AN ABSTRACT OF THE THESIS OF

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Monolayer cultures of Kc-H Drosophila cells were used to detect the effects of purine and pyrimidine compounds. Analysis of the end product of normal metabolism, uric acid, was done to detect effects of exogenous purine and pyrimidine derivatives. It was found that adenine, hypoxanthine, xanthine, and purine were toxic to Kc-H Drosophila cell cultures. A hyperproduction of uric acid was found as a result of adenine, hypoxanthine, and xanthine treatments which was reduced by thymine in both hypoxanthine and xanthine treated cells. These findings conclude that toxicity appears to be due both to an uric acid contribution from purine catabolism plus the ability of the compounds to feedback and starve cells of pyrimidines. This conclusion also was supported by the protective effects seen with guanine, cytosine, and thymidine treatments on adenine-treated cells. The hypothesis states that a possible regulatory connection exists between purine and pyrimidine synthesis probably due to the link of these pathways by their common substrate, phosphoribosylpyrophosphate (PP-ribose-P). Allopurinol, 8-azaguanine, aminopterin, and 2,6 diaminopurine were also tested for effects on Kc-H Drosophila cells. The results showed 2,6 diaminopurine, allopurinol, and aminopterin, by themselves, exerting no toxic effect on Drosophila cells. Allopurinol was found to inhibit hypoxanthine and xanthine toxic effects. Cells toxicity to 8-azaguanine

is probably due to the inhibition of xanthine oxidase and therefore, the inhibition of the normal degradation of purines. Aminopterin showed inhibition of thymidine protective effects toward adenine-treated cells. A synergistic effect, exerted by aminopterin and adenine is suggested to be the factor that inhibited thymidine protective effects on Drosophila adenine-treated cells and resulted in their death. The studies done with Hep-2 cells were used to compare the results of the present study with those already found in human cell lines.

TOXICITY OF NUCLEIC ACID BASES ON DROSOPHILA CELL CULTURES

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INTRODUCTION

Nucleotide compounds consist of a nitrogenous base that is either a purine or pyrimidine derivative, a sugar, and one or more phosphate groups. These compounds participate in nearly all biochemical processes (43). When the purines, adenine or guanine, link to deoxyribose-5-phosphate, they form two of the four basic units involved in the genetic code of the chromosomal polynucleotide, deoxyribonucleic acid (DNA), which is replicated during cell division. In combination with ribose the same purines form two of the structural units of ribonucleic acid (RNA), which includes the messenger RNA transcribed from DNA and the transfer RNA involved in protein synthesis (3).

In addition, nucleotide derivatives are activated intermediates in many biosynthetic reactions. For example, uridine diphosphate-glucose (UDP-glucose) and cytidine diphosphate-diacylglycerol (CDP-diacylglycerol) are precursors of glycogen and phosphoglyceride, respectively. Adenosine 5'-triphosphate (ATP), an adenine nucleotide, is also the universal currency of energy in biological systems. Adenine nucleotides are components of three major coenzymes: nicotinamide adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD), and coenzyme A (CoA). Nucleotides are metabolic regulators. Cyclic adenosine 5'-monophosphate (cyclic AMP) is a ubiquitous mediator of the action of many hormones. Covalent modifications introduced by ATP alter the activities of some enzymes as exemplified by the phosphorylation of glycogen and the adenylation of glutamine synthetase (43).

Evidently, purines and pyrimidines are involved in many aspects of cell intermediary metabolism, supply of high energy phosphates to various active transport systems, and nucleic acid synthesis.

Nearly all living organisms appear able to synthesize purine and pyrimidines from simpler precursors (30). The purines which are synthesized by the cells into purine nucleotides either originated by cellular biosynthesis from smaller molecules or formed from dietary purines. Preformed purines in food are liberated from their nucleotides by hydrolytic enzymes contained in pancreatic and intestinal secretions. Some of the nucleosides may be absorbed intact from digested food or may be degraded to the free purine bases (3).

Free purines and pyrimidines formed by degradation of nucleic acids are salvaged and reused for the synthesis of nucleotides and nucleic acids or may undergo degradation to yield nitrogenous and products that are excreted. The purines are ultimately degraded to allantoin and other end products by some vertebrates and to uric acid by man (43, 30) and insects (5, 16). Biochemistry of uric acid in insects is considered on Appendix I. In man overproduction of uric acid and its deposition in cartilage results in gout. Genetic failure in humans of the salvage of guanine and hypoxanthine results in the Lesch-Nyhan syndrome (30).

Additionally, nucleotides containing pyrimidines as the base moeity can be interconverted or degraded to uridine, thymidine, and cytidine in insects (5) and uridine monophosphate (UMP), cytidine triphosphate (CTP), and uridine triphosphate (UTP) in man, fungi, and bacteria (43, 30). A defect of pyrimidine metabolism, orotic aciduria, results from a gross deficiency of the bifunctional enzyme system orotatephosphoribosyltransferase and orotidylate decarboxylase. Deficiency of pyrimidine 5'-nucleotidase results in hemolytic anemia and prominent basophilic stippling of red cells (4).

