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Preface

This issue of the Southwestern Science Journal marks the second year of publication. The purpose of this journal is to provide a forum for student and faculty research and ideas. As the editor, I hope that this publication will stimulate the underclassmen enrolled at Southwestern to continue the tradition of research and investigation. All students interested in the pursuit of this tradition are encouraged to submit research and ideas for future issues.

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Detection of Amplification and Excision of Endogeneous and Gross Viral Genotype in Chronically Infected NIH-3T3 Cells

by Wayne W. Beam, Jr.

Introduction

Little initial notice was given to Peyton Rous's demonstration in 1910 that tumors could be produced in chickens from the cell-free filtrate of chicken sarcomas. But ever since Gross's finding that injection of cell-free filtrates from AKR leukemic mice into newborn AKR mice causes a high percentage of leukemia, numerous researchers have studied and questioned the viral causation of tumors. Today many tumor viruses are known, and it is evident that transformation of animal cells in culture (including human) can be achieved through integration of these viral genes into the host chromosomes. Although few viruses have been found to cause tumor formation in humans (and it is still in doubt whether viruses are a significant cause of human cancers), the relative simplicity of the virus particle itself has offered much information to the understanding of the fundamental genetic and molecular alterations which lead to the cancerous state of the cell.

Oncogenic viruses are those which can cause cell transformation of tumorogenesis upon infection. These viruses are divided into two categories, the DNA and RNA viruses. Oncogenic DNA viruses include the papovaviruses, to which the simian virus 40 (SV 40) belongs, the adenoviruses, which cause upper respiratory infections in man, and the herpesvirus, to which the Epstein-Barr (EB) virus belongs. Infection by these viruses involves viral integration into the cellular DNA which replicates and transcribes along with the host cell genome(1). It is presumed that one or more genes (onc genes) within the oncogenic virion is responsible for the transforming potential.

RNA viruses contain RNA-directed DNA polymerase within the virion, commonly known as reverse transcriptase; early upon infection, this enzyme transcribes the viral RNA into DNA, which then integrates into the parasitized cell genome, replicates, and transcribes along with it, leading to a constant production of virus. One notable group of RNA viruses are the "retroviruses," which are the only oncogenic RNA viruses(2). RNA retroviruses are classified into three groups according to the host range of the virus: ecotropic retroviruses (those which can re-infect the host cell), xenotropic retroviruses (those which cannot re-infect the host cell), and amphotropic retroviruses (those which can re-infect the host cell plus other compatible hosts).

It is well established that retroviruses obtained their oncogenic properties by incorporating cellular onc genes which have been evolutionarily conserved in many vertebrate species. That is, researchers have found onc genes that are commonly switched off in normal mammalian cells. Slowly transforming retroviruses have shown to activate cellular onc genes by integration into an adjacent position. The insertion sequences of these viruses may introduce a strong promotor for increased onc gene activation. Also, carcinogenic radiation of

chemicals may activate the onc genes(3).

It is possible that viral onc gene products differ in subtle but critical ways from cellular onc products. One such onc gene has been found to code for a protein kinase product, which binds to the inner cell membrane and phosphorylates the amino acid tyrosine of cellular proteins. The phosphorylation of proteins is one regulatory mechanism for growth control. Normal cell protein kinases are known to phosphorylate serine and threonine; therefore this enzyme (an oncogenic product) could greatly alter the growth regulation of cells(4).

Much attention has been focused upon the mechanisms by which carcinogens act in order to transform normal cells. Since the tumor state is passed on to both daughter cells after cell division, mutations in the DNA have been regarded as the likely affect of carcinogens. These carcinogens may be physical (i.e. ultraviolet light), chemical (i.e. polycyclic hydrocarbons), or biological (i.e. oncogenic viruses) in nature. The first two agents above are known to cause mutations in the DNA structure. If retroviruses disrupt DNA repair mechanisms, then their presence could increase the probability for cell transformation after exposure to carcinogens. Two proposed mechanisms include increased sensitivity to chemical or radiation carcinogenesis due to retrovirus infection, and DNA damage caused by the carcinogens could make available new viral integration sites which might alter cellular control processes. This transposition of movable elements, therefore DNA rearrangements, might be a general mechanism for tumor induction, possibly by the effects on cellular oncogenes. For this reason, one approach to carcinogenesis is under consideration: What affect, if any, do carcinogens cause upon endogenous or inserted viral genomes within the normal or infected cell?

Ultraviolet radiation (UVR) carcinogenesis has been studied extensively for several decades, particularly as the cause of skin cancer. The UVR wavelengths (280nm-320nm) are known to induce mutagenic thymine dimers and single strand breaks in DNA. But whether UVR acts by a direct or indirect action on DNA is not well understood. Other cellular organelles may be the initial target. But it seems that the susceptibility to the effects of UVR on a particular cell type depends on its DNA repair mechanisms; if errors are made, the change in the DNA sequence may trigger tumorgenesis(6).

UVR may cause DNA damage indirectly by interacting with chemicals in the cell and generating a carcinogenic potential that is not present with either agent alone (UVR or chemical). In 1970, Pond reported that short UV exposure to mouse skin, followed by repeated application croton oil, caused skin tumors; neither agent caused tumor formation alone(7). Mondal and Heidelberger demonstrated that a cloned line of C3H mouse fibroblasts were not transformed in vitro by UVR or by chemical treatment with TPA; however, UVR followed by a non-transforming or non-cytotoxic concentration of TPA in the culture medium resulted in transformation(8).

TPA (4β-phorbol 12β-myristrate 13α-acetate), a phorbol ester iso-

lated from croton oil, is an active tumor promoting agent. Most tumor promotors are non-carcinogenic alone and are believed to produce superoxide anion radicals; these radicals act as the mediators of gamma radiation and attack the DNA or other cellular components(9). TPA is also believed to be an effective viral inducing agent, although the mechanism for induction is not known(10).

The halogenated pyrimidines, BUDR (5-bromodeoxyuridine) and IUDR (5-iododeoxyuridine), are the most potent in vitro viral inducing agents known. Although the exact mechanism is disputed, most reporters believe that IUDR (or BUDR), present in the culture medium, is incorporated into the DNA in place of thymidine during the S-phase (DNA synthesis) of the cell cycle, creating a copy error after subsequent cell cycles(11). This copy error may be mutagenic or viral inducing, although again the mechanisms involved are not known. IUDR incorporation has been shown to increase the sensitivity of cells to irradiation, causing numerous single-stranded breaks. It is also believed that halogenated pyrimidine incorporation into host cell genomes alters the binding of a repressor-like protein to endogeneous viral sequences, allowing transcription and expression of otherwise repressed viral genes(12).

Although viral-infected cells contain minute amounts of free virus particles, several reporters have demonstrated that some carcinogens, such as UVR, cause increased excision of viral DNA from cellular DNA. UVR has been shown to induce low levels of infectious SV 40 viruses from SV 40-transformed Chinese hamster cells as well as infectious leukemic viruses from the C57BL mouse strain(13). Since UV alone causes transformation in some cell lines, it is believed that this and/or other carcinogens may give rise to amplification, excision, and subsequent multiple integration of viral genome back into the host cell DNA. It is this question upon which this study is based.

I work with the NIH-NV cell line, which are NIH-3T3 cells recently infected with Gross virus, an ecotropic RNA retrovirus. NIH-3T3 cells were used as a control group since this uninfected cell line has been found to naturally contain endogenous viral sequences. With these two cell lines, a comparative study of Gross and endogenous viral genome amplification and excision, stimulated by physical and/or chemical intrusion, was conducted. The cells in culture were exposed to three agents, alone or in combination: UV, TPA, and IUDR. After various time exposure, the cells were extracted by the method of Hirt, which selectively precipitates the cellular DNA in the presence of sodium dodecyl sulphate (SDS) and 1M NaCl, while RNA and extrachromosomal viral DNA remain in the supernatant(14). These two fractions were separated and analyzed separately. The precipitate, or Hirt pellet, was cut with restriction endonucleases Eco R1 and Pst 1 for detection of integrated viral amplification; the Hirt supernatants were analyzed for a relative comparison in the amounts of excised viral genomes from the host cell genome.

Materials and Methods

Cell lines and cell cultures. All NIH-3T3 and NIH-NV cells were cultured in plastic petri dishes (Falcon 100X20mm style) at 37°C in humidified air with 5% CO_2.

Experiment 1. Confluent plates were divided (at random) into sets of ten; eight sets NIH-3T3 and eight sets of NIH-NV cells were treated as follows:

SET	NUMBER OF PLATES	TREATMENT
1.	10	Control, normal medium(MEM)
2.	10	UV 4sec, MEM
3.	10	UV 2sec, MEM
4.	10	UV 4sec, TPA in MEM
5.	10	UV 2sec, TPA in MEM
6.	10	IUDR 24hr, MEM
7.	10	IUDR 24hr, TPA in MEM
8.	10	TPA in MEM

MEM was supplemented with antibiotics, L-glutamine, and fetal calf serum. Culture medium was removed before UV exposure and immediately replaced thereafter. The medium was replaced at 24hr and 72hr, and the cells were extracted at 96hr. Both UV 4sec sets were extracted at 192hr to allow for cell recovery.

Experiment 2. One plate NIH-NV cells from set 2 (UV 4sec, MEM) and set 4 (UV 4 sec, TPA in MEM) were each split after 13 days into 18 plates: 9 maintaining the identical medium, and 9 switching the medium (MEM in place of TPA in MEM and vice versa); these plates were cultured 6 days and extracted. One plate NIH-3T3 from set 2 and set 4 were each split after 13 days into 9 plates; the plates were cultured 6 days in the same medium and extracted.

Experiment 3. 60 confluent plates of NIH-NV cells were divided into 12 sets, treated, and extracted as follows:

UV 4sec, MEM

Number	Number of Plates	Extraction Time
1.	5	Control
2.	5	0hr
3.	5	6hr
4.	5	12hr
5.	5	18hr
6.	5	24hr

TPA 24hr, UV 4sec, TPA in MEM

Number	Number of Plates	Extraction Time
1.	5	Control
2.	5	0hr
3.	5	6hr
4.	5	12hr
5.	5	18hr
6.	5	24hr

TPA concentration was 0.15 μg/ml culture medium.

Hirt Extraction. Procedures of Hirt were followed with several modifications. Each plate was rinsed with 15ml ice cold phosphate-buffer saline (without Mg^{+2}, Ca^{+2}, or serum); then 15 ml cold TE solution (10mM Tris-Cl pH 7.6, 10mM EDTA) were added to each plate 2min and removed. 3ml 60° C Hirt solution (TE with 1%SDS) were then added to each plate and allowed to stand at room temperature 5min. By gently swirling the dish, the lysate of 5 plates were pooled into a 30ml centrifuge tube (polypropylene, Oak Ridge type); the five plates were washed by a common 3ml Hirt solution transfer, and this was added to the 30ml tube. 5ml 5M NaCl (filtered) were added to each tube and heated briefly at 60° C to ensure even dissolving. Each tube was inverted 25 times and stored overnight (4°C). The following day, the tubes were ultracentrifuged (27K, 1hr); from each set, the supernatant was removed from the chromatin DNA pellet and placed in a 50ml plastic conical tube. (The pellet may be stored at 4°C).

Hirt Supernatant. Self-digested pronase was added to each supernatant to a final concentration of 50μg/ml and incubated (51°C, 3hr). The solution was concentrated by dialysis to 1ml, placed in a 10ml centrifuge tube, and extracted with redistilled phenol/ chloroform/ isoamyl alcohol (25:25:1). The bag was rinsed with 1ml TE and back extracted, combining the aqueous phases into a final 10ml centrifuge tube; the solution was precipitated with 95% EtOH (overnight, -20°C), and then centrifuged (Servall, 10K, 30min, 4°C) to pellet the extrachromosomal DNA.

The EtOH was decanted, and 1ml TE solution was added to dissolve the DNA. The solution was incubated with ribonuclease (10μg/ml, 2hr, 40°C); 50μl 10% SDS was added and then incubated with self-digested pronase (200μg/ml, 2hr, 51°C). This solution was extracted with phenol/chloroform/isoamyl alcohol (25:25:1) and the aqueous layer was removed and precipitated with 95% EtOH (overnight, -20°C), centrifuged (Servall, 10K, 30 min, 4°C), decanted, and the pellet lyophilized (5min). 150μl of a 4:1 Hirt:EP buffer (0.25 mg/ml bromphenol blue, 1% glycerol, 0.5% SDS) solution was added to each tube and rolled to dissolve the pellet; the sample was incubated (60°C, 5min) and centrifuged (Servall, 3K, 5min, 4°C) prior to gel electrophoresis.

Hirt Pellet. 10 ml TE was added to each Hirt pellet (corresponding to five plates from each treatment). The tube was incubated (10min, 51°C) and inverted often to dissolve the pellet. 2mg pronase was added and the pellet solution incubated for 3 additional hours; the solutions were then placed at room temperature (overnight). Each solution was phenol/chloroform/isoamyl alcohol (25:25:1) extracted, back extracted with 2 ml TE, and the combined aqueous layers extracted once again. The aqueous layer was transferred to a plastic, conical 50 ml tube; the tube was tilted and 25ml cold 95% EtOH was slowly added. The DNA was spooled onto a pipette and stored in a conical test tube with 95% EtOH (overnight, 4°C).

The alcohol was decanted and each pipette lyophilized (5min); 1.5ml ddH 0 was added to each tube and stored (overnight, 4°C). 1.5 ml (200mM Tris-Cl pH 7.2, 100mM NaCl, 10 mM MgCl , 2-mercaptoethanol) sterilized solution was added to each tube and lightly vortexed 15sec to unwind the DNA. 75 units Eco Rl restriction endonuclease were added to each and incubated (4hr, 37°C) with frequent swirling. After removing the pipette, 0.4ml was removed for optical density measurement; 2ml was placed in a 10ml centrifuge tube, precipitated with 95% EtOH (overnight, -20°C), and centrifuged (Servall, 10K, 15min, 4°C). After decanting the alcohol, the remaining 0.6ml in the conical tube was added, along with 1.4 ml TE rinse; this 2ml was precipitated with 95% EtOH (overnight, -20°C), centrifuged (Servall, 10K, 15min, 4°C), decanted, and the pellet lyophilized (5min).

By the optical density measurements (260nm and 280nm), sterile TE was added to give 100μg DNA/ml; 0.4 ml of this was placed in a separate 10ml centrifuge tube; 0.1ml ddH_2O, 0.75ml (60mM Tris-Cl pH 7.5, 30mM $MgCl_2$, 150mM $(NH_4)_2SO_4$, 300μg/ml bovine serum albumin) sterilized solution, and 50 units Pst 1 restriction endonuclease were added and incubated (4hr, 37°C). The solution was alcohol precipitated (overnight, -20°C), centrifuged (Servall, 10K, 15min, 4°C), decanted and lyophilized (5min). Eighty μl 4:1 Hirt:EP buffer were added, rolled to dissolve the pellet, incubated (60°C, 5min), and centrifuged (Servall, 3K, 5min, 4°C), prior to gel electrophoresis.

Gel Electrophoresis and Southern blot hybridization. 30 μl samples were added to a 0.6% agarose gel into 1mm comb slots. Electrophoresis was performed in EP buffer (40mM Tris-Cl pH 7.8, 1mM EDTA, 5mM sodium acetate) at 30V for 22hr. The gels were denatured by two 500ml washings with 0.25N HCl, rinsed with H_2O, two washings with Southern-OH (0.5M NaOH, 1M NaCl), rinsed with H_2O, two 500ml washings with Southern-Tris (0.5M Tris-Cl pH 7.6, 3M NaCl), and rinsed in 2XSSC (300mM NaCl, 30mM sodium citrate). After overnight transfer to nitrocellulose paper, the paper was baked (vacuum oven, 80°C) and prehybridized (2hr, 42°C). Each experimental group was hybridized to two separate Nick translated probes, pG100 and IS, for at least 48hr. The paper was washed repeatedly with 2XSSC and once with 0.2XSSC, dried, and exposed to Kodak XAR-5 X-ray film with Dupont Cronex Xtra-Life Lightning Plus intensifying screens at -80°C for 1 to 6 days.

Results and Discussion

Normal cells in culture form a monolayer on the plate, displaying growth control and contact inhibition mechanisms. Transformed and tumor cells in culture, however, do not exhibit contact inhibition and continue to grow and to divide into several cell layers. The mechanisms responsible for this loss of growth control are still not understood.

The NIH-3T3 cells in normal medium (Figure 1) exhibit a normal morphology; likewise, the NIH-NV cells in normal medium (Figure 2) show a similar shape. No transformation of the NIH-NV cells is apparent due to the chronic nature of the Gross virus infection.

NIH-NV cells treated with UV 4sec in normal medium (Figure 3) were not transformed; however, the picture shown was taken approximately 12 days after UV exposure to allow for cell recovery. Hence, this physical DNA-damaging agent killed a majority of these cells but did not transform the survivors. Also, the NIH-NV cells cultured with TPA (0.15μg/ml medium, Figure 4) were not transformed but exhibited a noticeable degree of dead cells.

The NIH-NV cells exposed to UV 4sec followed by TPA in the culture medium resulted in obvious changes in morphology (Figure 5). Each individual nuclei cannot be seen clearly or distinct from other nuclei, which demonstrates layering of the cells. This is one criterion for transformed and tumor cells in culture; whether these cells are actually tumorogenic is not known, but the change in the cell morphology is interesting. The "apparent" transformation of these cells follows a similar observation using UV and TPA synergistically as a tumor initiating and tumor promoting agent respectively(15). The control NIH-3T3 (Figure 6) under the same treatment were not affected as greatly s the Gross viral infected cells, but some alteration in the morphology is noticeable.

After electrophoresis on two separate gels for each extracted DNA sample, the separated fragments are transferred to a nitrocellulose filter (see Figure 7) by the Southern transfer technique. A high salt buffer travels through a paper wick, the gel, and the nitrocellulose filter, being absorbed by the paper towels. This transfers the DNA from the gel onto the filter; the filter is then baked in a vacuum oven to covalently bond the DNA onto the filter.

Each filter was then hybridized to one of two probes, either pG100 or IS (see Figure 7). pG100 probe is radioactively labelled plasmid DNA containing the entire Gross viral genome. IS, or insertion sequence, probe is radioactively labelled DNA from a segment of the LTR (long terminal repeat) sequence characteristic of all endogeneous viral sequences. Separate hybridization by these two probes allows the detection of Gross virus and endogeneous viral genomes in both the Hirt supernatant (containing the excised, extrachromosomal DNA) and the Hirt pellet (containing integrated Gross virus and endogeneous viral sequences in the chromatin DNA). The filters are exposed to X-ray film, and the presence of radioactivity produces bark bands representative of the location of hybridized DNA.

In order to determine viral sequence amplification in the chromatin DNA, the Hirt pellet was cut with two restriction endonucleases, Eco R1 and Pst 1 (see Figure 8). Integrated Gross virus contains two Pst 1 cleavage sites, located on either end of the viral gene at the LTR's. Endogeneous viral sequences also have two Pst 1 cleavage sites at the LTR's, but these also contain one or more Eco R1 cleavage sites within the viral genome. Therefore, after cleavage by these two enzymes and gel electrophoresis of the DNA extracts, one should expect approximately an 8.3kbp band corresponding to the Gross virus genome, plus one or more bands 6.7kbp or less, corresponding to endogeneous viral sequences. A sample of Hind III fragments of radioactively labeled λ phage DNA is placed on the gel for known molecular weight markers.

