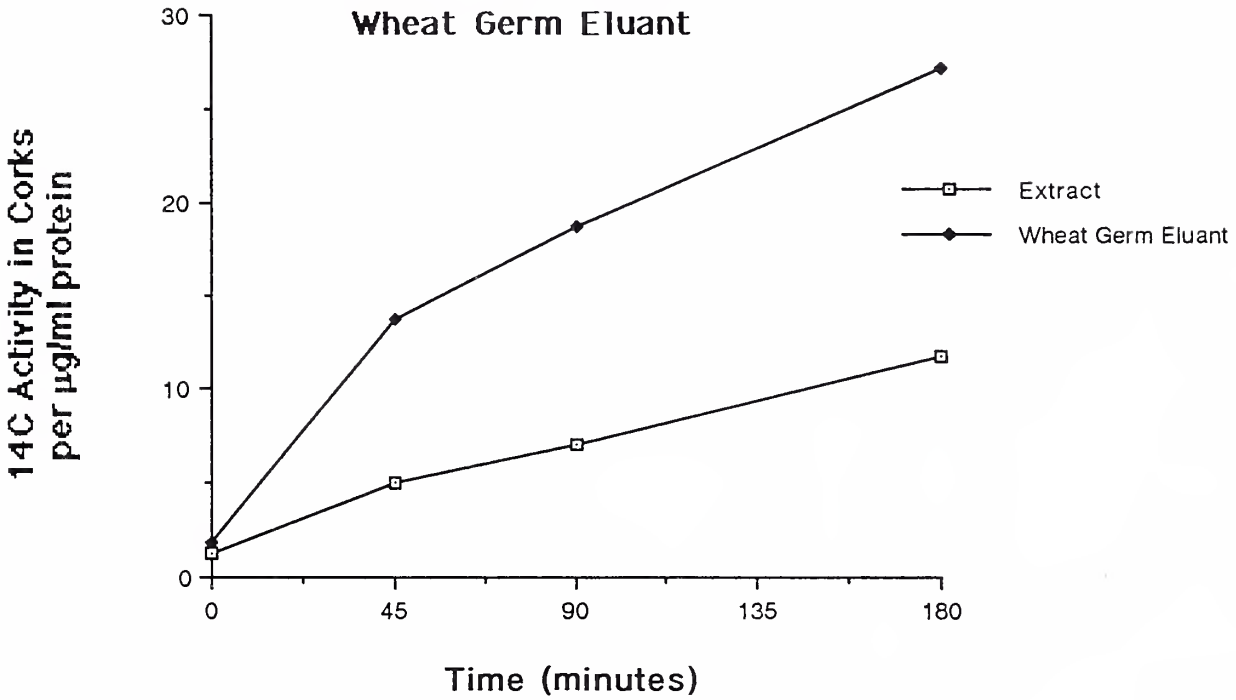


Figure 16. Decarboxylation Assay
Activity Accumulated in Corks
Cofactors and 10% Extract