Knowing the importance of purines and pyrimidines in cellular

biochemistry it has been vital to elucidate the mechanisms by which they and their analogs interact in biological systems. Surprisingly then, it is known that purines and pyrimidines exert a toxic effect in cultured mammalian cells, prokaryotes, and other systems.

Present concepts about purine and pyrimidine toxicity have been derived from the studies done with Escherichia coli (31), human lymphoblasts (22), neuroblastoma cells (42), HeLa carcinoma cells (45), human red blood cells (2), rabbit neutrophil cells (15), rats (40), mammalian cell culture (25, 36, 20, 11), L5178Y cells (14), Malus domestica explants (41), chinese hamster cells (44, 8, 39, 33, 34), mouse L1210 leukemia cells (32), S49 T-cell lymphoma (19), fibroblastic or lymphoid cells lines (46, 47, 49, 17, 12, 18), and Drosophila melanogaster (23, 26, 27).

Some of these studies illustrate the commonality of the synthesis and metabolism of nucleotide precursors in mammals, insects and even prokaryotes in regard to purine and pyrimidine toxic effects. For instance, de novo purine biosynthetic pathways in chinese hamster cells and Escherichia coli are both affected by exogenous adenine (28). The mechanism of adenine toxicity in Escherichia coli has been explained by Levine and Taylor (31). They observe that the ability of guanosine to prevent adenine toxicity indicates that adenine exerts its toxic effects by depleting guanine nucleotide pools. It is also known that adenine becomes lethal for chinese hamster fibroblasts when an adenosine analog (coformycin) is also present in the medium. The lethal effect is reversible by hypoxanthine which suggests that cells are starved of vitally needed inosine monophosphate (IMP) by simultaneous shutoff of the purine de novo biosynthesis pathway and the deamination of adenosine

and adenylate (44). Another source of adenine toxicity is the depression of pyrimidine as well as purine nucleotide synthesis that results from lowering the phosphoribosylyrophosphate (PP-ribose-P) levels (6).

Studies done in cultured L1210 leukemia cells (25) have shown that adenine enhances dThd (thymidine) mediated inhibition of cell growth and alterations of deoxyribonucleotide pools.

Additionally, it has been found that adenine and adenosine are toxic to human lymphoblast mutants defective in purine salvage enzymes (22). The results of this study showed that adenine and adenosine may be directly toxic to cells. It is suggested that there may exist purine receptors that are involved in mediating effects on cells. Alternatively these purines may directly interfere with an intracellular enzyme (or enzymes) to interrupt one or more pathways essential for normal growth.

Savaiano and Clifford (40) studying the effects of adenine, uracil, and uridine on rats found that there was a growth inhibitory effect due to adenine which was intensified by uridine and partially reversed by uracil. They suggest that the inhibitory effect of adenine was due to an interference with pyrimidine synthesis since the inhibitory effects could be reversed in microbial and mammalian cells with pyrimidine supplements.

Gudas et al., (19), using the S49 T-cell lymphoma system for the study of immunodeficiency disease showed that variants lacking hypoxanthine-guanine phosphoribosyl transferase (HGPRT) or adenine phosphoribosyl transferase (APRT) are sensitive to the killing action of adenosine. They pointed out that at low concentrations adenosine must be phosphorylated to deplete the cells of pyrimidine nucleotides and

pyrophosphoribosylphosphate (PP-ribose-P) and to promote the accumulation of orotate. These alterations account for one mechanism of adenosine toxicity.

Wen-Cheng Tseng et al., (45), in their studies with HeLa carcinoma cells, observed that an adenine analog, 9-B-D-arabino-furanosyl-2-fluroradenine (2-F-araA), inhibited the growth of HeLa cells by 50 % at a concentration of 0.25 μM and depressed the replication of herpes simplex virus Type 1 and Type 2 by 99 %. They stated that the cytotoxic action of 2-F-araA may be due, in part to a self potentiating inhibitor of DNA synthesis.

Green and Chan (18) have found that in the presence of $1x10^{-4}$ to $1x10^{-5}$M of adenosine, established cell lines of fibroblastic or lymphoid origin died of pyrimidine starvation. Less than lethal concentrations inhibited cell growth. Over a broad concentration range, the effects of adenosine were prevented by providing a suitable pyrimidine source.

Upchurch and Gabridge (46) examining the role of de novo purine metabolism in normal lung fibroblasts and Lesch-Nyhan fibroblasts (cells deficient in HGPRT) suggested that interference with de novo purine synthesis in cells played a critical role in the induction of cytotoxicity in Mycoplasma pneumoniae infected cells.

Ishii and Green (25) working with mouse 3T6 cells found that adenosine inhibited growth of these cells. They suggested that pyrimidine synthesis was blocked due to inhibition of the enzyme orotidylate phosphorylase.