The procedure for determining viral gene excision and amplification in the treated cells had several problems; in other words, clear results are few. In Experiment 1, the Hirt supernatant hybridized with pG100 probe gave three distinct bands near the 8.3kbp range. This clearly marks increased excision of Gross viral genome from the NIH-NV cells treated with UV 4sec and TPA, IUDR, and IUDR with TPA. Although IUDR and TPA alone did not alter the cell morphology, it was noted earlier that UV and TPA together "transformed" the NIH-NV cells in culture; here, the autoradiogram shows increased Gross viral genome excision within these transformed cells. The autoradiogram for IS probe show extensive hybridization, signifying endogeneous viral genome excision; however, no distinct bands are present and hence no conclusion may be reached identifying any particular viral sequence (see Figure 9).

The Hirt pellet hybridized with IS probe shows no distinct bands for excised endogeneous viral genes, although hybridization is apparent for cells treated with IUDR, IUDR and TPA, and TPA alone. The NIH-NV cells treated with IUDR and TPA show greater hybridization, so these two viral-inducing agents may work synergistically in culture.

The autoradiogram of the remaining filters hybridized with pG100 probe were not readable due to an improper washing technique; too much radioactivity remained on the filter, overshadowing any bands that might be present. If time had allowed, these filters could have been washed and exposed to X-ray film again. Therefore, the discussion of the remaining results will deal with hybridization with IS probe only.

The autoradiogram from Experiment 2, Hirt pellet, demonstrates results from the inappropriate washing of the membrane. The NIH-NV cells cultured with TPA 19 days after UV 4sec exposure shows several distinct bands, particularly near the 8.3kpb range; this may be integrated Gross viral genome, but a comparison of amplification between other treatments is not possible due to excessive radiation background.

Experiment 3, designed to show the kinetics involved in viral gene amplification and excision, met similar problems. Comparison of

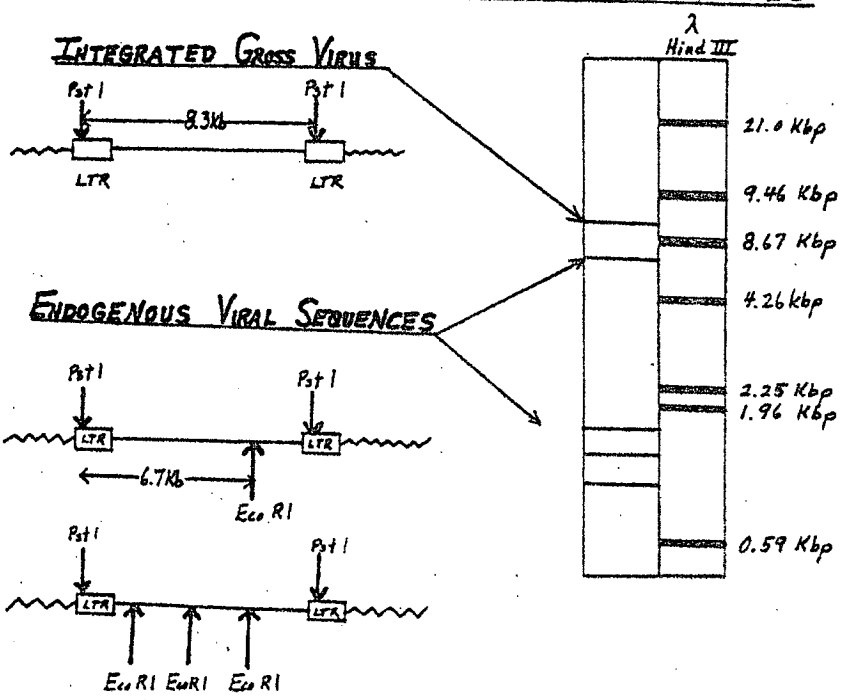

Figure 8

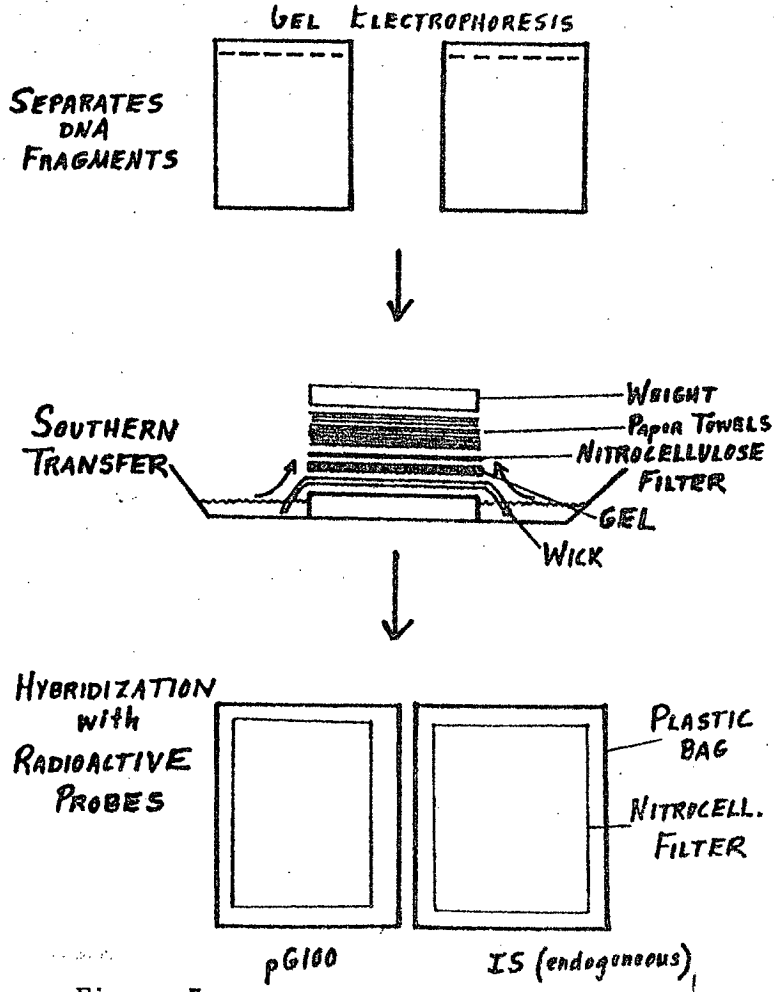

Figure 7

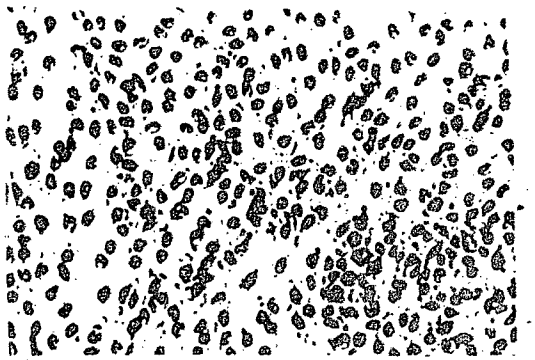

Figure 1. NIH-3T3 cells, normal medium

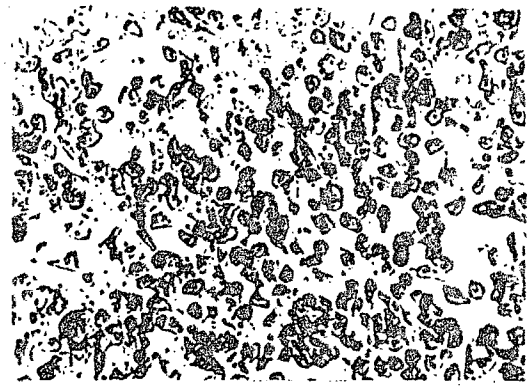

Figure 2. NIH-NV cells, normal medium

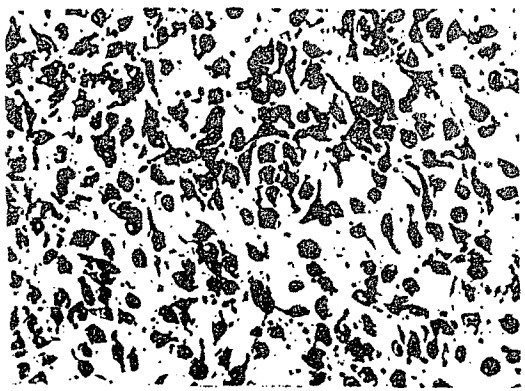

Figure 3. NIH-NV cells, UV 4sec, normal medium

Figure 4. NIH-NV cells, TPA (0.15 μg/ml medium)

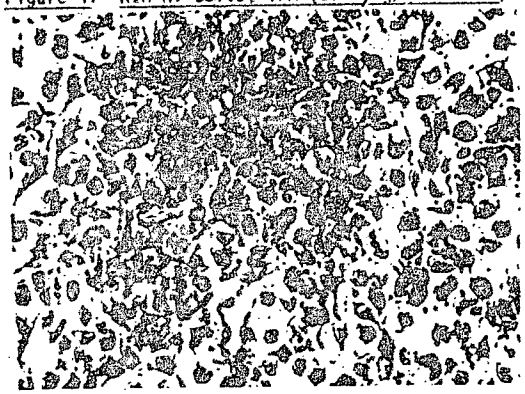

Figure 5. NIH-NV cells, UV 4sec, TPA (0.15 μg/ml medium)

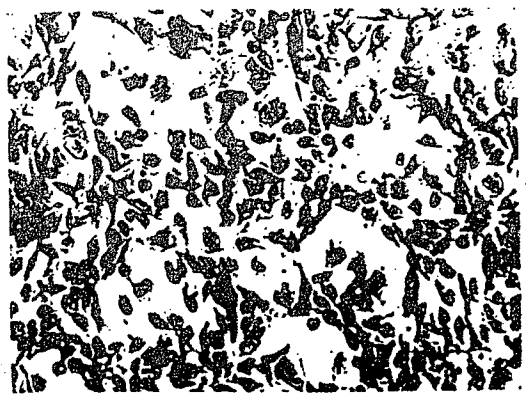

Figure 6. NIH-3T3 cells, UV 4sec, TPA (0.15 μg/ml medium)

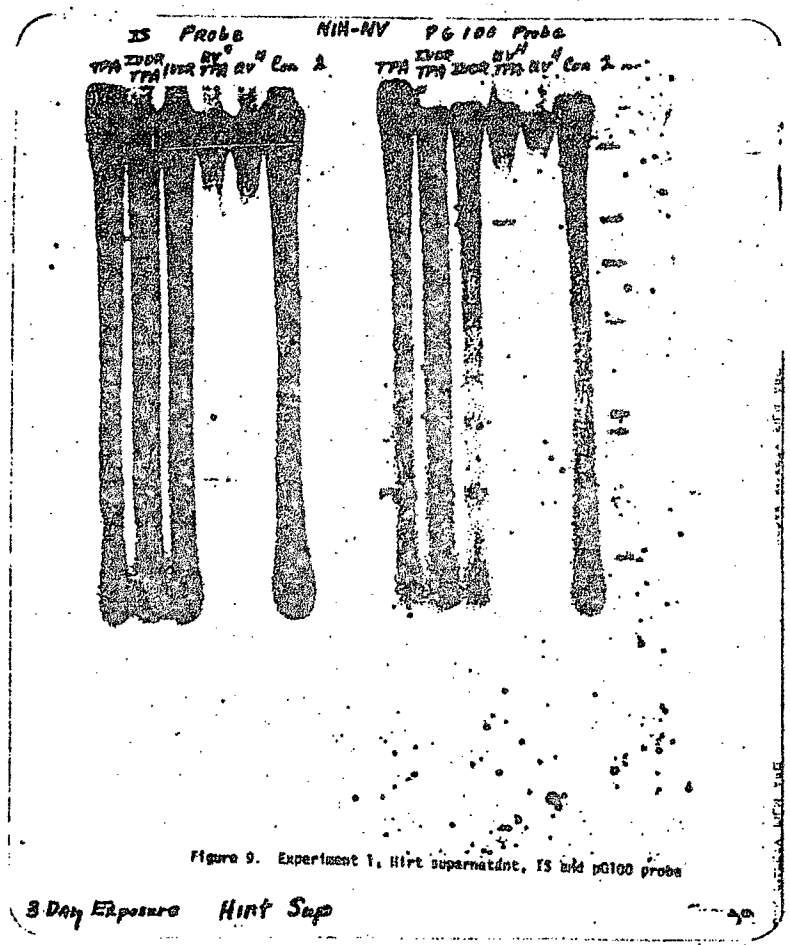

Figure 9. Experiment 1, Hirt supernatant, IS and pG100 probe

bands from the Hirt supernatant and Hirt pellet could have given some clues to whether the viral genes are first amplified and then excised or vice versa. The pG100 probe for both the supernatant and pellet DNA extracts failed; the IS probe did not hybridize appreciably with the Hirt pellet. The supernatant, hybridized with IS probe, did not show any appreciable excised endogeneous viral genes, but this may have been due to weak radioactivity or short time exposure.

These experiments show that UV and TPA had the greatest affect upon the morphology of the infected cells. This cocarcinogenic treatment also induced viral excision from the host DNA, as did IUDR and IUDR with TPA. Similar experiments may demonstrate a relationship between viral excision and carcinogenesis caused by physical and chemical intrusion. Research in this area may be used as a simple model for the mechanisms involved in tumor initiators and tumor promotors in carcinogenesis.

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The Effects of 2-Thiouracil on Tail Regeneration in Rana catesbeiana

by Rick Sullivan

The biological phenomenon of regeneration is the process whereby an organism replaces a lost or missing structure by adapting an existing structure to form a new one. Rana catesbeiana tadpoles are capable of tail regeneration and will replace a missing tail in a little over a month's time. It is the purpose of this investigation to study the processes that occur during tail regeneration and the effect of the drug, 2-thiouracil, on the regenerative processes. In order to fully understand how 2-thiouracil effects tail regeneration, one must understand intimately the stages and events that occur during regeneration.

When the tadpole's tail is removed, the start of an amazing morphological and physiological process begins. A clot forms on the wound surface within two to three minutes after the initial wound infliction. During the next 24 hours, the epithelial tissue surrounding the wound loses some of its integrity and migrates over the wound surface to form a protective barrier. This process begins as early as 15 minutes from wound infliction and is complete by 24 hours (Schmidt, 1968). This migration is necessary for regeneration and if it is blocked by a barrier, even a dermal one, regeneration will not take place (Goss, 1969). During this period, the stump of the tail shows an inflammation response to the trauma it has received. Blood supply, which has been drastically reduced, gradually returns to the normal rate of flow. With this increase in blood supply, a rise in the number of leukocytes and granulocytes can be seen also. These cells serve to cleanse the wound and rid the area of cellular debris.

In addition to the epithelial and vascular tissues, other surrounding tissues begin to undergo major changes. Muscle tissues contract and recede from the wound surface. Then, depending on the degree of injury, the muscle cells begin to break down and dedifferentiate. The myofibrils disappear and the syncitial fibers break down into individual cells. The striations disappear because of necrosis and the nuclear and cytoplasmic debris is lysed by macrophages (Hay, 1966). This muscle dedifferentiation is greatest near the distal ends of the tail stumps and least near the proximal ends.

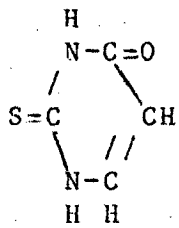
Nerve cells also begin to break down into their cellular components. Degeneration in mylinated nerves can be seen by 24 hours as the cells lose there integrity and the myelin sheaths become disorganized. The perineurium begins to break up or disappear, the nerve bundles separate, and the Schwann cells become isolated from one another (Norman and Schmidt, 1967). In addition to muscle and nerve tissue, connective tissue also dedifferentiates into fibroblasts or cells resembling fibroblasts in appearance and character (Schmidt, 1968).

After about a weeks time, these dedifferentiated cells from the various tissues in the stump begin to migrate to the end of the wound

and form a mound of cells which resemble embryonic mesenchymal tissues. Such cells undergo mitosis and form what is known as the blastema. The origin of blastema cells is an area of much debate. The most widely accepted theory is that the blastema cells are the result of the formed tissues dedifferentiating into a type of common denominator which facilitates mitosis and cellular proliferation. It is known that the epidermis contributes very little to the blastemal content and that the majority of cells come from the connective tissue elements (Thornton, 1956). During the formation of the blastema, the cellular nuclei enlarge and become vesicular. The nucleoli become more prominent and the cytoplasm takes on a basophilic staining reaction which is characteristic of growing cells active in DNA, RNA, and protein synthesis (Hay, 1966). The growth of the blastema into a conic protuberence is the time of most active cellular proliferation and the most critical phase in amphibian regeneration (Schmidt, 1968). After a substantial increase in the number of cells in the blastema, this structure flattens from its conical shape into a paddle shape and begins to grow. As it grows, under direction of the epithelium, nerves, and hormones, the lost structure is replaced by redifferentiation of cells in the blastema.

The epithelium which has migrated over the wound surface plays a major role in the growth process. The epithelium piles up on the wound surface and forms an apical cap, which functions much like the apical ectodermal ridge in embryonic limb development. This apical cap remains in close contact with the wound as its interaction with the stump can be seen in tongue-like projections which enter the stump (Goss, 1969). The apical cap determines the position of the blastema and it is thought it does this by various induction mechanisms (Hay, 1966). Nerves in the stump are also mandatory, in most cases, for regeneration to occur. It is not known what influence the nerves have on the regenerating tissues, but it is known that they are needed until the blastema tissues are capable of self-differentiation. It is thought that sensory nerves exert more influence than motor nerves, yet regeneration will occur in the absence of the former. It is known that nervous tissue exerts its greatest influence on the apical cap and the epidermis, which may account for the importance of the epidermis (Goss, 1969).

Hormones also effect the regenerative processes. 2-Thiouracil is an anti-metabolic drug which is in the thiocarbamide class (Devlin, 1982). The molecular structure of 2-thiouracil is:



It is capable of blocking thyroxin synthesis by preventing oxidation of iodide to iodine or by preventing the coupling of two diiodotyrosine molecules to form thyroxine (Orten and Neuhaus, 1975). Either

mode of action allows 2-thiouracil to block thyroxin synthesis in the thyroid gland which is active in R. catesbeiana tadpoles by the time they are about 9 mm long (Schotte, 1956). Another unique property of 2-thiouracil is its ability to be readily incorporated into nucleic acids instead of the natural bases (Mahler and Cordes, 1966). This aspect of thiouracil allows it to become involved in RNA mechanisms as it competes with the "natural base," uracil. By studying the effects of 2-thiouracil on tail regeneration in R. catesbeiana, one can gain insight into the regenerative processes as a whole as well as to the role that RNA plays in that process since RNA function will be hampered or disabled by the 2-thiouracil.