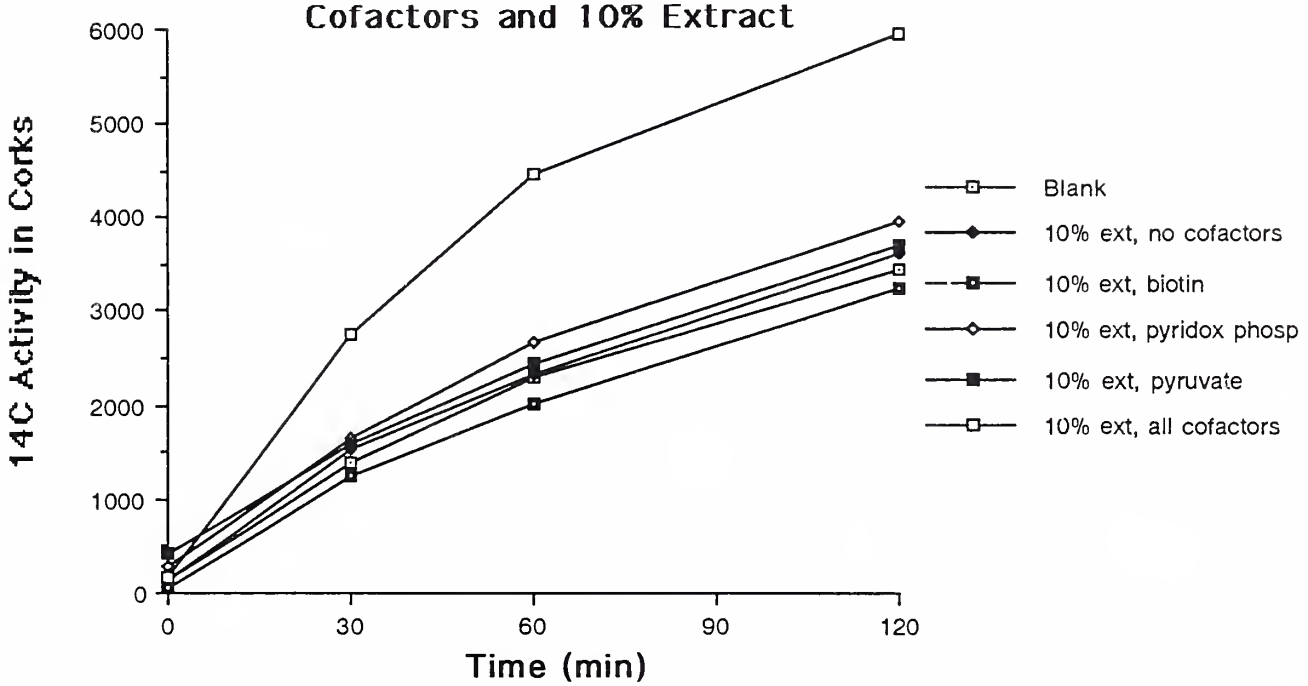


Figure 17. Decarboxylation Assay
Activity Accumulated in Corks
Cofactors and 50% Melanosomal Extract