According to Ho et al., (23), addition of purines (adenine and guanine) but not pyrimidines (cytosine and thymine) to the nutrient medium of Drosophila melanogaster affect its development. This study suggested that the supplementation of purines and pyrimidines in the

diet mediates different physiological effects on the metamorphosis of Drosophila melanogaster. It was found that adenine was detrimental to the flies. In addition, this study also showed that adenine changed high pressure liquid chromatography-measured purine-pyrimidine profiles in the larval and pupal stages, but had no effect on the profiles in adult flies. Ho et al., hypothesized two possibilities to account for the detrimental effects of adenine in these experiments. First, the ovulation by parental flies might have been impaired due to the ingestion of adenine, and second, the development of the larva may have been inhibited due to the ingestion of adenine.

It also has been shown that a deficiency of adeninephosphoribosyltransferase (APRT) activity is the primary biochemical defect of Drosophila selected for resistance to purine-induced lethality (26). Johnson and Friedman have investigated the mechanisms of purine-induced lethality in the wild type and the resistance to purine-mediated lethality in two mutants of Drosophila melanogaster. They claim that purine-induced lethality in wild type Drosophila might result from the anabolic metabolism of purine through a salvage pathway leading to the synthesis of a toxic nucleotide. They conclude that a deficiency for the majority of APRT activity permits Drosophila to survive high concentrations of purine. It is possible that purine, a competitive inhibitor of mammalian APRT, is an alternative substrate for APRT and that the product of this reaction is the lethal agent.

Most recently, Johnson and Friedman (27) have found that purine resistant Drosophila melanogaster result from the mutations in the APRT structural gene. They note that genetic mapping and complementation analysis demonstrate that purine resistance, deficiency of APRT

activity, and differences in the isoelectric point of APRT result from alterations at a single locus. They also say that level of APRT activity shows gene dose dependence in Aprt (purine resistant mutant) heterozygotes and in flies that are haploid for different alleles. As a conclusion of this study, it is suggested that Aprt contains the structural gene for APRT.

The present study was designed to correlate findings between the work of Ho, et al. on Drosophila flies with Drosophila cells in culture. It was of interest to elucidate if purine and pyrimidine bases affect Kc-H Drosophila cells in the same manner that they affected Drosophila flies and mainly to determine the range of adenine toxicity on Drosophila cells.

This work also would compare in situ with the in vitro studies using inhibitors of adenine metabolism and/or analogues to gain insights into toxicity mechanisms. Additionally, analysis of the end product of normal metabolism, uric acid, would be done to detect effects of exogenous adenine.

MATERIALS AND METHODS

Experimental Cells

The Kc-H Drosophila cell line was obtained from Dr. Terrel Johnson at Kansas State University, Manhattan, Kansas.

The Hep-2 cell line was obtained from the Kansas Department of Health at Topeka, Kansas.

Cell Culture

Kc-H Drosophila cells were grown in normal growth medium, D-22 medium plus 10 % calf serum (K. C. Biologicals, Lenexa, Kansas). This medium was prepared as described by Lucy Cherbas and Varda Chilo based on Echalier, 1976 (Appendix II). D-22 medium without serum was used to wash the cell monolayers before splitting and challenging them with purine antimetabolites, purine intermediates as well as with purine and pyrimidine bases. Analysis of the D-22 medium plus 10 % calf serum and D-22 without serum were done in the Department of Nutrition, University of California at Davis. The data from this analysis indicated that the samples tested had extremely low levels of purines and pyrimidines (Appendix III, IV and V).

To split the cells, flasks containing 1 ml of cell suspension and 4 ml of D-22 plus 10 % calf serum were incubated for 7 days until the cells were completely monolayered. The old medium was removed from the flasks and the cells were gently washed with 1 ml of D-22 without serum. Then, 5 ml of D-22 plus 10 % calf serum were added to the flasks to strongly wash cells down from the flasks. One ml of cell suspension was placed in a 25 cm^2 plastic tissue culture flask containing D-22 plus 10 % calf serum and the challenging reagent. A final volume of 5 ml was adjusted to reach the final desired concentration of test substance.

To make viable cell counts, 0.5 ml of Erythrosin B was mixed with 4.5 ml of D-22 serum-free medium (diluent medium). Then, 0.5 ml of cells were mixed with 4.5 ml of diluent medium. To make the final diluted cell suspension, 0.5 ml of diluted cells were mixed with 4.5 ml of diluted stain. To obtain total and viable counts, both sides of a Neubauer chamber were filled with the diluted cell suspension. The cells were allowed to settle for 1 minute and with the low power objective in place, the ruled area of the chamber was focussed. Cells that appeared in the four corner squares were enumerated. Single cells with well-defined nuclei and surrounding cytoplasm were counted. Clumps of cells in which individual nuclei and cytoplasm were easily visible, each cell was counted. When individual cells were not easily discernible as such, clumps were counted as a single cell. The total number of cells in all four corner squares were divided by four to find the average number of cells per square. The average number of cells per square times 10,000 (correction factor) X dilution factor (10) gave the cell count/ml.

Hep-2 cells were grown in Eagles Minimum Essential Medium (Gibco, Grand Island, NY) plus 10 % calf serum (K. C. Biologicals, Lenexa, KS), using standard mammalian cell culture technique (MC-702 Lab manual).