Materials and Methods

Fifty Rana catesbeiana tadpoles, obtained from Carolina Biological Supply Company, were divided into seven groups of five animals each. Each group was then put in a large culture dish (18 cm in diameter) which contained a different concentration of 2-thiouracil in deionized water. The concentrations used were .0025 M (0.321 g/liter), 10-3 M (0.128 g/liter), 10-4 M (0.0128 g/liter), 10-5 M (0.00128 g/liter), and 10-6 M (0.000128 g/liter). Two dishes containing only deionized water and five specimens each were used as controls. In one of these dishes, the animal's tails were left intact; in the other, the tails were removed. After being exposed to the solutions for 24 hours, the animals were placed in a dish containing ice water for ten minutes. After the animals became immobilized, their tails were amputated using a clean scalpel sterilized in an alcohol flame. About 1.5 cm was removed from each tail. Small, diagonal notches were cut in the dorsal or ventral tail fin in recognizable patterns so that individual animals could be identified later. After this operation, the animals were returned to their original containers. All animals were maintained at room temperature (70 $\pm$ 5 degrees F) and each group was fed approximately 0.15 g of "tadpole food" (Carolina Biological Supply Co.) per day. The solutions in the bowls were changed about every third day or when the water became cloudy. The regenerative processes in these animals were carefully watched and noted daily and measurements were made at least once a week as to the quantitative growth of the regenerate.

The remaining fifteen animals were placed in a clean, 4-liter, glass aquarium and kept under the same conditions as those just described. The tails of these tadpoles were removed in the manner described earlier. At crucial phases of the regenerative process, samples of regenerates were removed for histological study. These samples were fixed in 10% formalin solution, imbedded in paraffin, sectioned sagitally, and stained with hematoxylin and eosin using standard histological techniques. The four phases studied were: the tail before regeneration, the preblastema, the blastema, and the redifferentiating regenerate.

Results

Figure 1 is a plot of the total regenerated length of the tails

versus the concentration of 2-thiouracil. As the concentration increases, the amount of regeneration decreases. Figure 2 is a plot of the length regenerated per unit time. During the first week, all the tadpoles exhibited positive growth, then those in higher concentrations began to regress. In figures 1 and 2, each point is the average length obtained for the animals in the dish.

Discussion

2-Thiouracil, as previously stated, effects organisms in two ways. It acts to curb thyroxine synthesis and it competes with uracil for a position on the RNA chain. By looking at figures 1 and 2, one can see that as the concentration of 2-thiouracil increases, the amount of regeneration decreases. In the 0.0025 M and 10-3 M solutions, the tails actually regressed after the first week. In all of the solutions, the tails had positive growth during the first week, which was during the preblastema stage. During this stage, the tissues dedifferentiate to enhance mitosis and pluripotency (Goss, 1969). They then migrate toward the distal end of the stump where they multiply to form the blastema. This migration could account for the slight increase (less than 1 mm) in regeneration length. During the second week, the blastema elongates as the cells multiply and divide. During multiplication, there is extensive RNA and DNA synthesis. It seems that as RNA became increasingly more important, it also became more altered because of the 2-thiouracil. This drug interference is a probable cause for the slow and hampered regeneration in the treated animals. As the need for new cells increased, they did not appear; thus causing the slow or atypical regeneration.

Another interesting factor that can be seen on figures 1 and 2 is that the three lowest concentrations of 2-thiouracil all produced virtually the same results on the regenerates. The tails began to regenerate as normal, yet during the second week, the rate became incredibly slow as the cells began to divide. This seems to point out that the tadpole tissues are sensitive enough to respond to as little 2-thiouracil as 10-6 M, yet strong enough to withstand 10-4 M without mutation or regression. It can also be noted that the 10-4 M concentration produced the best results with regards to length of all the treated animals; however, there is a difference of less than 0.1 mm in these measurements and is probably not significant.

The effect that the 2-thiouracil had on the thyroid gland could be seen as the treated animals began to come to a metamorphic standstill. Those animals that had hind legs did not increase them in an appreciable length and those animals that did not have them did not grow them; however, the control animals continued to undergo metamorphosis and grow hind and even front limbs. With regards to the regenerative process, I feel that the blockage of the thyroid hormone had a negligible effect because of several reasons. Thyroxine, which was blocked, has an inhibiting effect on regeneration and thyroid hormones permanently inhibit limb regeneration (Hay, 1966). A thyroidectomy does not effect regeneration of tadpole tails, and in tadpoles, a hypothyroid condition is normal and favorable (Schmidt, 1968).

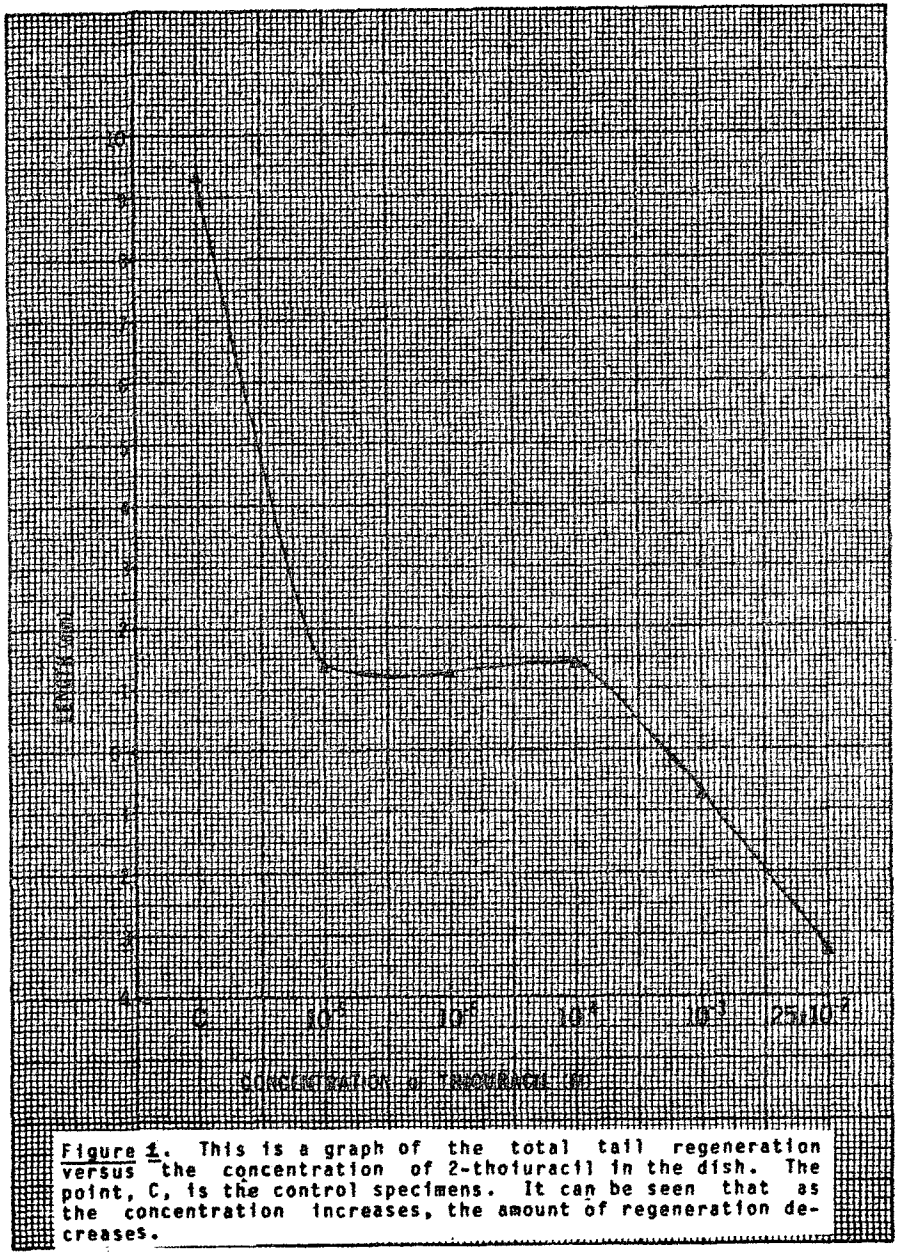


Figure 1. This is a graph of the total tail regeneration versus the concentration of 2-thiouracil in the dish. The point, C, is the control specimens. It can be seen that as the concentration increases, the amount of regeneration decreases.

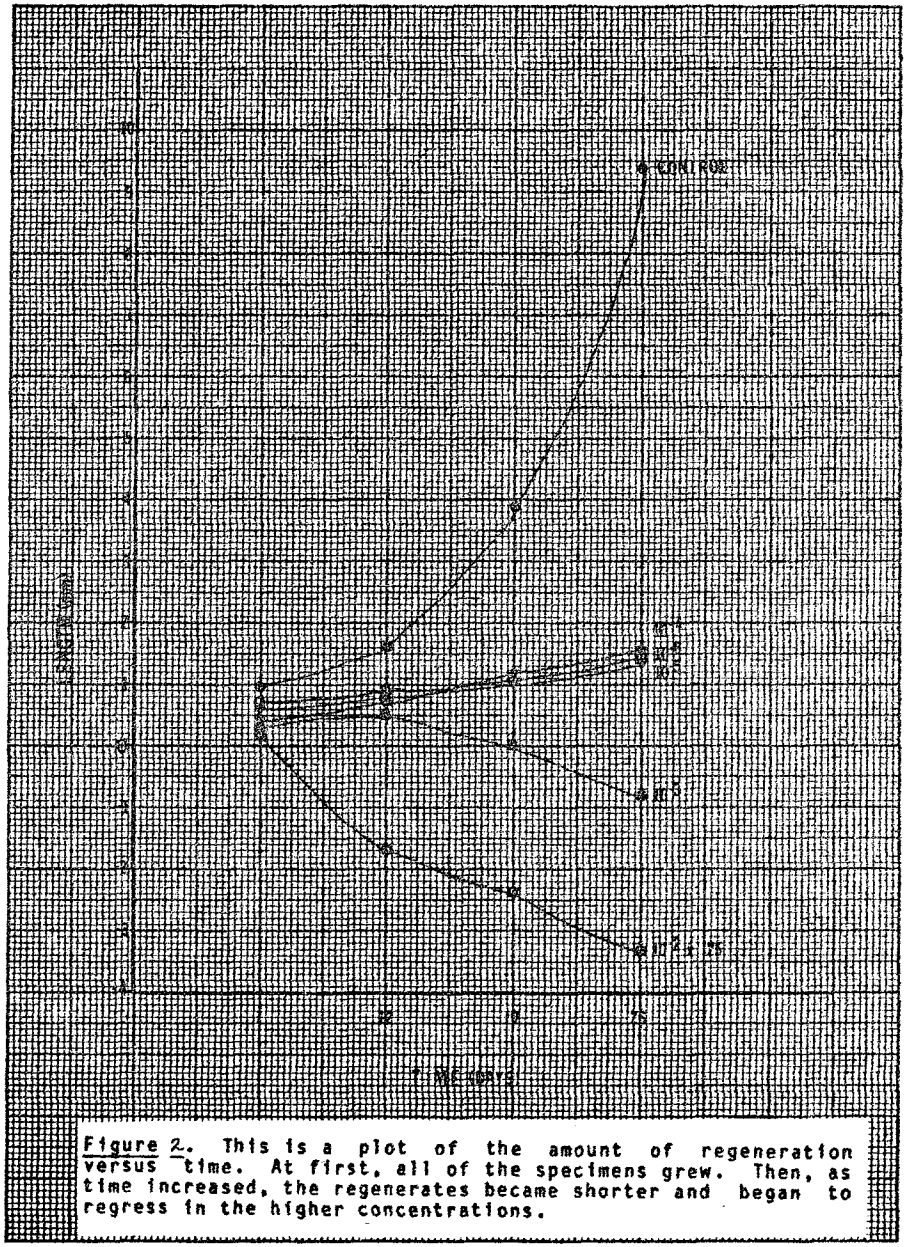


Figure 2. This is a plot of the amount of regeneration versus time. At first, all of the specimens grew. Then, as time increased, the regenerates became shorter and began to regress in the higher concentrations.

The events that occur after removal of the tadpole tail can also be seen elsewhere in the body. "The events which follow amputation of the amphibian limb appear to be identical with those following amputation of the tail" (Thornton, 1958). I feel it necessary to mention this point since most of my research has been done on the amphibian limb, especially the Urodele limb.

In this as well as any experiment, one must take the error factor into consideration. When one is dealing with chemicals and weighing them in amounts smaller than a milligram, extreme care must be taken. I did this by using an analytical balance. Another problem I encountered was in keeping individual animals separate. I tried to mark their tails; however, as the tails grew back, so did the marks. This was especially rapid in the control tadpoles and in those in lower concentrations of 2-thiouracil. This could account for the fact that the animals in the 10-4 M solution exemplified better progress than those in lesser concentrations. I was careful to feed the animals equal amounts by weighing the food, yet this is a minor point because starving and well-fed animals regenerate at the same rate (Hay, 1966). Measurements were made using a dissecting microscope equipped with an ocular micrometer which facilitated accurate growth measurements. Care was taken not to mix animals in common dishes while washing and cleaning the bowls.

I was not able to compare my results with any from previous experiments. As far as I can tell, this is an original experiment; however, other experiments using anti-metabolic drugs have yielded similar results. Chemicals that prevent cellular proliferation have been found to inhibit regeneration much like the 2-thiouracil did in this experiment.

Regeneration is an amazing biological process with many capabilities. The idea that during regeneration tissues that have differentiated to the extent of muscle tissue can dedifferentiate and become capable of mitosis and then redifferentiate is no less than phenomenal. In the quest for the mechanisms by which regeneration functions, we are brought down to the very molecular and chemical level of the genetic code, which may one day, through continued work on this and other subjects be broken. My research has alluded to the importance in regeneration of RNA formation and cellular proliferation along with a much better insight into the mechanisms by which regeneration occurs.

Acknowledgement

I would like to express my deepest appreciation to Dr. Robert L. Amy for his guidance in this endeavor. His understanding and interest in his students is to be commended.

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Electrophoresis of Trichoderma reesei Cellulases

by Dane Ciolino

Cellulose is the most abundant organic source of food, fuel, and chemicals naturally produced on earth. Being the primary constituent of plant cell walls, cellulose is produced photosynthetically at a rate of about 150 lbs. per day for each of the worlds 3.9 billion people (Spano et al. 1975). Cellulose is a polymer of beta-glucose linked linearly at the 1-4 position such that long molecular chains are formed with an average length of about 3,000 glucose units. Large scale commercial tapping of this abundant natural resource could liberate tremendous quantities of glucose to be used for such human needs as fuel alcohol manufacturing or as raw material for food production (Johnson 1983). However, such commercial utilization would mandate a relatively inexpensive and efficient means to unlink the long chains of glucose into readily usable monomers. While the unlinking of the cellulose polysaccharide could be accomplished with acid or high temperature degradation, this would be commercially inappropriate as the resulting sugars are wastefully decomposed (Spano et al. 1975). For this reason, cellulase, an enzyme which hydrolyzes the bonds of cellulose chains without decomposing the resulting sugars, could help meet man's future energy and nutritional needs.

Cellulase is a naturally occurring enzyme which plays an important role in the environment as well as in the life of a few animals, and many microorganisms. Cellulase producing symbiotic microbes are responsible for the digestion of cellulose in termites and ruminants. As polysaccharides are not readily absorbable through the animal digestive tract (like glucose and other monosaccharides) cellulase producing symbiotic microbes are essential to these animals in order for them to procure carbohydrate energy to fuel metabolism (Johnson, 1983). As fungi are heterotrophic and must rely on dissolved organic food materials, many secrete cellulase to break down large cellulose polysaccharides into smaller, more soluble units, which are able to pass more easily through their cell membrane. Because of their nutritional strategy, the cellulase secreting fungi play an important ecological role in the decomposition of organic matter and in the recycling of organic molecules (Johnson, 1983).

One such cellulase secreting fungus which is especially useful to man is in the genus of the ubiquitous soil dwelling fungus Trichoderma (Alexopolous 1979). This fungus, Trichoderma reesei, is one of the most productive sources of all microbial enzymes, especially cellulase (Ryu and Mandels, 1980). From this organism, the cellulase "complex" has been purified into three constituent enzymes: beta-glucosidase (cellobiase), endo- 1,4-beta-D-glucosidase (endoglucanse), and 1,4-beta-D-glucan cellobiohydrolase (exo-glucanase), which together compromise a system to convert cellulose to glucose (Gritzali and Brown, 1979). This cellulase complex probably hydrolyzes cellulose ultimately into glucose in much the following manner (Humphrey, 1979): The very long chains of cellulose are cleaved into shorter chains of amorphous cellulose and soluble oligosaccharides by the

endo-glucanases. These shorter chains have nonreducing ends from which the exo-glucanases can cleave cellobiose (a disaccharide) and a few glucose molecules. These cellobioses are then split into glucose monomers by the beta-glucosidases (cellobiase). The glucose is then able to easily pass across the fungus' cell membrane to be used in the metabolic process.

The purpose of the work described in this paper was to gain a general understanding about cellulases and the laboratory procedure of polyacrylamide gel electrophoresis. To this end, the aim of these experiments was to successfully localize Trichoderma reesei cellulases within a polyacrylamide gel after electrophoresis.

Materials and Methods

A medium (Appendix 1) in which a culture of the fungus Trichoderma reesei had grown at room temperature for five days was obtained from Dr. Terry Hill. The medium, which contained the cellulase complex, had been stored for 1 year at -20 C. For polyacrylamide gel electrophoresis (PAGE), some samples of the medium were concentrated approximately ten fold by dialysis against granular sucrose at 5 C. All samples for electrophoresis were mixed with an equal volume of a bromphenol blue-sucrose tracking dye solution (50 ml Peacock buffer, 70 g Sucrose, 10 mg Bromphenol Blue). One hundred microliters of this medium-tracking dye mixture was applied to each gel. For all PAGE runs, 6 mm x 10 cm gel tubes were used and they were performed in a Buchler Polyanalyst electrophoresis device.

Zonal PAGE

Polyacrylamide gel electrophoresis is a protein separation technique which electrically draws a protein containing sample solution through a polyacrylamide sieve (Gordon, 1973). This sieve is made by the crosslinkage of acrylamide and bisacrylamide within a gel. Separation of proteins is achieved through the differential rates of migration through the gel sieve among proteins of different anionic charges, molecular weights and structures.

The methods employed were those of Verachtert, et al. (1978). The gel included an artificial substrate, 5mM o-nitrophenyl-B-D-glucopyranosidase, in distilled water. This artificial substrate liberates a colored substance, o-nitrophenol, when acted upon by aryl-B-glucosidases (cellobiases), leaving yellow bands on the gel that facilitate the localization of the glucosidases. The runs were begun at a constant amperage of 2 milliamps/gel. After 10 minutes at this amperage, the current level was increased to 5 milliamps/gel. The buffer temperature throughout the run was maintained at approximately 15°C. The sample mixture was electrophoresed until the tracking dye reached the distal end of the gel. At that point the gels were removed from the apparatus and either incubated at 37°C., to permit reaction of B-glucosidases, or stained with a general protein stain (0.25% Coomasie Blue dye in methanol: acetic acid: water [5:1:5]) for 20-30 minutes. They were then destained overnight in a

solution of 7% acetic acid and 7% methanol in distilled water.