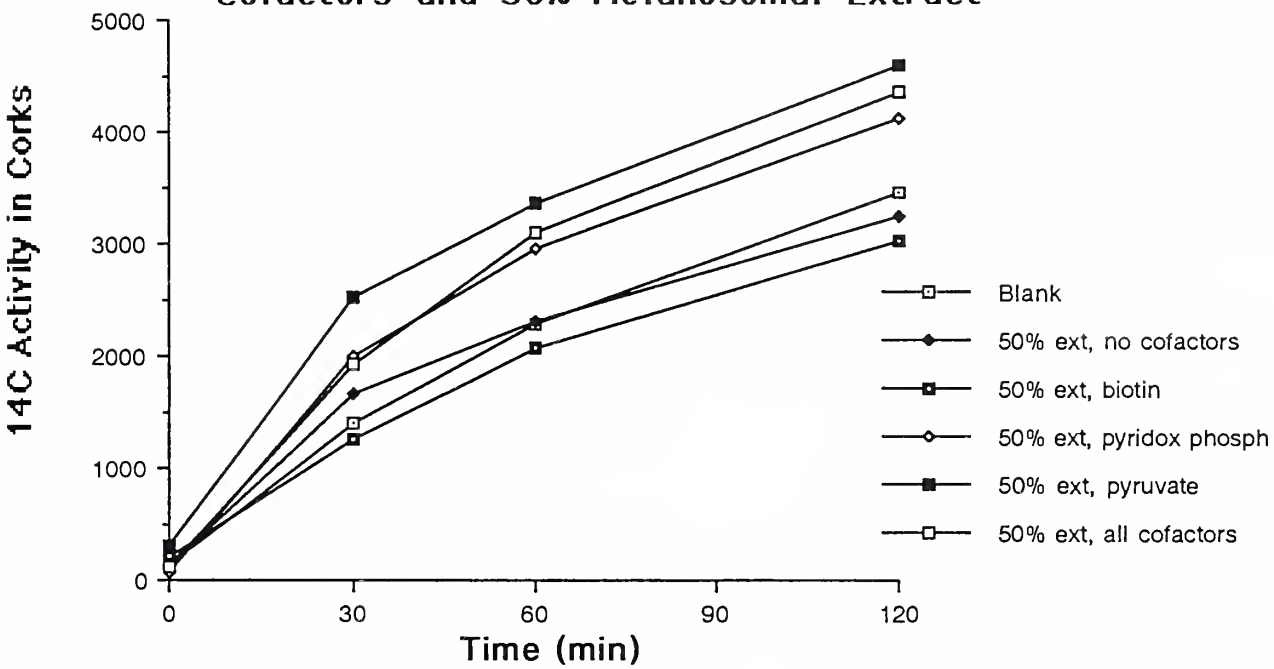


Figure 18. Decarboxylase Assay
Activity Remaining in Reaction Mixture
Cofactors and 10% Melanosomal Extract

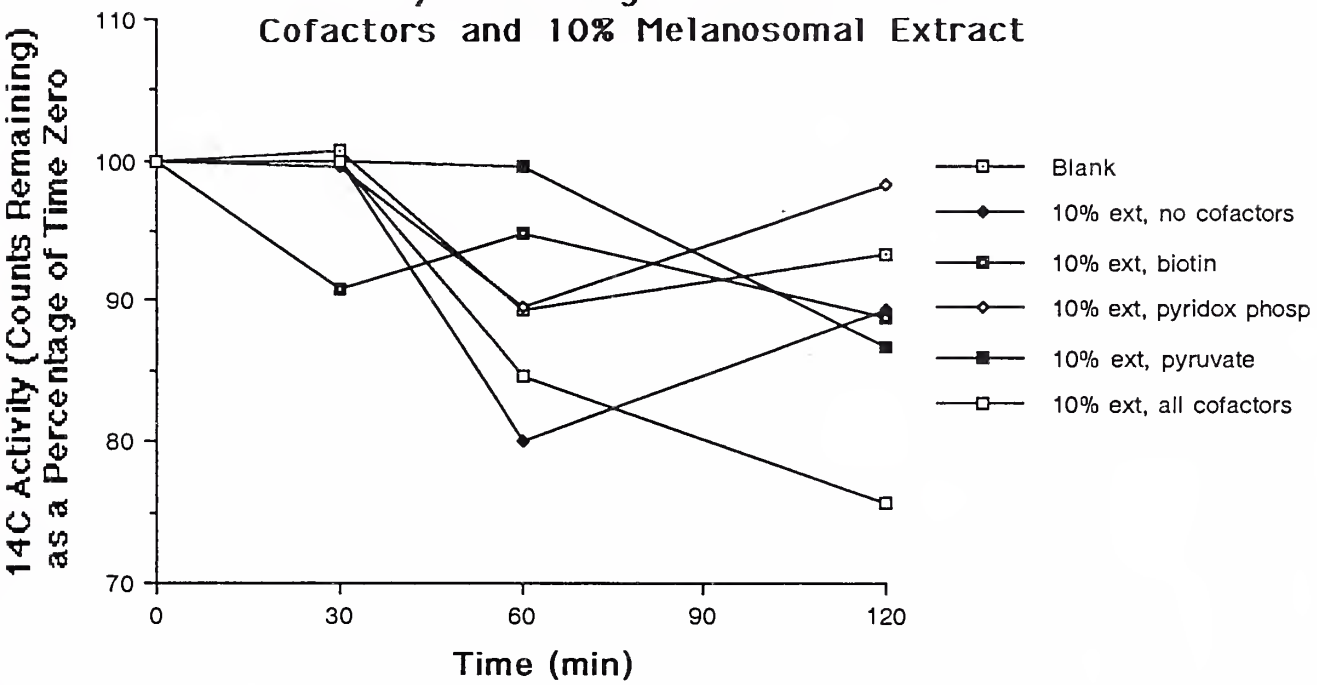


Figure 19. Decarboxylase Assay
Activity Remaining in Reaction Mixture
Cofactiors and 50% Melanosomal Extract