Stock Solutions

Adenine (Aldrich Chemical Co., Inc., Milwaukee, WI), cytosine (Nutritional Biochemicals Corporation, Cleveland, OH), guanine (Nutritional Biochemicals Corporation, Cleveland, OH), thymine (NBCO, Cleveland, OH), hypoxanthine (NBCO, Cleveland, OH), inosine (Sigma Chemical Company, St. Louis, MO), xanthine (NBCO, Cleveland, OH), purine (Sigma Chemical Company, St. Louis, MO) at 4.08×10^{-2}M were made in deionized

glass-distilled water, sterilized by autoclaving and stored in 25 ml quantities at 4°C.

Aminopterin (Sigma Chemical Company, St. Louis, MO and Fluka Chemical Corporation, Hauppauge, NY) at 1.9 ng/ml, .19 ng/ml, and .019 ng/ml were made in deionized glass-distilled water, sterilized by a filter membrane (0.22 µm) filtration, and stored at -20°C.

Azaguanine (Fluka Chemical Corporation, Hauppauge, NY) at 76 µg/ml was made in D-22 plus 10 % calf serum (K. C. Biologicals, Lenexa, KS), sterilized by a filter membrane (0.22 m) and immediately used.

Uric acid (Sigma Chemical Co., St. Louis, MO) at 2 mgs % was made in D-22 without serum, sterilized by membrane (0.22 µm) filtration and stored in 100 ml quantities at 4°C.

Gentamicin (Shering Corporation, Keneworth, NJ and United States Biochemical Corporation, Cleveland, OH) at 2.5 mgs/ml was made in D-22 medium, sterilized by a filter membrane (0.22 µm) filtration and stored in 10 ml quantities at 4°C.

Uric Acid Assay

For uric acid determinations, control and treated cells were strongly washed from the flasks. Cell suspensions were centrifuged at 5,000 rpm for 5 minutes. Pellets and supernatants were separated. The supernatant, was evaporated on a watch-glass and then resuspended in 1.2 ml of distilled water.

One-half ml of samples were placed into vials that contained the uricase and into clean dry tubes labeled total. Lateral shaking of the vials was done to dissolve the uricase. The vials with the samples were incubated at room temperature for 20 minutes. Then 8.5 ml of distilled water was added to the vials and tubes and mixed. One-half ml of sodium tungstate at 10 % concentration was added. The

vials and the tubes were mixed thoroughly and the mixture was filtered through Wathman No. 1 paper filter to obtain clear protein-free solutions. Two tubes were labeled for each sample as total and residual plus one tube labeled blank to which four ml of distilled water was added. Four ml of protein-free solution was added to the other tubes. Then 1.5 ml of sodium carbonate and 1 ml of phosphotungstate were added to all the tubes. The tubes were mixed by inversion several times. All the tubes were incubated at room temperature for 15 minutes. A Baush & Lomb spectophotometer with 700 μm wavelength filter was used to determine uric acid concentrations. The actual uric acid concentration was obtained by subtracting the residual uric acid concentration from the total uric acid concentration.

Experimental Protocol

In the first type of experiments, Kc-H Drosophila cells were exposed to the purine and pyrimidine bases adenine, cytosine, thymine, and guanine by themselves at $2x10^{-2}$ % and to a combination of bases; adenine plus thymine, adenine plus cytosine, adenine plus guanine, and adenine plus cytosine plus thymine plus guanine at $1.30x10^{-3}$M, respectively. Percentage of monolayering in cell culture was monitored and recorded each day during a seven day incubation period at 25°C. Non-treated cells were used as the control.

In the second experiment, the LD_{50} for adenine was determined using cells exposed to adenine at different concentration between $1.5x10^{-3}$M and $1.5x10^{-7}$M. Percentage of monolayering cells was recorded and cell counts as well as uric acid determinations at the end of the incubation period.

A third experiment used Kc-H Drosophila cells exposed to adenine at $1.5x10^{-3}$M for 6h, 12h, and 24h. The 7 day-incubation period was

RESULTS

Kc-H Drosophila cells exposed to bases

The monolayering process of cells is a good indicator of how the cells are dividing and multiplying to complete their normal life cycle. This process was monitored when Kc-H Drosophila cells were exposed to the purine and pyrimidine bases adenine, thymine, cytosine, and guanine as well as a combination of adenine plus the remaining bases by themselves to study the effect of these bases in cultured insect cells.

Kc-H Drosophila cells cultured in normal growth medium plus the bases, shows that adenine at 1.5×10^{-3}M is highly detrimental to the cells (Fig. 1) while guanine, cytosine, and thymine at the same concentration did not affect the normal growth of the cells. Cultures of Kc-H Drosophila cells that had been treated with a combination of adenine-cytosine, adenine-thymine, and adenine-guanine showed (Fig. 2) that one mechanism to overcome adenine toxicity is to add any of the other purine or pyrimidine bases to the cells growing in normal growth medium.