Later zonal PAGE runs were performed using the same methods, excluding the artificial substrate. A 10cm gel (measured from origin to dye front) obtained from one such run was sliced into ten 1 cm sections. Each of these sections was soaked overnight at 5°C. in 1.5 ml of distilled water in order to elute cellulases from the gel. The solution obtained was assayed for endoglucanase activity viscometrically using as a substrate 5 ml of 1.2% carboxymethyl cellulose (CMC) (Hercules Inc., type 7MF) in 0.018M Na citrate buffer, pH 5.0. Flow times of reaction mixtures were determined using size 300 Ostwald-Fenske viscometers at a temperature of 30°C., to which 1.0 ml of the enzyme solution had been added. Endoglucanase activity was expressed as increases in specific fluidity per hour x 1000. Specific fluidity is calculated as t0 / t - t0, where t = flow time of reaction mixture, and t0 = flow time of water as a reference fluid. For comparison, gels from the same run were stained with the Coomasie Blue stain for 20 min. and destained overnight with the 7% acetic acid destaining solution. The destained gels were scanned using a Gilford 2600 electrophotometer with a Gel Scanner attachment, coupled with a Hewlett-Packard X-Y Plotter. The step size was 0.1mm and the wavelength was set at 550nm.

Discontinuous PAGE

Discontinuous gel electrophoresis was performed on the same medium used in the previous zonal electrophoresis runs. The same electrophoresis apparatus and gel tubes were also used for these discontinuous runs. The running and stacking gels were prepared according to the procedure outlined in Hill, Cell Physiology Handout for Electrophoresis Lab. The temperature was maintained at approximately 15°C. and the amperage was constant at 2 milliamps/gel. The sample was electrophoresed until the dye front reached the distal end of the gel tube (appx 4.5 hrs.). After extruding the gels from the tubes, some were sliced into segments for assay of endoglucanase activity as described above, while others were stained with coomasie blue, using either of two methods: (1) staining and fixing the gels with the 0.025% coomasie blue - methanol stain as described above for 20 minutes and destaining overnight with the 7% acetic acid - 7% methanol. (2) Pre-fixing the gel with 12% trichloroacetic acid, staining overnight with 0.2% aqueous coomasie blue and destaining overnight with 12% TCA. After staining the gels were scanned with the Gilford Gel Scanning device.

Selective Enrichment for Cellulase

An experiment was performed in order to test the effectiveness of crystalline cellulose as an adsorbant for cellulase activity as part of an enrichment procedure. Approximately 75 ml of growth medium pH 7.0, was adjusted to pH 5.0 using 1N HCL. 2.4 g of Avicel (a crystalline cellulose) was added to 65 ml of the pH 5.0 growth medium and was stirred at 5° C. for 3 hours. This solution was then centrifuged.

The pellet containing cellulose was mixed with 50 ml of 6M Urea in order to elute the cellulase from the crystalline cellulose, and the supernatant (labeled first supernatant) was dialyzed against sucrose and concentrated 5-fold. The cellulase-urea mixture was then centrifuged, and the new pellet was discarded. The urea-containing supernatant (labeled "final supernatant") was dialyzed to remove urea, and then dialyzed against crystalline sucrose to concentrate approximately 15-fold. Samples of all these solutions (original medium, pH 5.0; first supernatant; final supernatant) were assayed for endoglucanase activity and analyzed by discontinuous PAGE. The three gels obtained form these discontinuous electrophoresis runs were stained using the pre-fixing - 0.25% aqueous coomassie method and were later scanned with the Gifford Gel Scanner.

Results

Zonal PAGE

In all of the electrophoresis runs where the artificial substrate 5nM o-nitrophenyl-B-D-glucopyranidase was included in the gel material, no significant localized change of coloration was noted. However, as evidenced by coomasie staining, there were proteins present in gels from simultaneous runs on the same medium. These stained gels exhibited two distinct bands, the front of the first band was located in the region approximately 2.5cm from the origin (relative mobility= .23) and the front of the second band was located in the region approximately 3.3 cm from the origin (relative mobility= .3). However, measurement proved difficult because these bands were poorly focused, and thus were not sharply defined.

In subsequent runs, the artificial substrate was excluded from the gel. As in the previous zonal electrophoresis runs, the protein bands were not clearly focused, and the demarcation between the two visible bands was hardly discernable. A scan of one such 11 cm gel stained with 0.025% coomasie blue (Figure 1) revealed two significant bands. The first peak, located approximately 2.0 cm from the origin (relative mobility= .18), was somewhat lighter than the second peak located at 2.9 cm from the origin (relative mobility= .26) Viscometry of a sliced gel from the same run (Table 1) roughly placed the majority of endoglucanase units in the vicinity of the two main peaks. See bar graph on Figure 1.

Discontinuous PAGE

Discontinuous PAGE proved to focus the protein bands in the gel more satisfactorily than did zonal electrophoresis. In the first of these runs (Figure 2), four distinct peaks were evident on the gel scan. The relative mobilities of these peaks were, .27 for the 1st peak, .35 for the second, .50 for the third, and the fourth had a rm of .52. Viscometry on 3mm sections from the 3rd, 4th, and 5th centimeters of a similar gel from the simultaneous run (Table 2) seemed to demonstrate that endoglucanase activity was reasonably higher in the general vicinity of the two smaller peaks on the corresponding stained

Table 1
Zonal PAGE

Gel Segment in Cm.	Cx Units/ML
1 origin	10.51
2	19.92
3	41.81
4	1.07
5	2.47
6	0.49
7	0.47
8	0.63
9	0.51
10 dye	0.25
Water Blank	0.07

Fig. 1

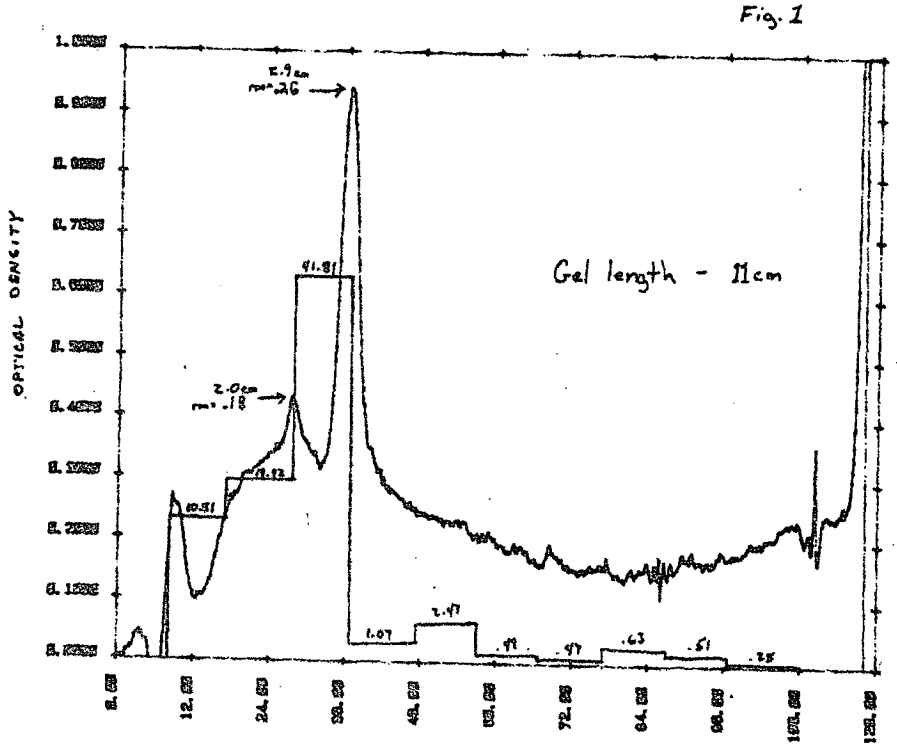


Table 2
Discontinuous PAGE

3rd, 4th, and 5th cm sections

3 mm Sections	Cx Units/ML
1 proximal	0.62
2	3.99
3	2.11
4	0.45
5	5.83
6	0.69
7	-17.78
8	2.75
9	1.03
10 distal	1.82
Water Blank	0.45

Table 3
Discontinuous PAGE

Sec.	Cx Units/ML	Sect. Size (cm)
1 dye	2.02	1
2	3.84	1
3	0.54	1
4	3.73	1
5	4.20	1
6	5.48	.5
7	24.80	.5
8	22.65	.5
9	49.11	.5
10	8.70	.5
11	3.79	.5
12	1.95	.5
13	21.07	.5
14 origin	17.38	.5
Water Blank	2.31	

Figure 2

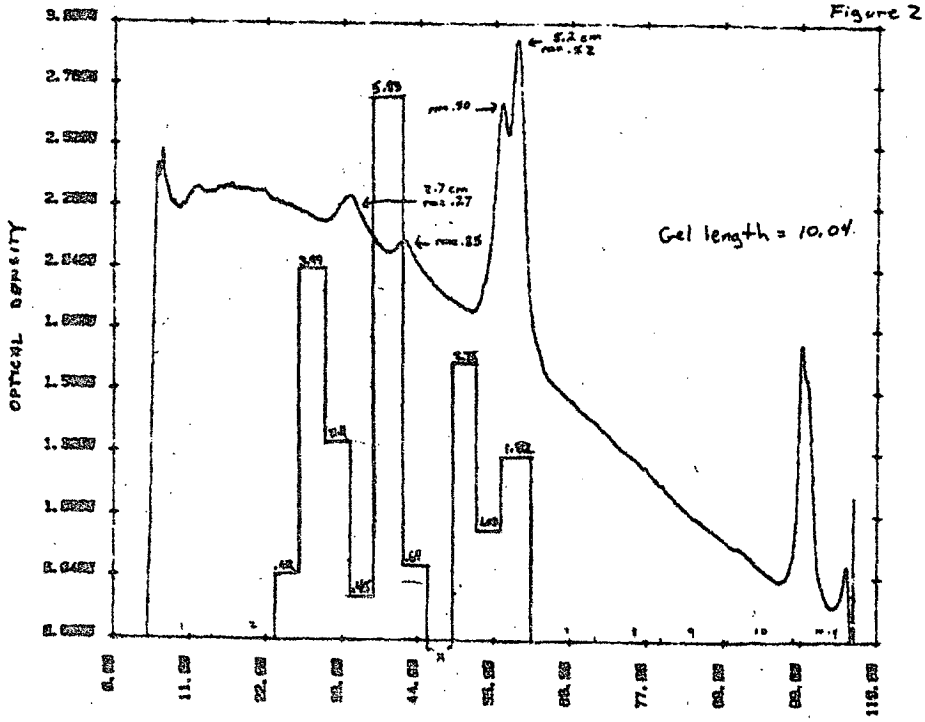


Table 4
Selective Enrichment for Cellulase

Solution	Cx Units/ml
Conc. Orig. Super.	22.63
Final Super.	173.24
Original Sol.	145.87
Water Blank	-0.50

Figure 3

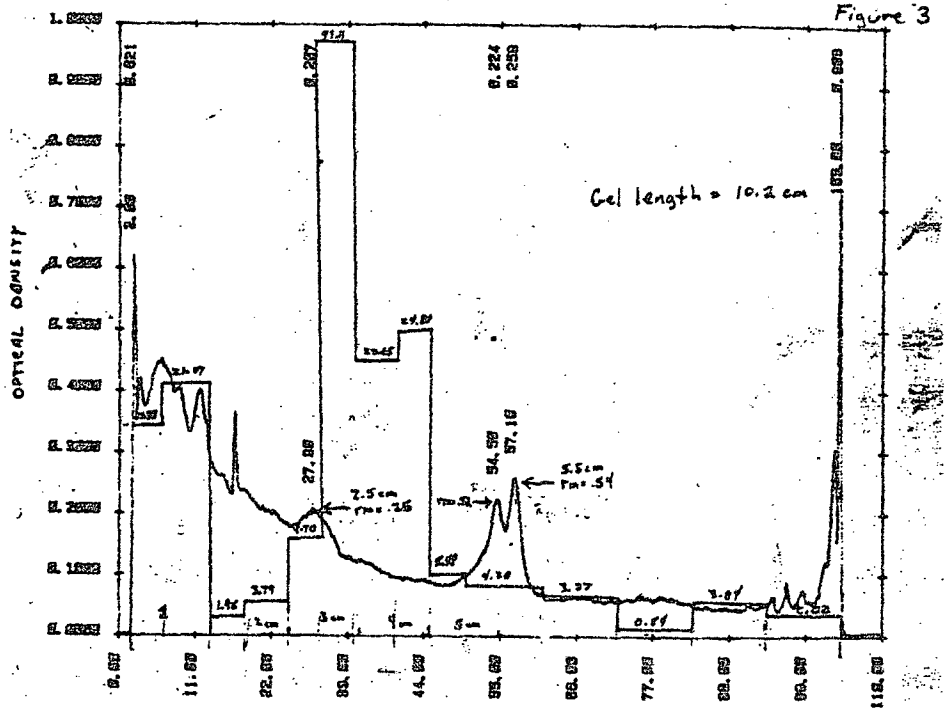


Figure 4

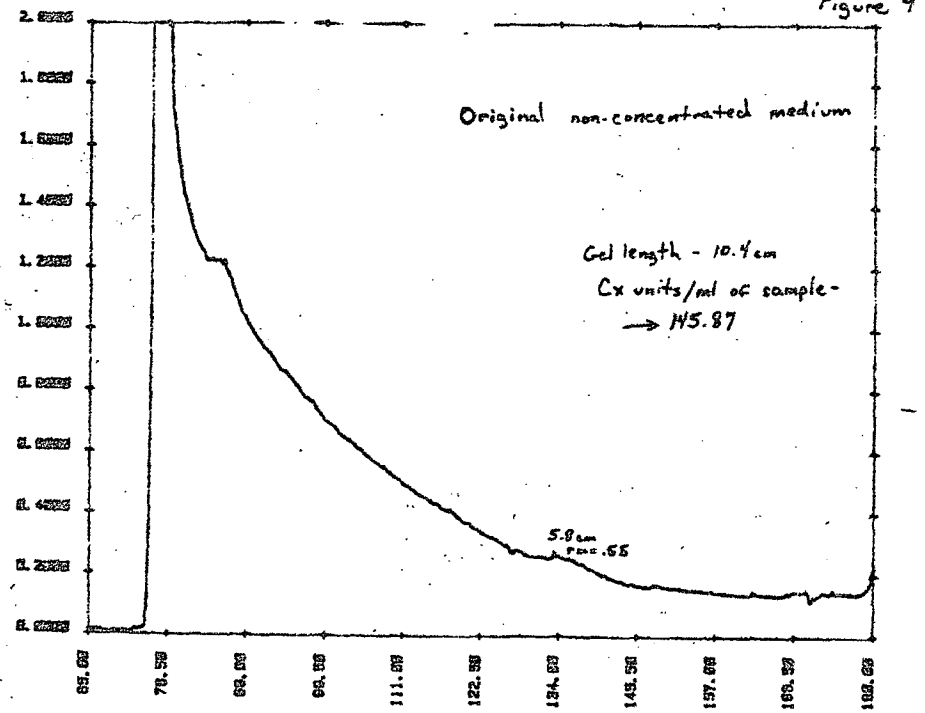


Figure 5

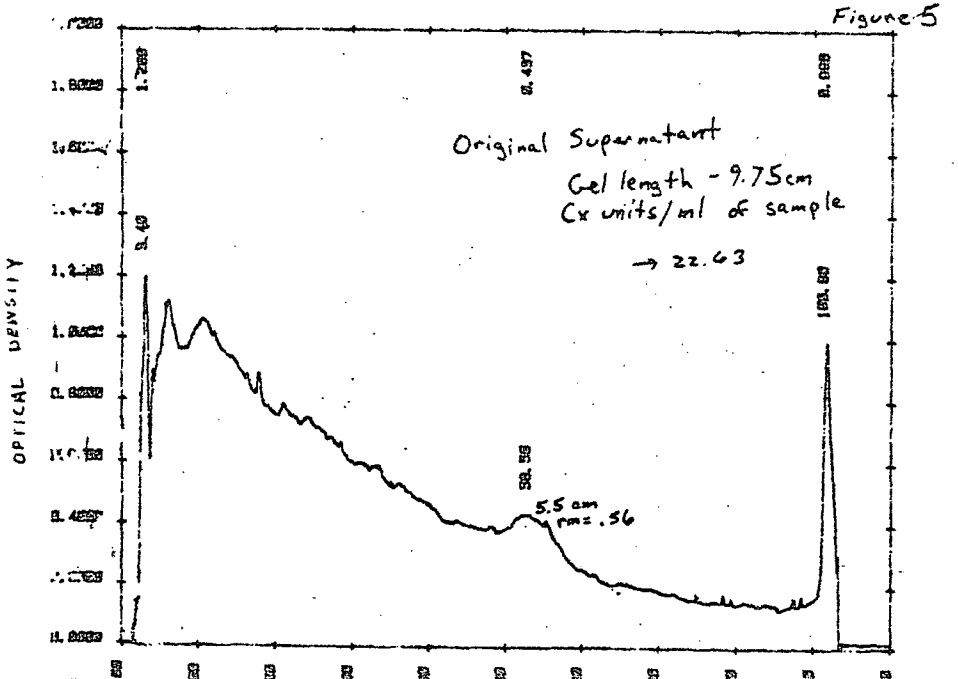
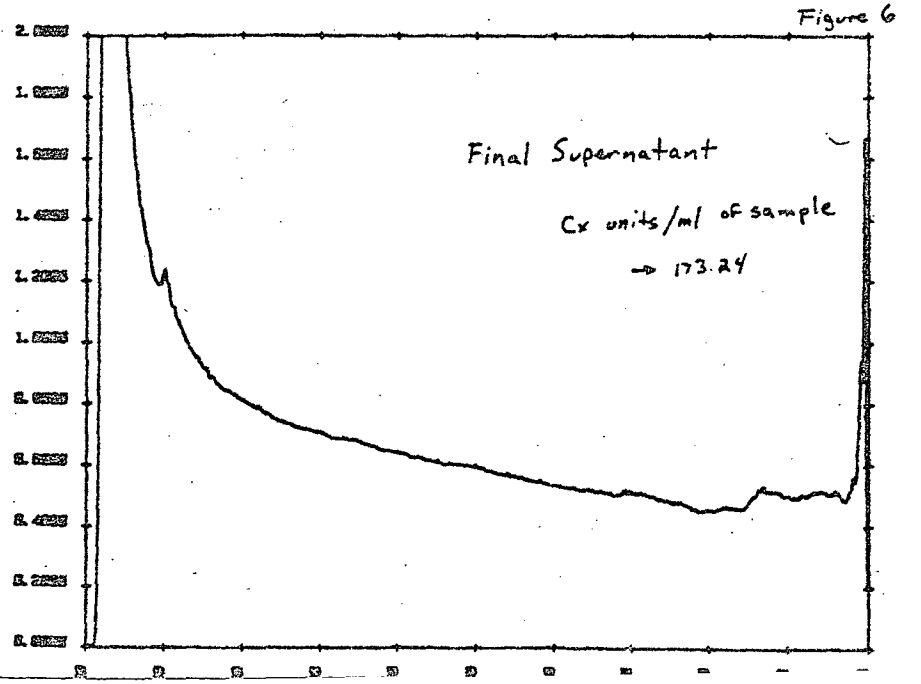


Figure 6



gel than it was in the area of the two largest peaks. (See bar graph on Figure 2) Similar results were obtained in later disc runs. In another such run, the gel scan discerned three major peaks (Figure 3). The first peak at 2.5 cm from the origin (relative mobility= .25), roughly corresponds to the first of the two single peaks in the earlier scan, the first of which occurs at 2.7 cm from the origin (relative mobility= .27; See Fig. 2). The second two peaks of figure 3, the highest of which occurs at 5.5 cm from the origin (relative mobility= .54), roughly correlate with the highest of the two peaks in the Figure 2, the highest of which is found at 5.2 cm from the origin (relative mobility= .52). Viscometry of gel segments from corresponding areas on a similar gel (Table 3) demonstrated that the bulk of endoglucanase activity was located in the region of 2.9 to 3.9 centimeters from the origin (relative mobility range= .28 to .38) (See also bar chart on Figure 3). It is interesting to note that the largest concentration of protein, as determined by gel optical density, is located around 5.1 cm from the origin (relative mobility= .50).