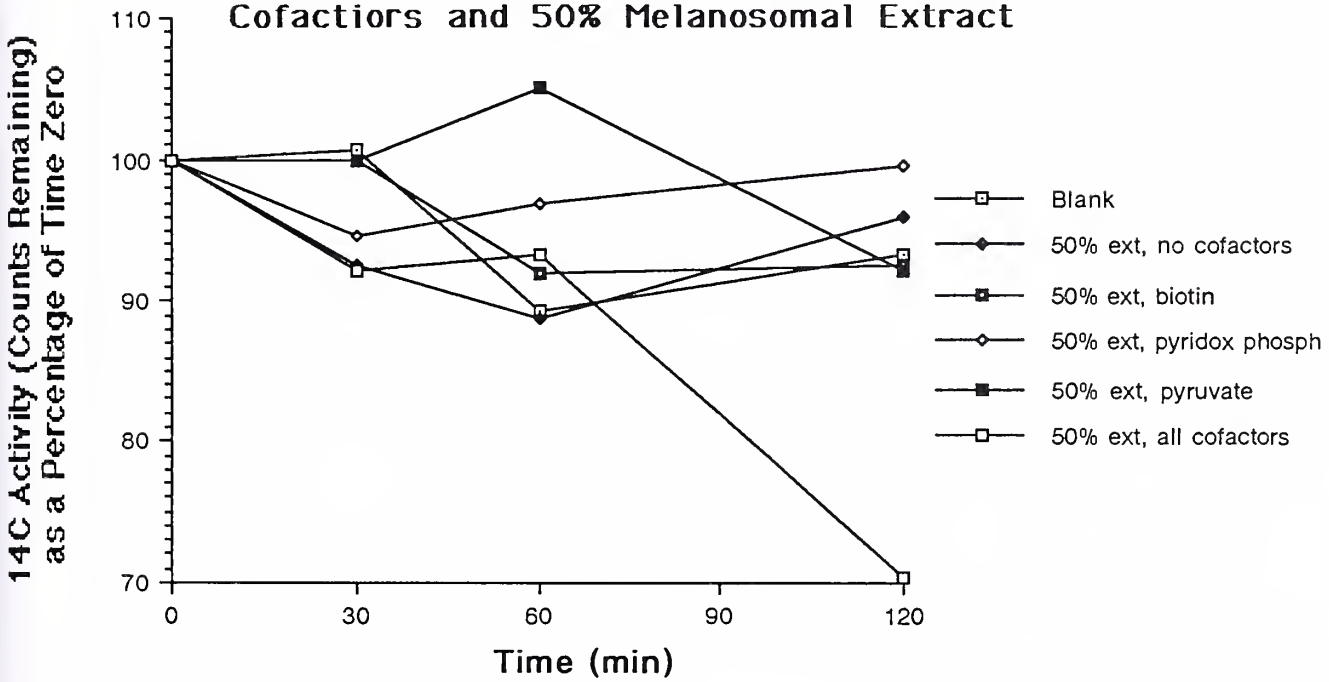


Figure 20. Protein Kinase Assay in the Presence of Magnesium

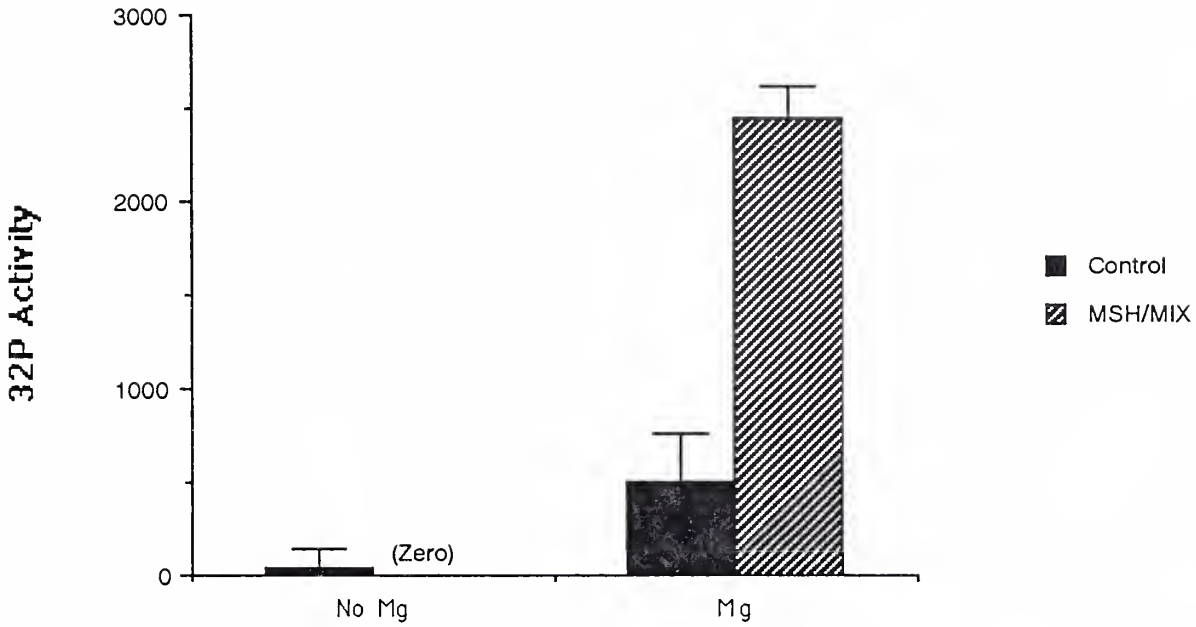


Figure 21. Protein Kinase Activity