LD_{50} for adenine

Kc-H Drosophila cells were exposed to different concentrations of adenine between 1.5×10^{-3}M to 1.5×10^{-7}M to detect the LD_{50}. The results in Table 1 demonstrate that the lethal dose in these cells was about 2.2×10^{-4}M. This value represents the dose of adenine that killed half of the cell population during the time of exposure to adenine (Table 1).

Recovery of cells exposed to adenine

This experiment used exposure of cells to adenine at 1.5×10^{-3}M for short and long periods of time as detailed in the materials and methods. It was found that another mechanism for Kc-H Drosophila cells

Fig. 1. Kc-H Drosophila cells cultured in normal growth medium plus purine and pyrimidine bases (1.5×10^{-3}M).

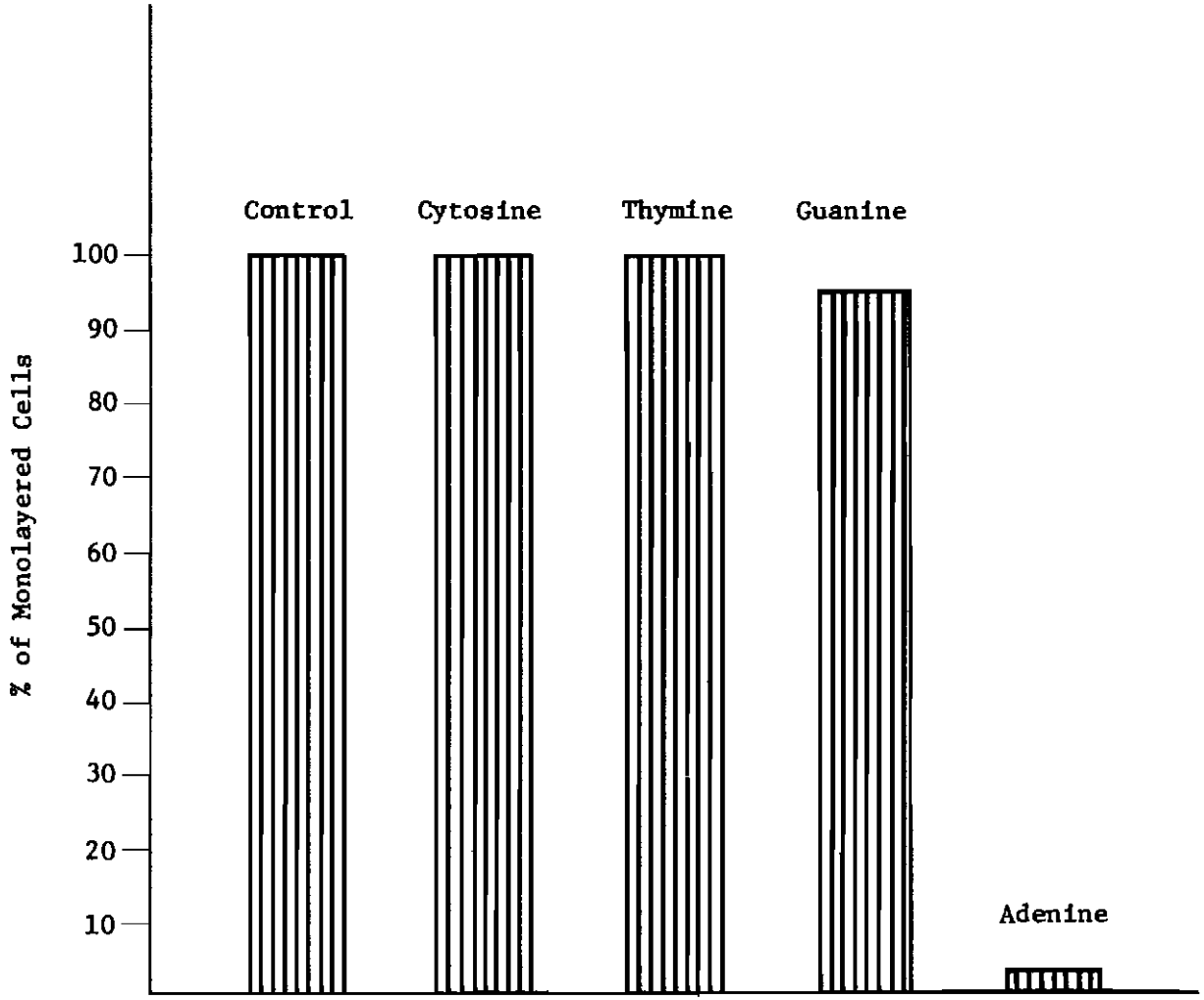

Control
Cytosine
Thymine
Guanine
Adenine
% of Monolayered Cells
100
90
80
70
60
50
40
30
20
10

Fig. 2. Cells treated with a combination of purine and pyrimidine bases.