Selective Enrichment for Cellulase

The gel scan from the pH 5 non-concentrated cellulase solution showed little or no localized protein (Figure 4). Viscometry of a sample portion however, demonstrated that there was in fact some endocellulase activity in the medium (Table 4). The scan of the final supernatant, like the scan of the pH 5 solution, showed no significant peaks or deflections (Figure 6). The assay for endoglucanase activity (Table 4) demonstrated that there was 173.24 units/ml of endo-glucanase activity in the final supernatant (urea sol. w/o crystalline cellulose). The gel scan of the first supernatant (post-centrifuged medium w/o crystalline cellulose) (Figure 5) showed only one significant peak, this located at 5.5 cm from the origin (relative mobility= .56). The endo-glucanase viscometric assay showed considerably less enzyme activity (only 22.63 Cx units/ml) in this solution than witnessed the other samples (Table 4).

Discussion

Zonal PAGE

The lack of coloration change in the gel containing the o-nitrophenyl-beta-D-glucopyranidase artificial substrate seems to indicate that there was no beta-glucosidase (cellobiase) activity in the electrophoresed sample. However, when a large portion of the medium was mixed with the artificial substrate, a color change was notable. It is possible that the extremely small quantity of medium that was electrophoresed (only 50 microliters) had not enough cellobiases present to liberate a quantity of o-nitrophenol significant enough to effect the visible coloration of the gel. The zonal PAGE runs done with this artificial substrate were performed with an unconcentrated medium sample. Perhaps if a larger, or a more concentrated sample had been electrophoresed, a better result would have been obtained. Also, if disc-PAGE were performed including the artificial substrate in the running gel, the cellulases would have been more concentrated within

the protein bands, and perhaps a greater quantity of o-nitrophenol would have been liberated in the area of concentrated cellobiase. The identity of the protein bands noted in the coomasie stained gel is difficult to speculate upon. However, a protein was relatively well concentrated in this area, and the lack of coloration in the corresponding region of the unstained gel may be indicative of the fact that the protein bands do not represent a large concentration of cellobiases.

The viscometric assay results from one such run performed without the artificial substrate indicated that a large concentration of endo-glucanases were in the region of the first, second, and third centimeters from the origin (10.51 Cx/ml in the 1st cm, 19.92 Cx/ml in the 2nd cm, and 41.81 Cx/ml in the 3rd cm). This roughly corresponds to the two main peaks in Figure 1, the first of which is located at 2.0 cm from the origin (rm= .18) and the second of which is located at 2.9 cm from the origin (rm= .26; See Figure 1 bar chart) The results of this particular experiment show little more than that the majority of the cellulase activity within the gel lies around the 1st, 2nd, and 3rd region of the gel. This demonstrates that the two peaks at relative mobilities .18 and .26 could be cellulase bands.

Discontinuous PAGE

Discontinuous PAGE runs proved more successful than zonal PAGE in separating the constituent proteins of the culture medium samples. Both of the disc PAGE runs demonstrated that there were at least three major protein bands present on the gel. The first of these major bands was in the relative mobility range of .27 to .25 (Figure 2, rm= .27; Figure 3, rm= .25). The second, and darker, set of bands was located in the relative mobility range of .52 to .54 (Fig. 2, rm= .52; Fig. 3, rm= .54). Given the similar appearance, and the reasonable correlation between the relative mobilities of both sets of bands in Fig. 2 and Fig. 3, it seems safe to conclude that the same proteins caused the bands in Figure 2 as caused the corresponding bands in Figure 3. While one might expect that the relative mobilities would be more precise between measured relative mobilities of the same electrophoresed protein, the slight discrepencies that do exist, might be accounted for by considering a number of factors. The gels came from two distinct PAGE runs. The gels used in each of the runs were not of exactly the same constituency. While the gels were mixed according to the same procedure, it is likely that there were very minor differences in conditions under which the gels polymerized that could have contributed to the differential sieving among gels (eg. temperature, solution measurement error). Also, the conditions of each of the runs might have been slightly different due to differences in such concerns as temperature fluctuation, running time, etc. In addition, comparison of measurements between stained gels and sliced gels was difficult considering the stained gels expanded as much as 37.5% from their original size. Compensation was made for the expansion, but naturally some error in such measurement is expected. Given the qualitative nature of this work, the very similar appearances of the bands, and the reasonable correlation between the relative mobili-

ties of similar bands, it is fair to assume that the same proteins are being observed in each case.

While the results from the viscometric assay do not conclusively demonstrate exactly where in the first five centimeters of a gel the endo-glucanase is located, the results do show that the majority of endo-glucanase activity is not in the region of the two darkest bands. Rather, it appears that endo-cellulase activity is more concentrated in the area of the smaller band(s) closer to the origin. See bar charts on Figures 2 and 3. This seems to show that the darkest bands in the gel are not cellulase proteins but rather, are indicative of some other protein present in the sample medium. The electrophoresis results from the selective enrichment samples seem to reinforce this conclusion.

Selective Enrichment for Cellulase

The scan of the gel in which the non-concentrated, original medium was electrophoresed exhibits only one interesting feature (Fig. 4). A very slight band seems evident at approximately 5.8 cm from the origin with a relative mobility of approximately 5.5. This appears to be in the range of the two larger peaks of the same approximate relative mobility range witnessed in Figures 2 and 3. The lightness of the band is probably due to the fact that the sample electrophoresed in the previous runs was concentrated while this sample was not. This non-concentrated sample had a level of endo-glucanase activity of 145.87 units/ml (Table 4). The gel in which the first supernatant (concentrated 5-fold) was electrophoresed (Fig. 5) shows a band with approximately the same relative mobility (rm= .56) as the band witnessed in the original sample (Fig. 5; rm= .55). This first supernatant was concentrated 10 fold and had a level endo-glucanase activity of 22.63 units/ml (Table 4). From this result, it is evident that the cellulase in the original sample did indeed bind to the Avicel cellulose and when this mixture was centrifuged, the cellulose and cellulase was removed from the original suspension. Because the band with a relative mobility of .56 is still evident at in the gel, even though the endo-glucanase has been largely removed from solution, it can be assumed that the band is not indicative of cellulase proteins but some other protein in the culture medium. This result is consistent with the conclusion drawn from Figures 2 and 3, that the darkest band in the gel is not cellulase.

The scan of the gel in which the second supernatant was electrophoresed shows no significant banding (Fig. 6). The fact that there is no band in the relative mobility range of appx 5.2 to 5.6 indicates that the protein which exhibited the most predominant banding in Figures 2, 3, 4, and 5, is either absent from the sample, or in a very small concentration. This may indicate that whatever the protein is in this relative mobility range, it does not bind to cellulose in significant quantities as would cellulase. This further reinforces the conclusion that the predominant bands in the relative mobility range of 5.2 to 5.6 are not cellulase. The assay for endo-glucanase activity in the second supernatant shows that there was only 173.24 units of

cellulase per ml (Table 4). Given that this sample was concentrated approximately 15 fold, this indicates that there was not as much cellulase in the sample as might have been expected. From this result we might conclude that the attempt to remove cellulase from the original solution was unsuccessful. However, given the lack of banding in the 5.2 to 5.6 relative mobility range, we have concluded that the attempt at removing cellulase from solution was probably successful. What might have caused this result is that the attempt to dislodge the cellulase enzyme from its substrate, cellulose, was unsuccessful. Perhaps the polar urea did not adequately disrupt the electro-chemical interactions between the enzyme and its substrate to the a degree necessary to free the enzyme. Or perhaps, the urea in some way injured the cellulase proteins to such a degree as to render them less functional. Whatever the cause, this does not alter the results of the final conclusion: cellulase is not the protein responsible for the darkest, most significant bands (rm range 5.2 to 5.6) in a coomasie stained gel in which T. reesei culture medium has been electrophoresed.

Appendix

Trichoderma reesei Culture Medium

Monosodium-l-glutamate	0.4 g
Whatman CF-11 cellulose	2.0 g
l-methionine (1.5%)	1.0 g
KCl (14.91%)	1.0 g
MgSO4 - 7H2O (0.05M)	1.0 ml
CaCl2 (0.5M)	1.0 ml
HEDTA (1%)	2.0 ml
KH2 PO4 (1.0M)	14.7 ml
* Metal Mix #4	10.0 ml

Adjusted to pH 5.3 and autoclaved.

* Metal Mix #4

Ground Together-

Fe (NH4)2 (SO4) - 7H2O	28.9 g
Zn SO4 - 7H2O	8.8 g
Mn SO4 - H2O	3.1 g

200 mg of the above dissolved in 100mg sulfosalicyic acid in 100ml of dist. H2O

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A Study of Photoreactivation in the Developing Embryo of Bracon hebetor

by Susan C. Eades

Introduction

Photoreactivation is the process by which living systems repair ultraviolet (UV) damage after subsequent exposure to light of a longer wavelength (Jagger, 1958). Though the bulk of experimentation in this area has dealt with bacteria and viruses this phenomenon has been demonstrated in several higher organisms including sand dollar gametes and zygotes (Cook and Reick, 1962), sea urchin eggs (Cook and Setlow, 1966), and several types of metazoan tissues (excepting mammals) (Cook and Mc Grath, 1967).

The study of UV damage and photoreactivation repair can be used as an analytical tool to explore processes that are occurring in the system under investigation. For this reason, photoreactivation has been studied in many embryonic systems in an effort to elucidate information concerning the myriad of changes that take place during embryogenesis. The purpose of this investigation is to provide an account of the pattern of photoreactivation in a series of developing embryos of the ectoparasitic wasp Bracon hebetor following UV irradiation. The probable chromophores associated with photoreactivable damage have been investigated by others and appear to be nucleic acids and nucleoproteins (Setlow, 1966). A correlation of photoreactivable damage with developmental events can lead to a better understanding of the role played by these materials during development. This in turn can lead to a better understanding of embryogenesis in general.

Materials and Methods

The organism used in this study was the embryo of the ectoparasitic wasp Bracon hebetor, of the stock H+. The adult females feed off the larvae of the mediterranean flour moth Ephestia kuhniella prior to oviposition, and then lay their eggs on the host. In this study, well fed females were removed from their hosts 4-12 hours before the experiment was to begin. At this time the females were given new hosts upon which to lay their eggs. One hour later the eggs were collected from the host and oriented with their concave side up on 2 microscope slides, with an equal number of eggs per slide. The age of the eggs was thus known within an interval of an hour.

The slides were placed in a covered container and put in an incubator at approximately 30°C and 60-70% relative humidity until the desired developmental stage was reached. At this point the eggs on both slides were exposed to ultraviolet radiation from a germicidal lamp at a known dose. One slide was then immediately placed back into the covered container and returned to the incubator. The other slide was placed under a black lamp and exposed for 1 hour. This slide was then also put in the light proof container in the incubator. The slides

were examined for hatchability with the aid of a dissecting microscope (x30) approximately 2 days after they had been laid.

The ultraviolet (UV) radiation source was a General Electric 15 - watt germicidal lamp, with a transmission of almost exclusively 2537 Å radiation (Koller, 1962). The photoreactivating (PR) light source was a General Electric 15 watt 360BL lamp with a spectrum ranging from 3200 Å to 4400 Å, with a peak near 3600 Å (Koller, 1962).

Eggs of three different developmental stages were used: 4-5 hours, 11-12 hours, and 24-25 hours of development. At each age, 5 groups were exposed (a minimum of 30 eggs per group), each to a different dose of UV, in an attempt to determine a range of UV sensitivity and photoreactivability. The percentage hatched was plotted against the dose received for eggs exposed to UV and PR light, and those exposed only to UV light. Unirradiated eggs were kept as a hatchability control, yielding a hatchability rate of 74.25%.

Results

The results obtained in this experiment have been presented graphically in Fig. 2-5 and summarized in Table 1. The developmental stages chosen for the study were 4-5 hours, 11-12 hours, and 24-25 hours. These particular times were chosen in an effort to investigate photoreactivation in an early stage, a middle stage, and a late stage of development. The Bracon embryo has a normal gestation period of approximately 30 hours when incubated at 30°C.

The typical Bracon egg is oviod, averaging 550μ in length and 100μ at its greatest width. The dorsal side is concave and the ventral side is slightly convex. The newly laid egg is composed mainly of yellow-white yolk.

Fig. 1 An early Bracon egg.

Figures 2-4 give a comparison of the effect on hatchability of exposure to UV light, and exposure to both UV and photoreactivating (PR) light. It is evident that though different stages show different sensitivities, the eggs exposed to both UV and PR light did not reach the hatchability rate of unirradiated eggs at any of the stages.

The figures in Table 1 were calculated from the UV/UV + PR light graphs. The LD 50 value is the dose at which 50% of the eggs live (hatch) and 50% die. The dose-reduction factor (DRF) is a value which relates photoreactivation and UV damage in such a way that the capabilities of the PR light can be evaluated. It is the ratio of the LD 50 values for UV treatment and UV and PR light treatment. As the DRF approaches 1, there is less photoreactive repair taking place. Figure 5 offers a graphical comparison of the effects of UV and UV + PR light among the 3 stages studied. The LD 50 values were used as a basis for

this comparison. Apparently UV sensitivity decreases as development proceeds. Along with this, however, is an apparent decrease in the ability of PR light to effectively reduce damage. It took relatively little more radiation for fatal damage to occur in eggs 24-25 hours than for the same amount to be fatally damaged without PR treatment. In comparison, the eggs 4-5 hours old and 11-12 hours old showed significant recovery after exposure to PR light. These same conclusions can be drawn from the DRF values.

Discussion

The results of this study show that photoreactivation is a method employed by Bracon embryos to repair UV damage. The variability among the stages in UV sensitivity and in the ability to repair the damage implies that the UV chromophores associated with photoreactivable damage may vary in their importance over the gestation period. Setlow has done a great deal of work on the identification of the important chromophores, and has concluded that the nitrogenous bases of DNA are probably the most sensitive targets (Setlow, 1966).

A similar conclusion can be reached by inference. The information-containing macromolecules, protein and nucleic acids, are essential to the survival of a living system. A single cell may contain several thousand different protein molecules, plus a thousand copies of each type. Thus the destruction of a number of particular proteins would probably not kill the cell. Proteins are synthesized by translation of a genetic code from a strand of ribonucleic acid (RNA). These strands of RNA are transcribed from strands of deoxyribonucleic acids (DNA). As long as DNA is present, any RNA that is destroyed can be replaced through transcription of the DNA template. The destruction of DNA, however, would severly impair the cell, for the strands are coded with the genetic specifications of all the necessary components of the cell. The destruction of this material would lead to developmental arrest. Thus one can infer that a component of DNA is probably the most sensitive target of ultraviolet radiation (reasoning adapted from Hanawalt, 1969).

Many different types of UV-induced damage have been proposed, such as chain breaks, denaturation, DNA crosslinks, and nucleic acid-protein crosslinks (Setlow, 1966). The most important types of damage relating to photoreactivation, however, is the formation of pyrimidine dimers. It was discovered in the 1950's that covalent dimers between adjacent thymines are a significant photoproduct of UV-irradiated DNA. (Hanawalt, 1969). The effect of these dimers is the distortion of the shape of the DNA molecule by interference with normal base pairing. This results in the disruption of the replication of daughter DNA strands (Stanier et al, 1976). Thymine is the one nitrogenous base found in DNA that is not in RNA; this serves to support the hypothesis that DNA is the sensitive chromophore.

There is considerable evidence that the mechanism of photoreactivation is the activation of an enzyme capable of splitting UV-induced pyrimidine dimers. The presence of such an enzyme has been demonstrated in

TABLE 1

Age, principle morphology, LD_{50} values for UV/UV+PR light, and dose-reduction factors for UV-irradiated Brecon embryos tested for photoreactivation.

Age (hours)	Principle morphology*	LD_{50} (ergs/mm^2) UV	LD_{50} (ergs/mm^2) UV+PR	DRF
4-5	Nuclei in periphery (syncytium) Incipient blastoderm	80	160	.50
11-12	Blastoderm Gastrulation initiated	100	180	.56
24-25	Most organ systems complete	350	480	.73

*from Amy, 1961.

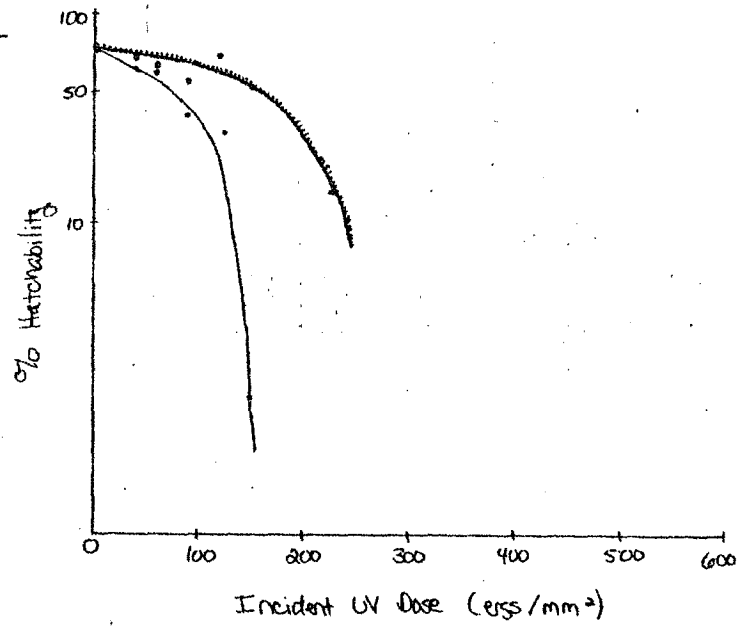

Fig. 2. Dose-hatchability curves for eggs irradiated at 4-5 hours of age.
——— : eggs irradiated with 2537Å UV only.
▪▪▪▪▪▪ : eggs irradiated with 2537Å UV and immediately exposed to 1 hour of PR light (3600Å).
Each point represents at least 30 eggs; control hatchability = 74.25%.