Comparison of EGTA, Mg, and Ca

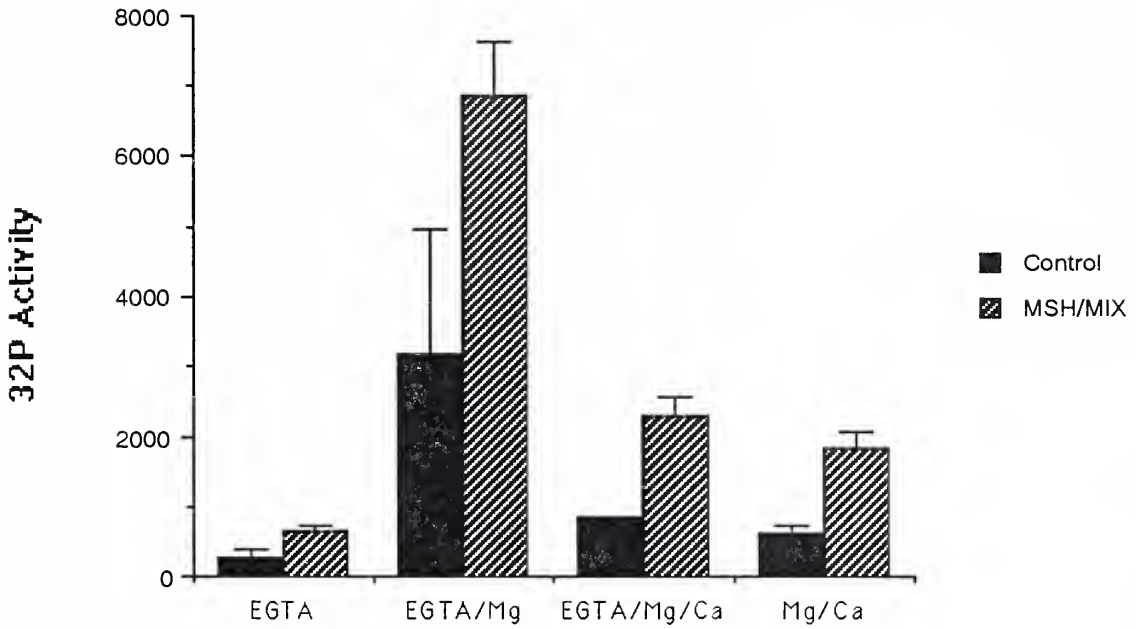


Figure 22. Protein Kinase Activity:
A Comparison of EGTA, Mg, Ca, Calmodulin

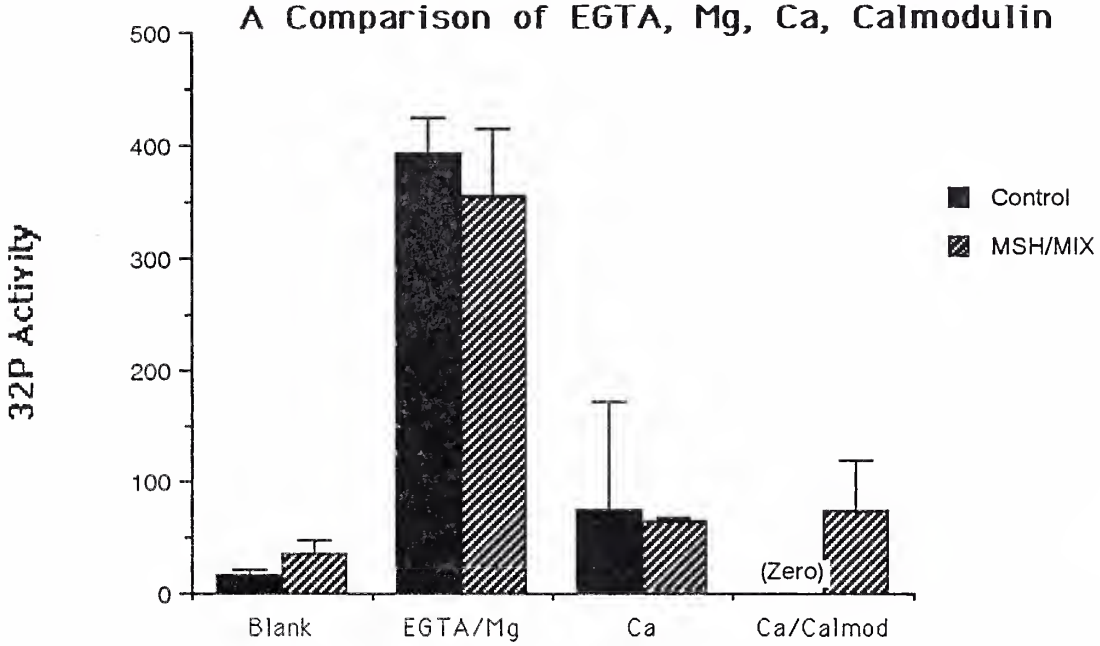