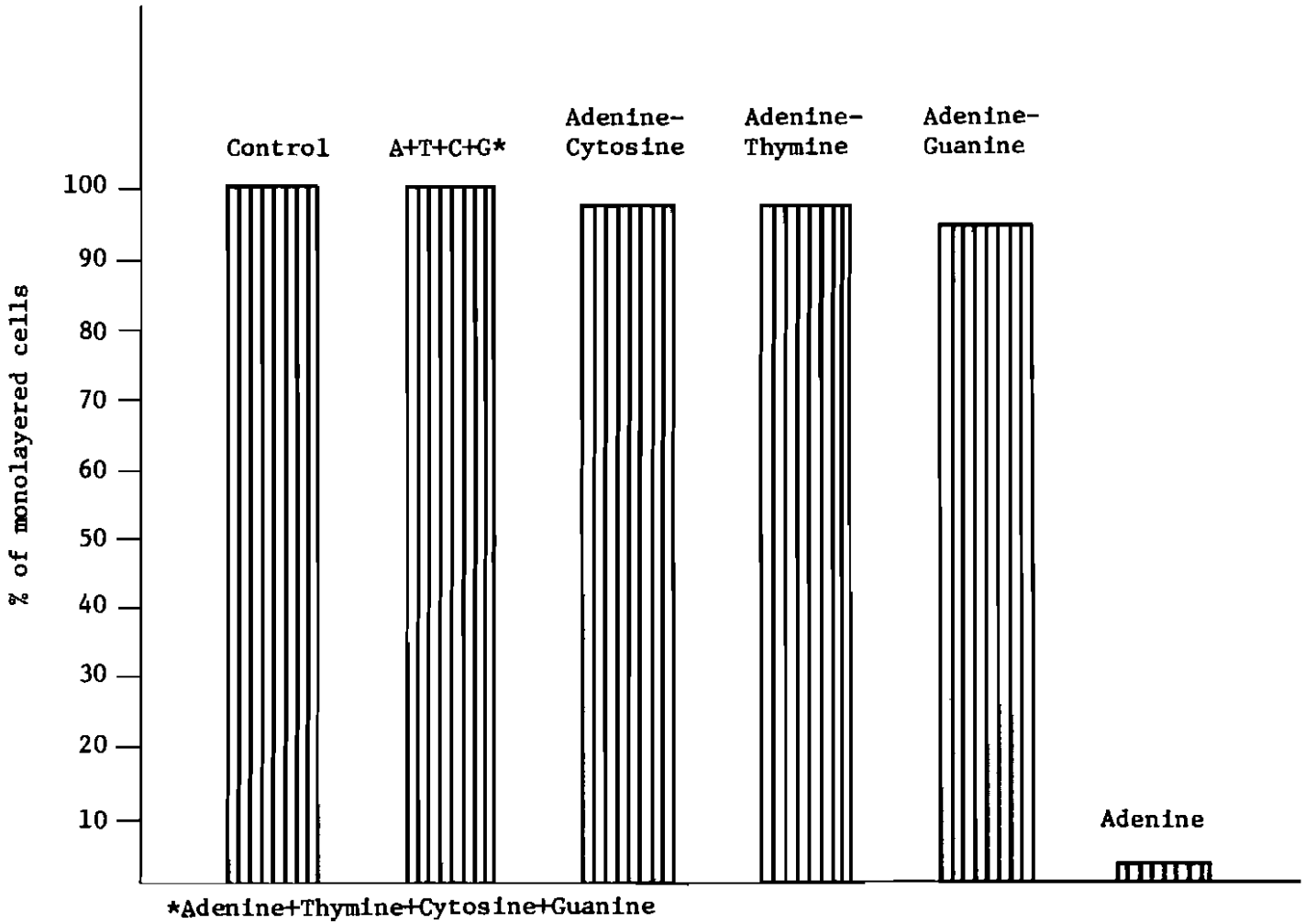

Control
A+T+C+G*
Adenine-Cytosine
Adenine-Thymine
Adenine-Guanine
Adenine
% of monolayered cells
100
90
80
70
60
50
40
30
20
10
*Adenine+Thymine+Cytosine+Guanine

Table 1. LD_{50} for Adenine

Adenine Concentrations	Mortality Ratio*1	Percent Dead*2
$1.5x10^{-3}M$	5.35/6.015	89 %
$2.96x10^{-4}M$	4.865/7.91	62 %
$1.5x10^{-4}M$	3.745/9.04	41 %
$2.96x10^{-5}M$	2.985/11.51	26 %
$1.5x10^{-5}M$	2.035/13.045	16 %
$2.96x10^{-6}M$	1.360/27.72	5 %
$1.5x10^{-6}M$	0.785/16.52	47 %
$1.5x10^{-7}M$	0.31/18.423	1.6 %

*1 Mortality ratio: relation of the cumulated values of the total number of dead cells and the cumulated values of the total number of survived cells.

*2 Percent dead: This value was determined by dividing the cumulated values of the total number of dead cells by the cumulated values of the total number of cells.

LD_{50}: $2.2x10^{-4}M$ was determined by the interpolation of the % of cells affected at concentrations next above 50 % and the % cells affected at concentrations next below 50 %.

to overcome adenine toxicity beside supplementation with either purine or pyrimidine is re-exposing adenine-treated cells to normal growth medium (D-22 plus 10 % calf serum). This also showed that toxicity is based on length of adenine exposure. The longer the cells were stressed the fewer cells were alive at the end of the test.