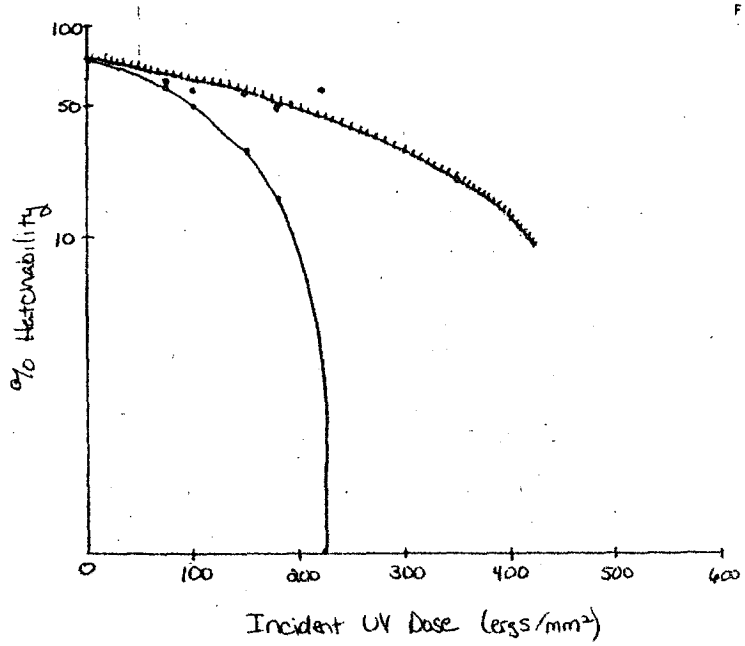

Fig. 3. Dose-hatchability curves for eggs irradiated at 11-12 hours of age.
——— : eggs irradiated with 2537Å UV only.
▪▪▪▪▪▪ : eggs irradiated with 2537Å UV and immediately exposed to 1 hour of PR light (3600Å).
Each point represents at least 30 eggs; control hatchability = 74.25%.

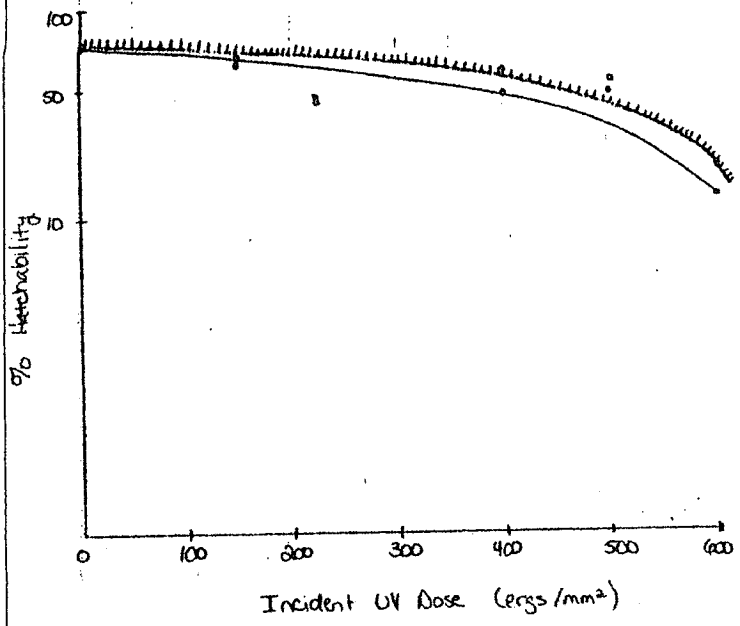

Fig. 4. Dose-hatchability curves for eggs irradiated at 24-25 hours of age.
——— : eggs irradiated with 2537Å UV only.
wwwwww : eggs irradiated with 2537Å UV and immediately exposed to 1 hour of PR light (3600Å).
Each point represents at least 30 eggs; control hatchability = 74.25%.

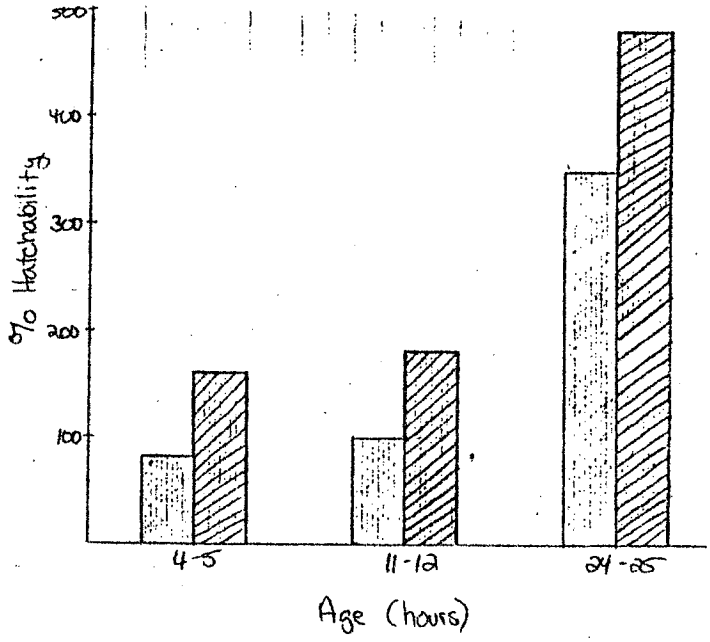

Fig. 5. A graph of the LD_{50} values for eggs of 3 developmental stages.
□ : eggs irradiated with 2537Å UV only.
▨ : eggs irradiated with 2537Å UV and immediately exposed to 1 hour of PR light (3600Å).

sea urchin eggs (Cook and Setlow, 1966), several species of bacteria (Setlow, 1966), and several types of metazoan tissues (Cook and McGrath, 1967). Light of wavelengths between 3100 Å and 4400 Å serves as an energy source to activate the enzyme. The enzyme is complexed with the dimer and upon activation, cleaves the unwanted bonds between the adjacent bases, restoring the original nucleotide sequence (Grosch, 1979).

With the idea in mind that photoreactivation is a method of repairing UV-induced damage to nucleic acids, the results obtained from this experiment can provide some information on the role of nucleic acids in development. Amy (1964) investigated UV sensitivity in the Habrobracon embryo and found that sensitivity is related to the developmental stage of the embryo at the time of exposure. Since any damage that can be corrected by a photoreactivating light most likely occurred in nucleic acids, the pattern of photoreactivation can be used to determine the importance of nucleic acids, especially DNA, at a given developmental period.

Herein lies the value of the dose-reduction factor (DRF). A constant DRF signifies that UV damage is similar at each age, meaning that the mechanisms of UV damage are the same, though the sensitivities to this damage may differ. A change in the DRF signals a change in the type of damage. Therefore the results of this experiment seem to show that the chromophores responsible for photoreactivable damage, most likely nucleic acids, are not involved in a constant manner during development.

Dissimilar results were found by Amy in his investigation of photoreactivation in the Habrobracon embryo (1965). His DRF values were fairly constant, except at the very early stages of development when the eggs were oriented in such a way that the UV could not easily penetrate to the nuclei. Excluding these exceptions, his average DRF value was .30. At the age of 25 hours, the value jumped to .50. A similar jump was seen in the 24-25 hour embryos of my experiment which went from an average DRF value of .53 to .76. The reason for the difference in the constancy of the DRF values between the two experiments is probably due to the time he was able to spend on his experiment as opposed to what I spent on mine. He studied the entire gestation period thoroughly, so that a small difference in the DRF value of one stage was not as significant as a similar change within the three stages I studied. Also, the larger number of eggs he used in his experiment (42,000 total) made his results more statistically reliable.

The higher DRF value at 24-25 hours in both experiments indicates that UV damage is less responsive to PR light after the embryo has essentially completed organogenesis. This indicates the importance of DNA in directing the processes of morphogenesis, most of which have occurred by 24-25 hours. However, any results obtained at this stage must be interpreted cautiously, because as the embryo becomes more and more definitive, hatchability becomes a less reliable criterion (Amy, 1965).

Figure 5 gives a graphical representation of the changes in sensitivity to UV radiation of the 3 stages. The results indicate that the embryo is less sensitive to all types of UV-damage, not just photoreactivable damage, as it approaches its definitive stage. This is perhaps due to an increased effectiveness of alternative repair systems at later stages, or, more likely, that the UV chromophores do not have the same influence upon the embryo as they did at earlier stages. This again suggests the importance of nucleic acids in directing morphogenetic events. Once the embryo has developed its rudimentary organs, damage to nucleic acids will not necessarily impair hatchability, though it may produce mutagenic effects in the larvae. Since photoreactivable damage also declines in the later stage, perhaps the large dose of UV radiation necessary to have an effect induces irreversible damage to chromophores other than nucleic acids which are capable of alternative repair at lower doses.

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Physiology of Thermoregulation

by Jean Dabezies

Before discussing thermoregulation, there are several facts which must be pointed out. The core of the body is of a higher temperature than the skin; therefore the surface must be at a lower temperature than the inner parts. The heat produced must be transferred to the surface before it can be transferred to the environment. The body is cooled by the blood(1).

There has been found a circadian cycle of core temperature fluctuations of birds and animals. Diurnal animals show a temperature peak during the day, with the minimum at night. Nocturnal animals are opposite. Further investigation has shown that this circadian cycle will continue if the organism is at rest; the cycle is not a result of activity. This cycle can be reversed by reversing the dark and light periods, although this cycle is not governed by illumination. In the presence of uniform light conditions, the cycle of core temperature variation continues with the same timing; it is also endogenous(2).

In order to have a maintenance of temperature, heat loss must equal heat gain. Heat loss can be accomplished by conduction, radiation, and evaporation. Conduction is only a useful means of heat loss if the environment is at a lower temperature than the skin. Radiation is only good if the external radiation (i.e., the sun) is not too strong. This equilibrium is stressed in times of severe work loads when 10 times the heat must be dissipated(3).

In hypothermic conditions, conditions in which the core temperature could be lowered, evaporation is not an important mechanism of heat loss. There are two responses that an organism can make under hypothermic conditions. There can be an increase in heat production to maintain a certain core temperature or there can be a lowering of core temperature resulting in hibernation. Heat production occurs by the following three processes: 1) increased muscular activity, 2) involuntary muscle contraction (shivering), and 3) non shivering thermogenesis (increased metabolic rate)(4).

In all animals there is a temperature called the critical temperature, below which, metabolic rate will increase. It has been found that arctic animals have a lower critical temperature than tropical animals. This is a result of lower conductance of arctic animals due to greater insulation. The insulation also increases the thermoneutral zone. The thermoneutral zone is the temperature range within which metabolic heat production is not affected.

There are several ways in which animals have adapted to hypothermic environments. Small animals take advantage of microclimates (burrows) or hibernate. Swimming animals have blubber to reduce heat loss. Birds reduce exposure to the extremities and decrease circulation to the skin(5).

Hibernation is the drop in body temperature of animals to the level of their surroundings. The metabolic rate, heart rate, respiration and other functions are slowed resulting in less energy consumption. The hibernator gains weight to create reserves for extended periods of unfavorable conditions. Some of this is what is known as brown fat. Brown adipose tissue is specialized for heat production and its abundance is usually correlated with the capacity for calorigenic response to catecholamines(6). This fat is used to help bring animals out of hibernation. It produces heat by the oxidation of fatty acids. Adaptation to the cold leads to an increase in the amount of fat and a series of changes (biochemical and ultrastructural) that can be observed in the tissue. There is an increase in the number of catalase-positive particles within the brown fat in relation to the number of mitochondria after cold adaptation. It has been shown that these catalase-positive particles are true peroxisomes. Peroxisomal reactions carry no provisions for the retrieval of energy through oxidative phosphorylation and catalyze an essentially wasteful form of respiration or exactly the heat-evolving quality of brown fat metabolism. Most animals which hibernate are small(7).

Torpor is similar to hibernation. Doves and hummingbirds become inactive at night. The temperature is lowered but only to a certain point at which temperature the metabolic rate will increase; therefore body temperature is maintained somewhat(8).

In hyperthermic environments, evaporation becomes a key aspect in heat balance. When environmental temperature equals body temperature, conductance is zero and radiation in near zero. There are several ways in which an animal can respond to hyperthermic environments. One response is increased circulation to the skin. Another response is sticking out and exposing increased surface areas of naked or thinly furred areas(9).

Seals and whales have flippers rich in blood supply with little blubber. In warm waters heat can dissipate through the flippers. However in cold waters, cool venous blood would quickly decrease the core temperature. For the maintenance of core temperature, a heat exchanger has evolved in seals and whales. In cool waters hypothermia is prevented by surrounding arteries with veins. The cool venous blood is warmed by the arterial blood. In warmer waters when heat dissipation is required, increased blood supply causes the diameter of the central artery to increase. This causes the surrounding veins to collapse and therefore the blood returns via more cutaneous veins, bypassing the heat exchanger(10).

In man a similar process is seen though there is no true heat exchanger. Most venous blood from the limbs return by way of the deep veins. In warm suroundings, much venous blood returns through superficial veins(11).

In times of greater hyperthermic conditions, evaporation plays a greater part in thermoregulation. Humans perspire; dogs pant; cats and marsupials lick their fur and limbs; birds have a gular flutter,

which is the rapid oscillation of the floor of the mouth and upper throat. Evaporation occurs on panting animals as warm air flows over moist surfaces. However, in panting animals it was believed that the muscle work involved in panting would have produced greater amounts of heat than it would have dissipated. It has been shown that panting animals have elastic properties in their respiratory tract. By using the resonance frequency of the respiratory tract, less heat is produced by muscle action and thus heat is dissipated(12).

Estivation is inactivity usually during the summer. Snails will estivate during times of drought. Squirrels go into burrows and become inactive during the hottest months of the summer to avoid the heat(13).

In desert environments, animals have evolved several adaptations to cope with the harsh climate. One such adaptation is seen in the camel. It evaporates less than expected. It stores heat during the day resulting in increased body temperature. The increase in body temperature decreases further heat flow from the environment. The fur acts as a substantial barrier to heat gain from the environment; if the fur is shaved 50% more evaporation occurs. At night the heat is released. Although there is a greater variation in body temperature, this adaptation allows for protection against hypothermia during the day and hyperthermia at night(14).

In the ground squirrel there is another behavioral adaptation for extreme hyperthermic environments. The ground squirrel defies expected behavior by running around and being active during the day. It can be seen ducking in and out of burrows. This allows it to dissipate body heat and avoid hyperthermia. This dissipation is avoided by the squirrel's large relative surface area(15).

The brain is the organ that is most sensitive to hyperthermia. An African gazelle, after running from a predator, can increase its body temperature to dangerously high levels. To help cool the blood flowing to the brain, the gazelle has a blood system called a rete. Arterial blood flows to the brain from a network of vessels called the circle of Willis. This network is located in a pool of venous blood in the cavernous sinus and acts as a heat exchanger. The venous blood comes from the nasal veins and is cooler because of the heat loss across the nasal membranes. In fact blood flow to the tongue and nasal mucosa is closely related to body temperature and the rate of breathing. Venous blood cooled by sweating on the face of humans could cool the cerebral arterial blood, though there is no rete in humans(17). (See Fig. 1 and 2).

Cold-blooded animals have made several adaptations to their various environments. Terrestrial animals have dark skin to attract solar radiation and thus gain heat in cool conditions. In warm conditions they increase exposed surface area to help dissipate heat. Aquatic homeotherms such as the tuna and the shark which are large fast-swimming fish, maintain a core temperature though their surface temperature is that of the surrounding waters. This is accomplished

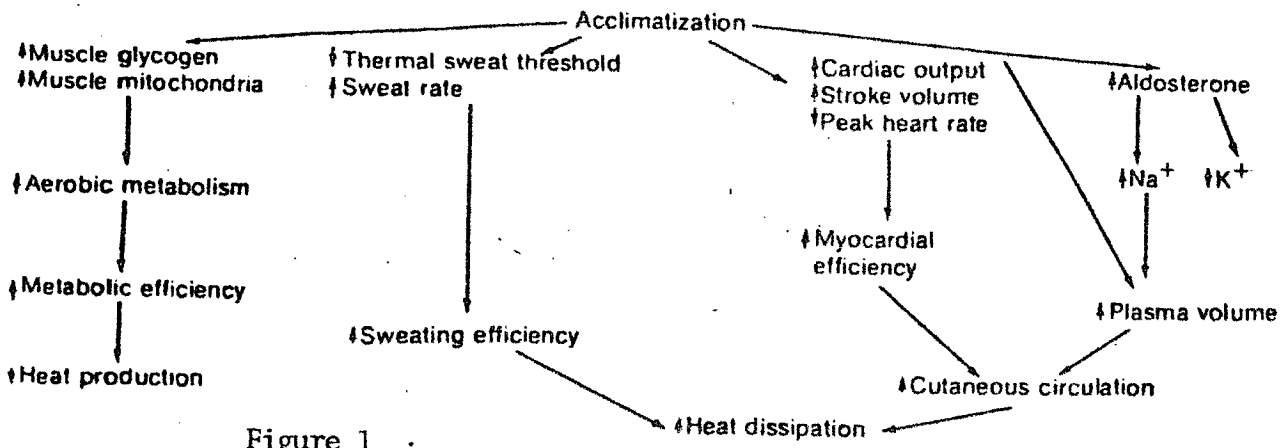

Figure 1

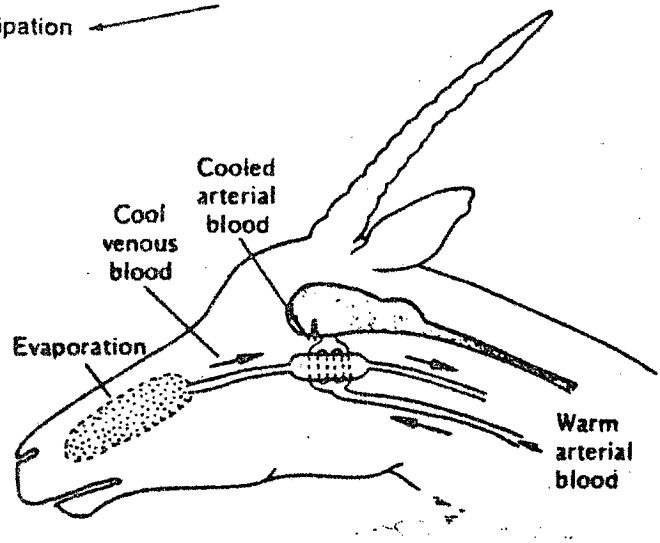

Figure 2

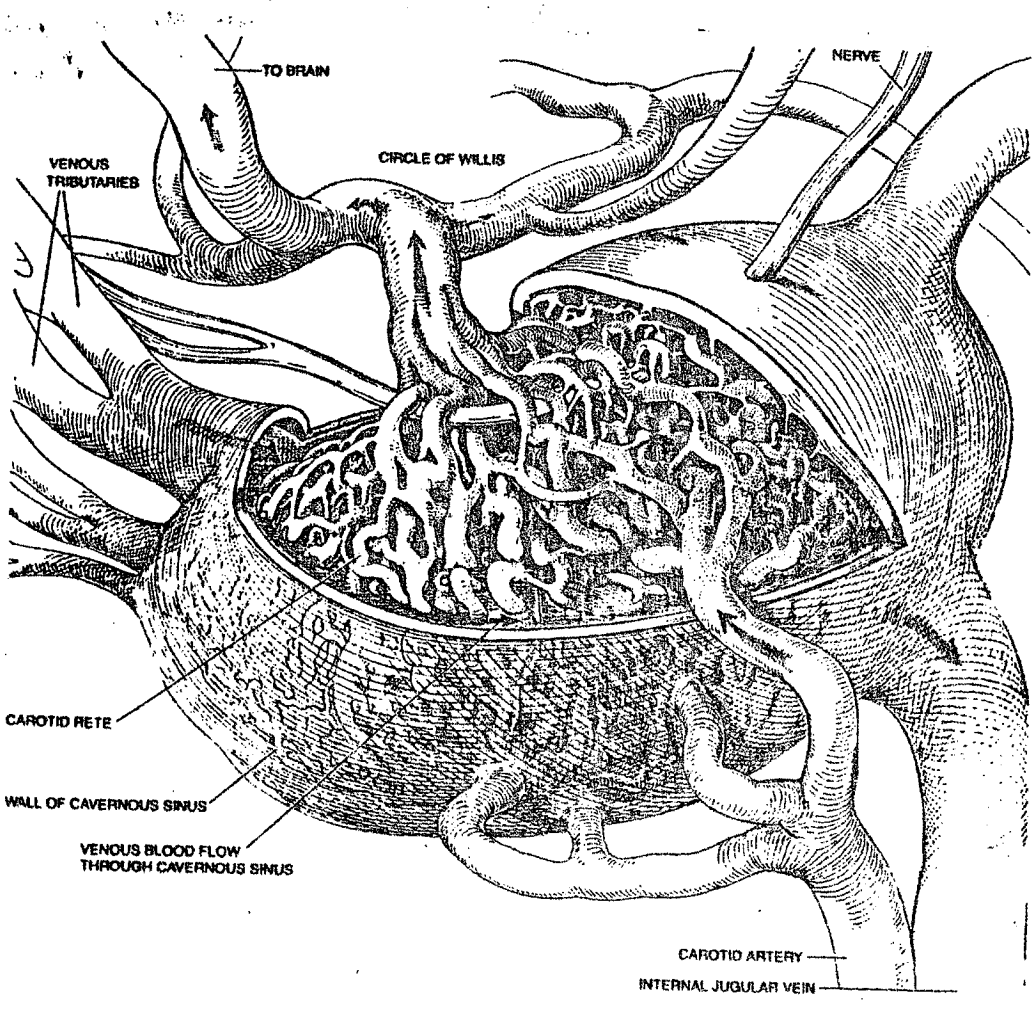

Figure 3

by the use of a counter current heat exchanger between the gills and the tissue(18).