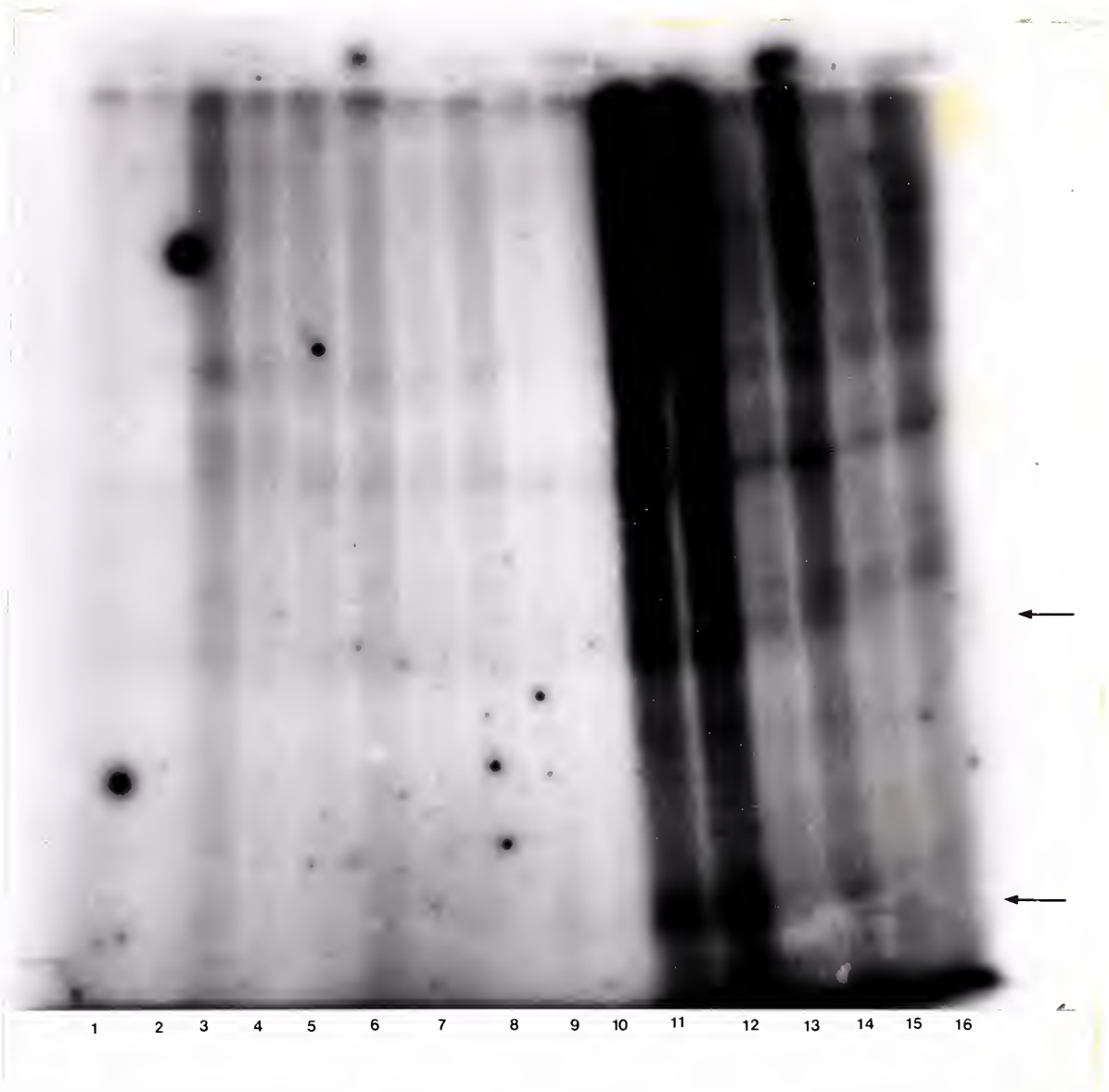


Figure 23



Lanes for Autoradiogram

Coated vesicles
Lanes 1,2- EGTA
Lanes 3,4- EGTA + Mg
Lanes 5,6- EGTA + Mg + Ca
Lanes 7,8- Mg + Ca

Coated vesicles incubated with MSH
Lanes 9,10- EGTA
Lanes 11,12- EGTA + Mg
Lanes 13,14- EGTA + Mg + Ca
Lanes 15,16- Mg + Ca

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