Kc-H Drosophila cells exposed to adenine and its relationship to uric acid

Cells exposed to adenine at 1.5×10^{-3}M for 12h, 24h, and 48h were tested for uric acid production. Uric acid determinations done in both cell pellets and supernatants showed (Fig. 3) that the amount of uric acid was greater in the supernatants than in the cell pellets. Figure 3 also shows that the supernatant concentrations of uric acid in adenine-treated cells decreased with time.

An earlier experiment (Table 2) found that the amount of uric acid/cell is greater at any concentration of adenine than in the control. This table also shows that the highest concentration of adenine tested (1.5×10^{-3}M) was detrimental to the cells since 51 % of the cells died when they were exposed to this concentration. This result complements the finding that adenine-treated cells could be recovered once they are supplemented with normal growth medium. The rationale for the explanation of these results are that adenine-treated cells loose their capacity of attachment to the culture flask, and therefore, their monolayering capacity, but not their ability to grow and survive in their appropriate environment.

Kc-H Drosophila cells exposed to purine antimetabolites

The cells were treated with aminopterin (0.57 to 5.7 µg/ml); aminopterin at the same concentrations plus adenine at 1.5×10^{-3}M;

Fig. 3. Uric Acid Production by Kc-H Drosophila Adenine (1.5×10^{-3}M)-Treated Cells.

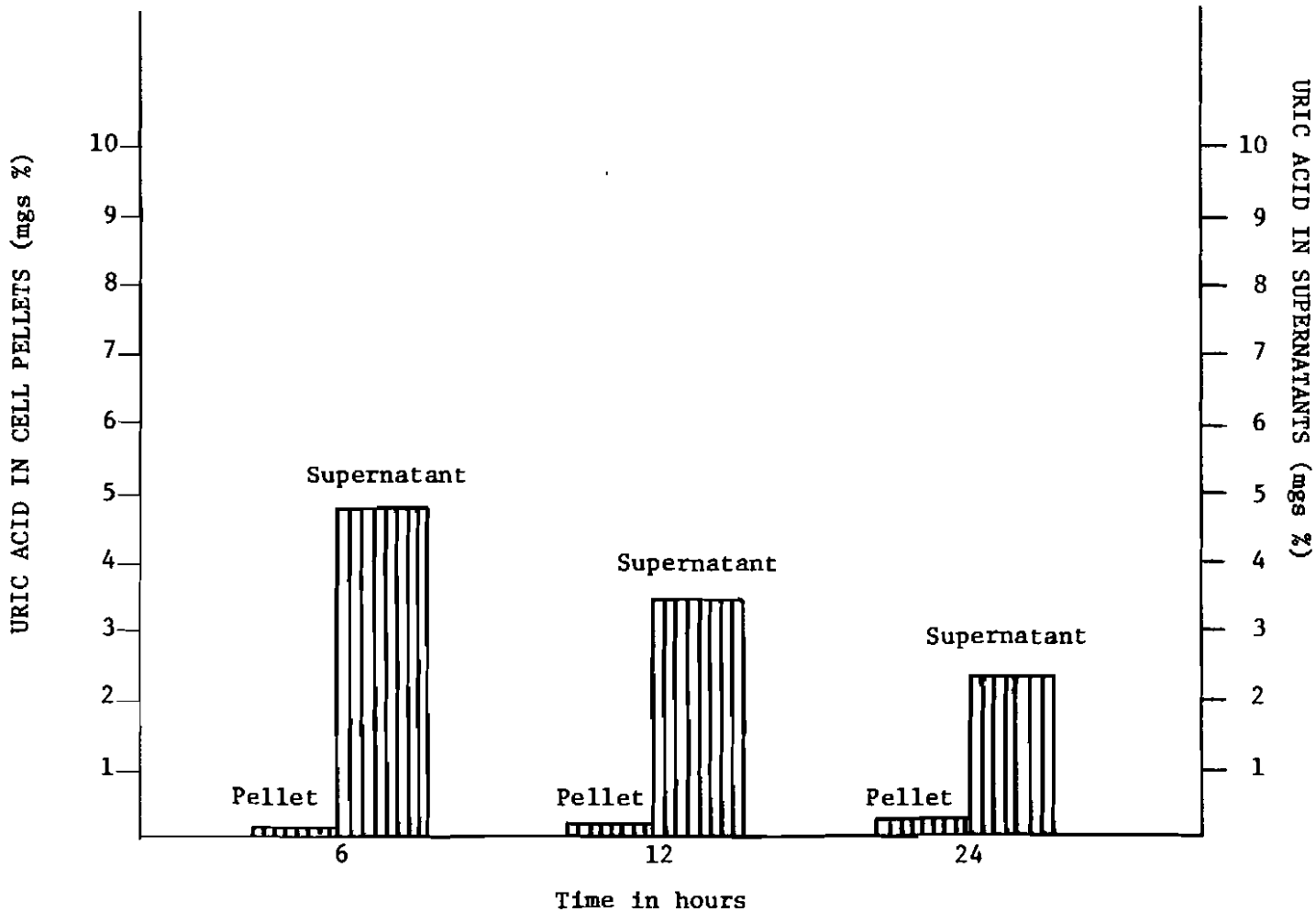

URIC ACID IN CELL PELLETS (mgs %)
URIC ACID IN SUPERNATANTS (mgs %)
10
9
8
7
6
5
4
3
2
1
Supernatant
Supernatant
Supernatant
Pellet
Pellet
Pellet
6
12
24
Time in hours

Table 2. The effects of various adenine concentrations on the viability and uric acid levels of cultured Kc-H Drosophila cells.