The swordfish is a cold-blooded aquatic animal, but unlike the tuna or the shark, does not maintain a high level of continuous activity. Its muscle and viscera are close to the water temperature. They spend the night near the surface, but descend as much as 600m during the day. The temperature change may be as great as 19 degrees C in less than 2 hours. These large and abrupt changes in temperature that the swordfish experience would cool the brain and affect the central nervous system processes in the fish. But the swordfish has developed a heater which warms the brain and eye. There is a mass of specialized tissue and a vascular heat exchanger which warms organs to temperatures significantly above that of the surrounding waters and thus reduces the dangerous temperature fluctuations(19).

Temperature regulation requires autonomic, endocrine, and skeletomotor responses. The hypothalamus is well suited for this task. Stimulation of the anterior hypothalamus causes supression of shivering and cutaneous vasodilation (drop in body temperature). Stimulation of the posterior hypothalamus produces a set of opposite responses that function to generate or conserve heat(20).

The hypothalamus and the process of temperature regulation fits the model of a control system. A control system regulates a controlled variable (temperature) that is maintained within a certain range. One way of regulating the controlled variable is to measure it by means of a feedback detection and to compare it with a desired value of set point. This is accomplished by an integrator or an error detection that generates an error signal when the measurement of the controlled variable does not match the set point signal. The error signal then drives controlling elements that adjust the controlled system in the desired direction. In thermoregulation, normal body temperature is the set point. The integrator and many controlling elements seem to be in the hypothalamus. Feedback detectors collect body temperature information from peripheral temperature receptors located throughout the body and central temperature receptors concentrated in the hypothalamus(21).

Fever is an increase in body temperature usually associated with bacterial or viral infection. Substances called pyrogens, present in bacteria, are what cause the temperature to increase. This is not, however, a failure of the thermoregulatory mechanism. It is as if the pyrogen causes an increase in the body thermostat. In these conditions there is a higher set point temperature. It has been shown in studies with lizards that the rise in temperature increases the survivability of the individual(22).

Most of the thermoreceptors are in the hypothalamus. They read the temperature of the blood passing through the hypothalamus. But the hypothalamus is in the center of the core of the body and is one of the last places that the temperature changes occur. It is therefore necessary to have peripheral thermoreceptors to have a more accu-

rate judge of the body's thermal equilibrium. In humans nervous input of facial cutaneous thermoreceptors control the amount of sweating in the face(23). Skin temperature overall is an important input to the thermoregulatory center and skin temperature affects sweat rates at even elevated temperatures. Sweating rates on both upper and lower body surfaces are proportional to the lower body skin temperature(24).

There is a theory known as the Monamine Theory of thermoregulation which states that the release of putative neurotransmitters norepinephrine (NE), epinephrine (E), and serotonin (5-HT) in the hypothalamus affects body temperatures. When 5-HT was injected into the cerebral ventrical of an unanesthetized cat, hypothermia developed. Injection of NE or E to the same region caused hypothermia. The site of action of these substances was localized in the anterior-preoptic hypothalamus. This accounts for the functional control, but does not explain the intrinsic control (set point).

The set point for body temperature, it is believed, may be controlled by the ratio of Na to Ca ions in the posterior hypothalamus. When a solution containing excess Na ions was injected into the posterior hypothalamus, hyperthermia developed. These cations were without effect when injected into other regions of the hypothalamus. When the set point is changed by altering the Na to Ca ion ratio, an animal can thermoregulate around the new artificial set point(25).

Rats with brain levels of NE depleted by alpha-methyl p-tyrosine, had lower than normal rectal temperatures in the cold and higher than normal rectal temperatures in the heat. Monkeys and rats injected with a serotonin depleter, 5,6-dihydroxytryptamine, experienced an increase in body temperature and were unable to maintain a normal body temperature in warm or cold conditions(26).

Acclimatizaton is a physiologic process by which an individual becomes able to tolerate extremes of temperature safely and comfortably. Adaptation takes place that involves the cardiovascular, endocrine and exocrine systems(27). Acclimatization can occur for both cold environments as well as warm. It has been shown that long term exposure to cold can enhance the release of thyroxine and thus increase body heat by increasing tissue metabolism. In cold adapted organisms, there is brown adipose tissue, the principle function of which is heat production. This helps avoid hypothermia. As was mentioned earlier, the brain is the organ that is the most sensitive to temperature changes. The heat production is brought about under physiological conditions by the release of norepinephrine from sympathetic nerve terminals directly innervating the brown adipocytes. The interaction of this neurohormone with cell surface receptors triggers a series of metabolic events in the adipocyte. One of the earliest events is the activation of the adenylate cyclase-cAMP system. As a consequence, glycolysis, lipolysis, fatty acid oxidation, and mitochondrial oxygen consumption are all stimulated. It is also well established that brown adipose tissue is an important site of nonshivering thermogenesis(28).

Acclimatization to heat stress is closely related to physical fitness. In fact some of the physiological changes are similar to those made in physical training. The goal in acclimatization to heat stress is less heat production and greater heat dissipation. There is an increase in muscular glycogen, muscle mitochondria and metabolic efficiency. There is less heat production. Sweat rate goes up, and the thermal sweat threshold, the temperature at which sweating starts, goes down. Sweating efficiency increases to increase heat dissipation. Cardiac output and stroke volume increase while peak heart rate decreases which results in increased myocardial efficiency. Aldosterone levels increase resulting in increased Na ion levels and decreased K ion levels. This results in increased plasma volume. Increased plasma volume along with increased myocardial efficiency results in increased cutaneous circulation. Increased cutaneous circulation and increased sweating efficiency result in increased heat dissipation(29).

In this paper I have discussed thermoregulation in somewhat broad terms. I have attempted to show general thermoregulatory problems as well as specific adaptations to these problems. Through the physiological changes in acclimatization, not all individuals react as effeciently to changes in core temperature. Most of what was discussed in this paper was directed towards animals and though not all organisms can regulate their own temperature, the temperture of the surrounding environment is important to all organisms.

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Symbolic Differentiation Using LISP

by Jim Golden

The rules governing differentiation can be given in 6 forms: the power rule, the sum rule, the product rule, the quotient rule, rules concerning trigonometric functions, and the chain rule. Unlike integration or theorem proving, these rules can be seen as relatively definite and finite. Though there are only a few rules, the task of differentiation via a computer language would be a formidable and arduous one, and even then all possible cases could not be represented. The ability to cover all possible cases is the advantage of symbolic mathematics. In the realm of symbolic mathematics LISP and its descendant languages stand alone.

Overview of LISP

A symbol-manipulation program is one that uses symbolic expressions to remember and work with data. A symbol-manipulation program is one in which there are a number of sections that recognize particular symbolic expressions, tear apart old ones, and assemble new ones. (see fig. 1.)

```
(DEFINE DFUNC (L)
    (COND (EQ (CAR L) (' TAN))
(LIST (' *) (LIST ('  SEC 2)) (CADR L)) (DIFF (CADR L)))))
```

Fig. 1. A symbolic subroutine in LISP that breaks apart a list to find the tangent function and evaluate its derivative.

Using such a rule involves taking a list apart, finding the conditions for the rule and checking to see if those conditions are on a list of believed assertions.

In the English language letters make up words which make up sentences which make up paragraphs, etc. In LISP the fundamental things formed from bits are word like objects called atoms. Groups of atoms form lists from which higher level lists are formed. Atoms and lists collectively are called symbolic expressions or S-expressions. S-expressions are the objects manipulated by LISP. (see fig. 2.)

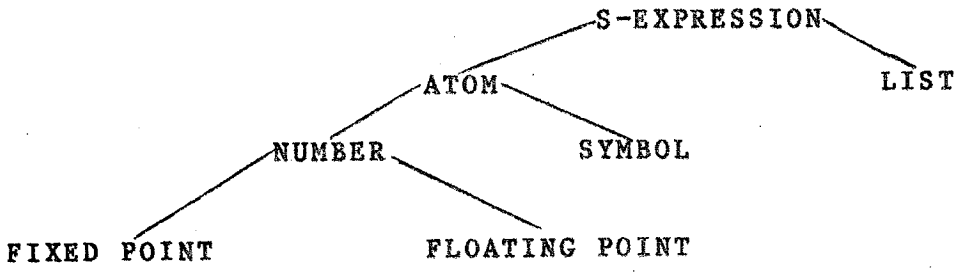


Fig. 2. The various types of LISP objects and their relationships

In LISP the function to be performed is always given first followed by the arguments that the function works with. For example; in this particular form of LISP to add 3 and 4 the programmer inputs (+ 3 4) and the computer responds 7. To multiply 3 and 4 type (* 3 4) and 12 is returned.

Other mathematical expressions or S-expressions in LISP include -, /, max, and min. Originally the functions were written out in English. For this particular LISP interpreter I redefined them in mathematical/computational symbols for easier manipulation. LISP can handle both fixed-point and floating-point numbers.

To understand the way LISP can perform derivatives we must look at some of the ways LISP takes apart S-expressions and manipulates them. Take for example the following sentence: "Differentiation is for freshmen." To take this sentence apart LISP uses the expressions CAR and CDR. CAR returns the first element in a list.

```
(CAR ('( DIFFERENTIATION IS FOR FRESHMEN)))
DIFFERENTIATION
(CAR ('( A B C)))
A
```

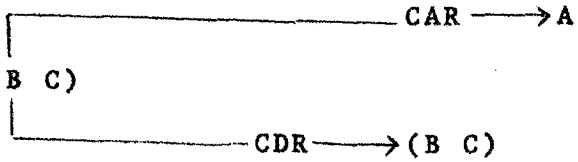
CDR does the complimentary of CAR. It returns a list containing all but the first element.

```
(CDR ('( TWAS BRILLIG AND THE SLYTHY TOVES)))
(BRILLIG IN THE SLYTHY TOVES)
(CDR ('( A B C)))
(B C)
```

CDR, unlike CAR always returns a list (denoted by the parenthesis). When CDR is applied to a list with only one element it returns the empty list denoted by (). To take the actual value instead of the list, we use (CAR (CDR L)).

```
(CAR (CDR ('( A B C))))
B
```

Fig. 3.



It should be obvious upon seeing some of these LISP functions that LISP is a powerful tool and the right language to learn for symbolic manipulations. LISP was designed for symbol manipulation from the start and in fact LISP is an acronym for List Processing Language.

Not only does LISP have functions that are used to break up lists, there are functions such as LIST, APPEND and CONSTRUCT that put lists together.

Some of the more important functions in LISP that make differentiation possible are the user defined functions. LISP contains a special function labeled DEFUN that is used to define new functions for

easier manipulation of lists. In this particular form of LISP the function is DEFPROP. To avoid confusion in a large program DEFPROP has been changed to DEFINE. DEFPROP itself was used to define DEFINE. Let's take a look at the advantages of DEFINE over DEFPROP and see how the two are put together.

```
(DEFPROP DEFINE FEXPR
  (LAMBDA (L)
    (EVAL
        (LIST (' DEFPROP) (CAR L) (' EXPR)
        (LIST (' LAMBDA) (CAR (CDR L))
                (CAR (CDR (CDR L)))))))))
```

This function takes the working power of DEFPROP and defines it in the simplified form of DEFINE. For example; let's say we want to break apart a large list such as "Fast computers are always nice." We want just the phrase "always nice." Using CDR over and over again we can get "always nice."

```
(CAR (CDR (CDR (CDR ('( FAST COMPUTERS ARE ALWAYS NICE))))))
```

This is both awkward and inconvenient. It would be nice to have a single function that broke apart the list in the way we want it. using the power of DEFINE we can have such a function.

```
(DEFINE CADDDR (L)
        (CAR (CDR (CDR (CDR L)))))
```

and now to get "always nice" we simply type

```
(CADDDR ('( FAST COMPUTERS ARE ALWAYS NICE)))
```

Using DEFINE I set up a file of functions that I would use to create the differentiation program.

Now that we have the functions that break down the S-expressions we can take a look at the individual pieces. COND (condition) is the function to use. The format for COND is to test a piece of a list and give the proper result. (see fig. 4.)

```
(COND (<test 1>.....<result 1>)
      (<test 2>.....<result 2>)
        .                .
        .                .
        .                .
      (<test N>.....<result N>))
```

Fig. 4. The format for COND.

Here is an example of how DEFINE and COND are used together to start the main function of the derivative program. This section of the program is called DIFF for differential. It is here that the function to be differentiated is first broken down then sent to the respective rule for evaluation.

```
(DEFINE DIFF (L)
    (COND   (( EQ L (' X)) 1)
            ((EQ (CAR L) (' )) (POWER L))
            ((EQ (CAR L) (' +)) (SUM L))
```

```
((EQ (CAR L) (' *)) (PROD L))
((EQ (CAR L) (' /)) (QUOT L))
(T (DFUNC L))))
```

This algorithm looks at the first element of each list to test what rules should be used (remember LISP functions are written in infix notation). If the value is simply X a value of 1 is returned, if the value is a + then the sum rule is used to evaluate the function, if * the the product rule, etc. If none of the above then the program assumes that the value is a trigonometric function. This is easier than having to define all the trig functions.

Before we look at the program any further it is important to look at the compiler/interpreter and how it modifies LISP.

The Compiler

The compiler I used was originally the MICROLISP program for the Apple II. One of the main purposes for my work was to design a LISP interpreter for the Digital PDP-11.

Many of the algorithms and functions had to be redefined for the PDP-11. Most of this was simply redefining smaller functions or replacing symbols.

The important difference is the use of the LOAD function. When the interpreter was completed and run a question mark was returned. a LISP function can be inputed. This is perfectly satisfactory for small functions such as +, or *, but to use user defined functions and large intelligent programs this is unsatisfactory. A LOAD function was placed in the interpreter. Programs could be written under the EDIT mode using the extension .LSP. When a program was to be used by the interpreter it could be loaded. For example; upon running the final version of the interpreter (LISP3.BAS) a question mark was returned. Then the LISP function LOAD was used like any other LISP function.

```
? (LOAD DIFF4.LSP) <return>
```

The interpreter would then return a list of all user defined functions, then the function for evaluation could be given in the following form:

```
(DERIVATIVE ('( TAN X)))
```

or

```
(DERIVATIVE ('( X  2)))
```

DERIVATIVES USING LISP, DIFF4.LSP

The LISP program for taking derivatives is divided into five main sections. The first section is a group of user defined functions that will be used to set up the skeleton of the program. These include CADR, CDDR, CADDR, LENGTH, ADD1, etc. To define all these functions in the actual interpreter would take up a lot of storage space and some of these functions are rarely used. It is much easier to set these up in the program to be loaded.

The next section is the subroutine to convert infix notation to LISP's prefix notation. Because LISP operates in prefix notation all problems to be evaluated must be in that form. The original program (DIFF3.LSP) was setup without the INF-TO-PRE function. When submitting a problem for differentiation the problem had to be in LISP notation. For example; to evaluate d/dx((tan x)2) the correct form was (DIFF('((TAN X) 2))). This is confusing to look at and is a source of error to the programmer not used to LISP notation. Using INF-TO-PRE the new form is (DIFF('((TAN X) 2))).

The fifth section of the program is the opposite of INF-TO-PRE, that is prefix to infix or TOINFIX. This section converts the finished derivative back into infix notation so that it is easier to read.

We have already seen the section of the program called DIFF which is the derivation directory to other subroutines. This section gives us a literal derivative in LISP notation. The problem with this section of the program is that it returns all values in the derivative, including 1 and 0. For example; evaluating d/dx(X2) we get 2*X, but in actuality we get 2*(X1). To clarify the answer we need to get rid of the 1. A fourth section to this program takes care of this. This subroutine is SIMPLIFY. It is similar to DIFF in that it is a directory of recursive subroutines, and uses many of the same programming techniques.

```
(DEFINE SIMPLIFY (L)
        (COND   ((EQ (CAR L)(' *))(MSIMP L))
                ((EQ (CAR L)(' ))(PSIMP L))
                ((EQ (CAR L)(' +))(SSIMP L))
                ((EQ (CAR L)(' /))(QSIMP L))
                ((EQ (CAR L)(' -))(BSIMP L))
                (T L))))
```

MSIMP is an acronym for multiply-simple, PSIMP for power-simple, etc. SIMPLIFY checks the first character of each and sends the function to be simplified to the appropriate subroutine.

In each subroutine the S-expression is torn apart and evaluated. In MSIMP there are commands that check for 1's and 0's.

```
(COND   ((= M 1) N)
        ((= N 1) M)
        ((= N 0) 0)
        ((= M 0) 0)
```

If M is equal to 1, return just the value of N. If N=1, then just return the value of M, etc. PSIMP, SSIMP, BSIMP and QSIMP are all similar and have similar test patterns.

The final definition of DERIVATIVE uses infix, prefix, DIFF, and SIMPLIFY:

```
(DEFINE DERIVATIVE (L)
        (TOINFIX (SIMPLIFY (DIFF (INF-TO-PRE L)))))
```

This program fully expresses the ease and power of symbolic ma-

thematics expressed in LISP. Many more programs can be formed using DIFF4.LSP as a model. The DIFF subroutine can be replaced with programs that do Laplace transforms, integration, or trigonometric simplification. LISP is both simple and powerful and should be added to the repetoire of any serious programmer.

Appendix: A Listing of DIFF4.LSP

```
(DEFPROP DEFINE FEXPR
   (LAMBDA (L)
      (EVAL
        (LIST (' DEFPROP) (CAR L) (' EXPR)
          (LIST (' LAMBDA) (CAR (CDR L)) (CAR (CDR (CDR L))))))))
(DEFINE ADD1 (N)
    (+ N 1))
(DEFINE SUB1 (N)
    (- N 1))
(DEFINE MINUS (N)
    (- 0 N))
(DEFINE CADR (L)
    (CAR (CDR L)))
(DEFINE CDDR (L)
    (CDR (CDR L)))
(DEFINE CADDR (L)
    (CAR (CDR (CDR L))))
(DEFINE LENGTH (L)
    (PROG (M A)
        (SETQ M L)
        (SETQ A 0)
        LOOP
        (COND ((NOT M) (RETURN A))
              (T T))
        (SETQ A (ADD1 A))
        (SETQ M (CDR M))
        (GO LOOP)))
(DEFINE DERIVATIVE (L)
    (TOINFIX (SIMPLIFY (DIFF (INF-TO-PRE L)))))
(DEFINE DIFF (L)
    (COND ((EQ L (' X)) 1)
          ((NUMBERP L) 0)
          ((EQ (CAR L) (' )) (POWER L))
          ((EQ (CAR L) (' +)) (SUM L))
          ((EQ (CAR L) (' *)) (PROD L))
          ((EQ (CAR L) (' /)) (QUOT L))
          (T (DFUNC L))))
(DEFINE POWER (L)
    (LIST (' *)
        (LIST (' *)
            (CADDR L) (LIST (' ) (CADR L) (SUB1 (CADDR L))))
                (DIFF (CADR L)))))
(DEFINE SUM (L)
    (LIST (' +) (DIFF (CADR L)) (DIFF (CADDR L)))
        )
(DEFINE PROD (L)
    (LIST (' +)
```

```
        (LIST (' *) (CADR L) (DIFF (CADDR L)))
        (LIST (' *) (CADDR L) (DIFF (CADR L)))
        ))
(DEFINE QUOT (L)
     (LIST (' /)
           (LIST (' -)
                 (LIST (' *) (CADDR L) (DIFF (CADR L)))
                 (LIST (' *) (CADR L) (DIFF (CADDR L))))
           (LIST (' ) (CADDR L) 2)))
(DEFINE DFUNC (L)
    (COND ((EQ (CAR L) (' TAN))
               (LIST (' *)
                     (LIST (' )
                           (LIST (' SEC) (CADR L)) 2) (DIFF (CADR L))))
          ((EQ (CAR L) (' SIN))
               (LIST (' *) (LIST (' COS) (CADR L)) (DIFF (CADR L))))
          ((EQ (CAR L) (' COS))
               (LIST (' *)
                     (LIST (' MINUS)
                           (LIST (' SIN) (CADR L))) (DIFF (CADR L))))
          ((EQ (CAR L) (' SEC))
               (LIST (' *)
                     (LIST (' *)
                           (LIST (' SEC) (CADR L)) (LIST (' TAN) (CADR L)))
                              (DIFF (CADR L))))
          (T (' ??))))
(DEFINE SIMPLIFY (L)
    (COND ((EQ (CAR L) (' *)) (MSIMP L))
          ((EQ (CAR L) (' )) (PSIMP L))
          ((EQ (CAR L) (' +)) (SSIMP L))
          ((EQ (CAR L) (' /)) (QSIMP L))
          ((EQ (CAR L) (' -)) (BSIMP L))
          (T L)))
(DEFINE MSIMP (L)
    (PROG (M N)
        (SETQ M (SIMPLIFY (CADR L)))
        (SETQ N (SIMPLIFY (CADDR L)))
        (COND ((= M 1) N)
              ((= N 1) M)
              ((= N 0) 0)
              ((= M 0) 0)
              (T (LIST (' *) M N)))))
(DEFINE PSIMP (L)
    (PROG (M N)
        (SETQ M (SIMPLIFY (CADR L)))
        (SETQ N (SIMPLIFY (CADDR L)))
        (COND ((= N 1) M)
              ((= N 0) 1)
              (T (LIST (' ) M N)))))
(DEFINE SSIMP (L)
    (PROG (M N)
        (SETQ M (SIMPLIFY (CADR L)))
        (SETQ N (SIMPLIFY (CADDR L)))
```

```
        (COND ((= M 0) N)
              ((= N 0) M)
              (T (LIST (' +) M N)))))
(DEFINE BSIMP (L)
    (PROG (M N)
        (SETQ M (SIMPLIFY (CADR L)))
        (SETQ N (SIMPLIFY (CADDR L)))
        (COND ((= N 0) M)
              (T (LIST (' -) M N)))))
(DEFINE QSIMP (L)
    (PROG (M N)
        (SETQ M (SIMPLIFY (CADR L)))
        (SETQ N (SIMPLIFY (CADDR L)))
        (COND ((= N 1) M)
              (T (LIST (' /) M N)))))
(DEFINE WEIGHT (OPERATOR)
    (COND ((EQ OPERATOR (' DUMMY)) 0)
          ((EQ OPERATOR (' +)) 1)
          ((EQ OPERATOR (' MINUS)) 1)
          ((EQ OPERATOR (' *)) 2)
          ((EQ OPERATOR (' /)) 2)
          ((EQ OPERATOR (' )) 3)
          (T T)))
(DEFINE INF-TO-PRE (AE)
    (PROG (OPERANDS OPERATORS)
        (COND ((ATOM AE) (RETURN AE))
              (T T))
        (COND ((= (LENGTH AE) 2)
                  (RETURN (LIST (CAR AE) (INF-TO-PRE (CADR AE)))))
              (T T))
        (SETQ OPERATORS (LIST (' DUMMY)))
        STUFF
        (SETQ OPERANDS (CONS (COND ((ATOM (CAR AE)) (CAR AE))
                                   (T (INF-TO-PRE (CAR AE))))
                             OPERANDS))
        (SETQ AE (CDR AE))
        SCAN
        (COND ((AND (NOT AE)
                    (EQ (CAR OPERATORS) (' DUMMY)))
                    (RETURN (CAR OPERANDS)))
              (T T))
        (COND ((OR (NOT AE)
                   (NOT (> (WEIGHT (CAR AE))
                                   (WEIGHT (CAR OPERATORS)))))
               (PROGN (SETQ OPERANDS
                    (CONS (LIST (CAR OPERATORS)
                                (CADR OPERANDS)
                                (CAR OPERANDS))
                          (CDDR OPERANDS)))
                    (SETQ OPERATORS (CDR OPERATORS))
               (GO SCAN)))
              (T (PROGN (SETQ OPERATORS (CONS (CAR AE)
                                              OPERATORS))
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                    (SETQ AE (CDR AE))
                (GO STUFF))))))
(DEFINE TOINFIX (L)
    (COND ((ATOM L) L)
          ((OPERP (CAR L))
               (LIST (TOINFIX (CADR L)) (CAR L) (TOINFIX (CADDR L))))
          (T (LIST (CAR L) (TOINFIX (CADR L))))))
(DEFINE OPERP (A)
   (COND ((EQ A (' +)) T)
         ((EQ A (' -)) T)
         ((EQ A (' *)) T)
         ((EQ A (' /)) T)
         ((EQ A (' )) T)
         (T NIL)))
```

The Moore-Penrose Inversion

by Terri Wilhite

In this paper, we consider a generalized inverse of an mxn matrix A with real entries. A special generalized inverse, the Moore-Penrose inverse, will be considered. We will make use of the following ideas from Linear Algebra.

From the study of nxn matrices and their inverses, we know that for a matrix A, if there exists a matrix B such that AB=I and BA=I, then we say that A is invertible, and B is the inverse of A. It is easily shown that B is unique. Usually, the inverse of A is denoted A^{-1}. The transpose of A is denoted by A^t. The rank of a matrix is defined as the dimension (number of linearly independent rows) of the row space.

We may wonder whether there exists an inverse for a non-square matrix A_{mxn} such that AB=I and BA=I. In general, we cannot find a matrix B_{nxm} such that AB=I and BA=I.

However, for an mxn matrix A, there is a unique nxm matrix X which satisfies all of the following properties, which is a generalized inverse referred to as the Moore-Penrose inverse (M-P inverse for short).

(1) $AXA = A$
(2) $XAX = X$
(3) $(AX)^t = AX$
(4) $(XA)^t = XA$

Theorem 1: Let A be an mxn matrix. If B and C satisfy the four equations above, then B = C.

Proof: Let B and C satisfy the above properties. We shall show that B = C by using the above properties.

$B = BAB = B(AB)^t = BB^tA^t = BB^t(ACA)^t = BB^tA^tC^tA^t = B(AB)^t(AC)^t = BABAC = BAC = BACAC = BA(CA)^tC = (BA)^t(CA)^tC = A^tB^tA^tC^tC^t = (ABA)^tC^tC = A^tC^tC = (CA)^tC = CAC = C.$

Thus, we have shown that B = C. Therefore, a matrix that satisfies all four properties of the M-P inverse is unique. This matrix will be denoted A^+. Note: For an invertible matrix A, $A^+ = A^{-1}$.

We now develop a method to find A^+ for any matrix A with rank r.

Lemma 1: Let A be an mxn matrix A with full row rank (rank m). Then $A^+ = A^t(AA^t)^{-1}$.

Proof: We must show that $A^t(AA^t)^{-1}$ satisfies all four properties of the M-P inverse. Note that $A[A^t(AA^t)^{-1}] = [AA^t(AA^t)^{-1}] = I$.

(1) Show $A[A^t(AA^t)^{-1}]A = A$.
$A[A^t(AA^t)^{-1}]A = IA = A$.

(2) Show $[A^t(AA^t)^{-1}]A[A^t(AA^t)^{-1}] = [A^t(AA^t)^{-1}]$.
$[A^t(AA^t)^{-1}]A[A^t(AA^t)^{-1}] = [A^t(AA^t)^{-1}]I = [A^t(AA^t)^{-1}]$.

(3) Show $(A[A^t(AA^t)^{-1}])^t = A[A^t(AA^t)^{-1}]$.
$(A[A^t(AA^t)^{-1}])^t = I^t = I$.

(4) Show $([A^t(AA^t)^{-1}]A)^t = [A^t(AA^t)^{-1}]A$.
$([A^t(AA^t)^{-1}]A)^t = A^t[(AA^t)^{-1}]^t[A^t]^t = A^t[(AA^t)^{-1}]^tA$.
If we show that $[(AA^t)^{-1}]^t = (AA^t)^{-1}$,
then the equation is true.
$[(AA^t)^{-1}]^t = [(AA^t)^t]^{-1} = [(A^t]^tA^t)^{-1} = (AA^t)^{-1}$.

Lemma 2: Let A be an mxn matrix A with full column rank (rank n). Then $A^+ = (A^tA)^{-1}A^t$.

Proof: Similarly. Note that $[(A^tA)^{-1}A^t]A = I$.

Corollary: If A is an nxl matrix (or lxn), then $A^+ = (1/A^tA)^t$ (or $A^+ = (1/AA^t)A^t$).

Now we must consider the case where A does not have full row or column rank. To find A^+, we must use a technique called rank factorization. We factor A_{mxn} into matrices B_{mxr} and C_{rxn} such that A=BC and B and C have rank r.

Theorem 2: If A=BC where B is mxr, C is rxn, and B and C have rank r, then $A^+ = C^+B^+$.

Proof: First recall from Lemma 2 that if B_{mxr} has rank r then $B^+B=I$ and from Lemma 1 that if C_{rxn} has rank r then $CC^+=I$.

(1) Show $BC[C^+B^+]BC = BC$.
$BC[C^+B^+]BC = B(CC^+)(B^+B)C = BIIC = BC$.

(2) Show $[C^+B^+]BC[C^+B^+] = C^+B^+$.
$[C^+B^+]BC[C^+B^+] = C^+(B^+B)(CC^+)B^+ = C^+IIB^+ = C^+B^+$.

(3) Show $(BC[C^+B^+])^t = BC[C^+B^+]$.
$(BC[C^+B^+])^t = (B(CC^+)B^+)^t = (BB^+)^t = BB^+$
(Property (3) of the M-P inverse).
$BB^+ = BIB^+ = BCC^+B^+$.

(4) Show $([C^+B^+]BC)^t = [C^+B^+]BC$.
$([C^+B^+]BC)^t = (C^+(B^+B)C)^t = (C^+C)^t = C^+C$
(Property (4) of the M-P inverse.
$C^+C = C^+IC = C^+BB^+C$.

Now that the theorem has been established, we develop a method for factoring A into B and C. As stated previously, this method is called rank factorization. Consider a matrix A with r linearly independent row vectors and d linearly dependent row vectors such that:

$$A = \begin{bmatrix} L_1 \\ L \\ \cdot \\ \cdot \\ L_r \\ D_1 \\ \cdot \\ D_i \\ \cdot \\ D_d \end{bmatrix} = \begin{bmatrix} L_{11} & L_{12} & L_{13} \cdots L_{1n} \\ L_{21} & L_{22} & L_{23} \cdots L_{2n} \\ \cdot & \cdot & \cdot \cdots \\ \cdot & \cdot & \cdot \cdots \\ L_{r1} & L_{r2} & L_{r3} \cdots L_{rn} \\ D_{11} & D_{12} & D_{13} \cdots D_{1n} \\ \cdot & \cdot & \cdot \cdots \\ D_{i1} & D_{i2} & D_{i3} \cdots D_{in} \\ \cdot & \cdot & \cdot \cdots \\ D_{d1} & D_{d2} & D_{d3} \cdots D_{dn} \end{bmatrix},$$

where $D_i = k_{i1}L_1 + k_{i2}L_2 + \ldots + k_{ir}L_r$ and $i = 1,\ldots,d$. Note: We can assume A is in the above form, since if it is not, we can rearrange A to the above form, compute the M-P inverse, and then perform the corresponding rearrangement on B. Let C consist of the linearly independent rows of A.

$$C = \begin{bmatrix} L_{11} \ldots L_{1n} \\ L_{21} \ldots L_{2n} \\ \cdot \quad \cdots \\ L_{r1} \ldots L_{rn} \end{bmatrix}$$

Then B may be expressed by

$$B = \begin{bmatrix} & I_r & \\ K_{11} & K_{12} & \cdots K_{1r} \\ K_{21} & K_{22} & \cdots K_{2r} \\ \cdot & \cdot & \cdots \\ K_{d1} & K_{d2} & \cdots K_{dr} \end{bmatrix}$$

where I_r is the identity matrix of order r. It is easily shown by direct computation that BC=A. We conclude by giving a numerical example of rank factorization of a matrix A and by finding A^+.

Let A be the matrix $\begin{bmatrix} 1 & 2 & 3 \\ 1 & 0 & 2 \\ 2 & 2 & 5 \\ 0 & 2 & 1 \end{bmatrix}$

C will be the matrix consisting of the linearly independent row vectors of A.

$$C = \begin{bmatrix} 1 & 2 & 3 \\ 1 & 0 & 2 \end{bmatrix}$$

B will be of the form $\begin{bmatrix} a & b \\ c & d \\ e & f \\ g & h \end{bmatrix}$

such that BC=A. Solving for all the variables in B will give

$$B = \begin{bmatrix} 1 & 0 \\ 0 & 1 \\ 1 & 1 \\ 1 & -1 \end{bmatrix}$$

$$\text{and } BC = \begin{bmatrix} 1 & 0 \\ 0 & 1 \\ 1 & 1 \\ 1 & -1 \end{bmatrix} \begin{bmatrix} 1 & 2 & 3 \\ 1 & 0 & 2 \end{bmatrix} = \begin{bmatrix} 1 & 2 & 3 \\ 1 & 0 & 2 \\ 2 & 2 & 5 \\ 0 & 2 & 1 \end{bmatrix} = A$$

Now that A has been factored into B and C, we know that $A^+ = C^+B^+$. From Lemma 1, we know that $C^+ = C^t(CC^t)^{-1}$ and from Lemma 2 we know that $B^+ = (B^tB)^{-1}B^t$. Therefore, we have:

$$C = 1/21 \begin{bmatrix} -2 & 7 \\ 10 & -14 \\ 1 & 7 \end{bmatrix} \quad \text{and } B = 1/3 \begin{bmatrix} 1 & 0 & 1 & 1 \\ 0 & 1 & 1 & -1 \end{bmatrix}$$

$$\text{Thus } A = 1/63 \begin{bmatrix} -2 & 7 & 5 & -9 \\ 10 & -14 & -4 & 24 \\ 1 & 7 & 8 & -6 \end{bmatrix}$$

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Dr. Helmuth Gilow on "Toxic Waste Disposal"

by Janet Freytes

The controversy of determining the best way to dispose of toxic wastes is of utmost importance in our society today. Not long ago,some industries tried to build an incinerator here in Memphis; however, this project was unsuccessful because of the frequent complaints by Memphis residents. Toxic waste disposal is considered, by many, to be a dilemma that could have adverse effects in our economy as well as in our enviroment. Regarding this subject, we asked Dr. Helmuth Gilow, Professor of Chemistry at Southwestern At Memphis, to share his views during a short interview.

J.F.: What constitutes toxic chemical wastes?

Dr. Gilow: Toxic chemical waste generally refers to the by-products generated from chemical manufacturing industries. These residues,which tend to accumulate quickly, are of no use and usually are extremely harmful. Nuclear wastes are a special problem.

J.F.: Is there an ideal way to get rid of toxic chemical wastes?

Dr. Gilow: As of yet, there is no such thing as an ideal method of toxic waste disposal. The best way is by means of incineration. Although it has its drawbacks, (it is expensive and may cause air pollution if not done properly), it usually yields good results.

J.F.: If this is a good means for toxic chemical waste disposal, why does the public oppose this measure?

Dr. Gilow: People don't want to live next to an incinerator for fear of toxic chemicals and air pollution endangering their enviroment. A young politician in Minnesota found this out after a five year study where he held meetings and forums with this state residents. He supported public debates on the different techniques of toxic chemical waste disposal and held a study over the areas where the incinerators were to be constructed.The conclusion after this detailed study was that people agreed that no matter what method of toxic waste disposal was used, it should not be located in the state of Minnesota.

J.F.: What other methods can be used for toxic waste disposal?

Dr. Gilow: Some toxic chemical wastes are stored in isolated areas inside granite or salt mines. Here they accumulate until they are incinerated or transfered to other mines. Yet, another rather current disposal technique has been proposed by several countries in Europe. They have suggested that an incinerator be located on a ship so that the incineration is carried out at sea

where it would not affect the inhabitants of any country. Nonetheless environmentalists are opposed to this measure since it may endanger the marine ecosystem.

J.F.: What do you feel can be done to resolve this predicament?

Dr. Gilow : I believe we may have to pay a high price for the development of an effective toxic waste disposal technique. The major obstacle in the development of such a needed measure is an economic one, but there are technical problems involved in it as well. Unless these difficulties are overcome we may have to pay a much higher price - that of a less inhabitable enviroment.