Adenine Concentrations	Total cells	Viable cells	Dead cells	% dead cells	Uric acid/cell
1.5×10^{-3}M	0.6375×10^{6}	0.312×10^{6}	0.325×10^{6}	51 %	9.45×10^{-6} mgs %
2.96×10^{-4}M	3.48×10^{6}	2.35×10^{6}	1.12×10^{6}	32.3 %	1.6×10^{-6} mgs %
1.5×10^{-4}M	3.012×10^{6}	2.25×10^{6}	0.762×10^{6}	25.3 %	1.38×10^{-6} mgs %
2.96×10^{-5}M	4.18×10^{6}	3.23×10^{6}	0.95×10^{6}	22.7 %	1.07×10^{-6} mgs %
1.5×10^{-5}M	3.16×10^{6}	2.48×10^{6}	0.675×10^{6}	21.3 %	1.1×10^{-6} mgs %
2.96×10^{-6}M	2.88×10^{6}	2.30×10^{6}	0.575×10^{6}	19.9 %	1.1×10^{-6} mgs %
1.5×10^{-7}M	3.0×10^{6}	2.68×10^{6}	0.312×10^{6}	10.4 %	0.6×10^{-6} mgs %
Control	6.95×10^{6}	5.76×10^{6}	0.187×10^{6}	2.6 %	0.12×10^{-6} mgs %

allopurinol at 8.3×10^{-6}M and azaguanine at 76 µg/ml. It was found that any concentration of aminopterin, the cells exhibited their normal growth with the formation of confluent monolayers. Aminopterin did not help the cells to overcome the adenine toxicity. This experiment also showed that azaguanine at 76 µg/ml is highly detrimental to the Kc-H Drosophila cells. From studies of cells exposed to a combination of allopurinol-adenine, and allopurinol-purine intermediates it was found that allopurinol seems to help the Kc-H Drosophila cells to overcome xanthine and hypoxanthine toxicity but not adenine toxicity (Table 4).

Kc-H Drosophila cells exposed to purine intermediates

Cells exposed to hypoxanthine, xanthine, and inosine at 3.38×10^{-3}M showed that they were susceptible to hypoxanthine and xanthine but not to inosine (Table 3). Uric acid determinations in the supernatants found that the amount of uric acid was greater in hypoxanthine and xanthine-treated cells than that of inosine and control cells (Fig. 4).

Cells exposed to the intermediates at 1.5×10^{-3}M, respectively plus thymine at 1.5×10^{-3}M showed that thymine helps the cells overcome the detrimental effects of hypoxanthine and xanthine. Uric acid determinations showed that the amounts of uric acid production decreased with the addition of thymine to the flasks containing the cells growing in normal growth medium plus hypoxanthine and xanthine (Fig. 5).

Cells were also tested for sensitivity in normal growth medium to purine and 2,6 diaminopurine at 1.5×10^{-3}M, respectively. Cell susceptibility to purine was demonstrated while 2,6 diaminopurine did not affect normal cell growth (Table 4).

Kc-H Drosophila cells exposed to Thymidine

The results showed that cells were highly sensitive to thymidine

Table 3. Comparative effects of various purines and pyrimidines on the growth of Kc-H Drosophila cells and Hep-2 cells.

Treatment	Kc-H Drosophila cells	Hep-2 cells
Adenine ($1.5x10^{-3}M$)	Toxic	Toxic
Guanine ($1.5x10^{-3}M$)	Not Toxic	Partially toxic*
Cytosine ($1.5x10^{-3}M$)	Not Toxic	Not Toxic
Thymine ($1.5x10^{-3}M$)	Not Toxic	Not Toxic
Hypoxanthine ($3.38x10^{-3}M$)	Toxic	Not Toxic
Inosine ($3.38x10^{-3}M$)	Not Toxic	Not Toxic
Xanthine ($3.38x10^{-3}M$)	Toxic	Not Toxic
Allopurinol ($8.3x10^{-6}M$)	Not Toxic	Not Toxic
Thymidine (50 µM/ml)	Toxic	Toxic
Aminopterin (76 µg/ml)	Not Toxic	Partially toxic*
Azaguanine (76 µg/ml)	Toxic	Toxic
Adenine + Thymine ($1.5x10^{-3}M$ ea.)	Thymine helped to overcome adenine toxicity	Thymine helped to overcome adenine toxicity

*25-30 % of controls

Fig. 4. Uric acid production (mgs %) by inosine, hypoxanthine, and xanthine Kc-H Drosophila treated cells.

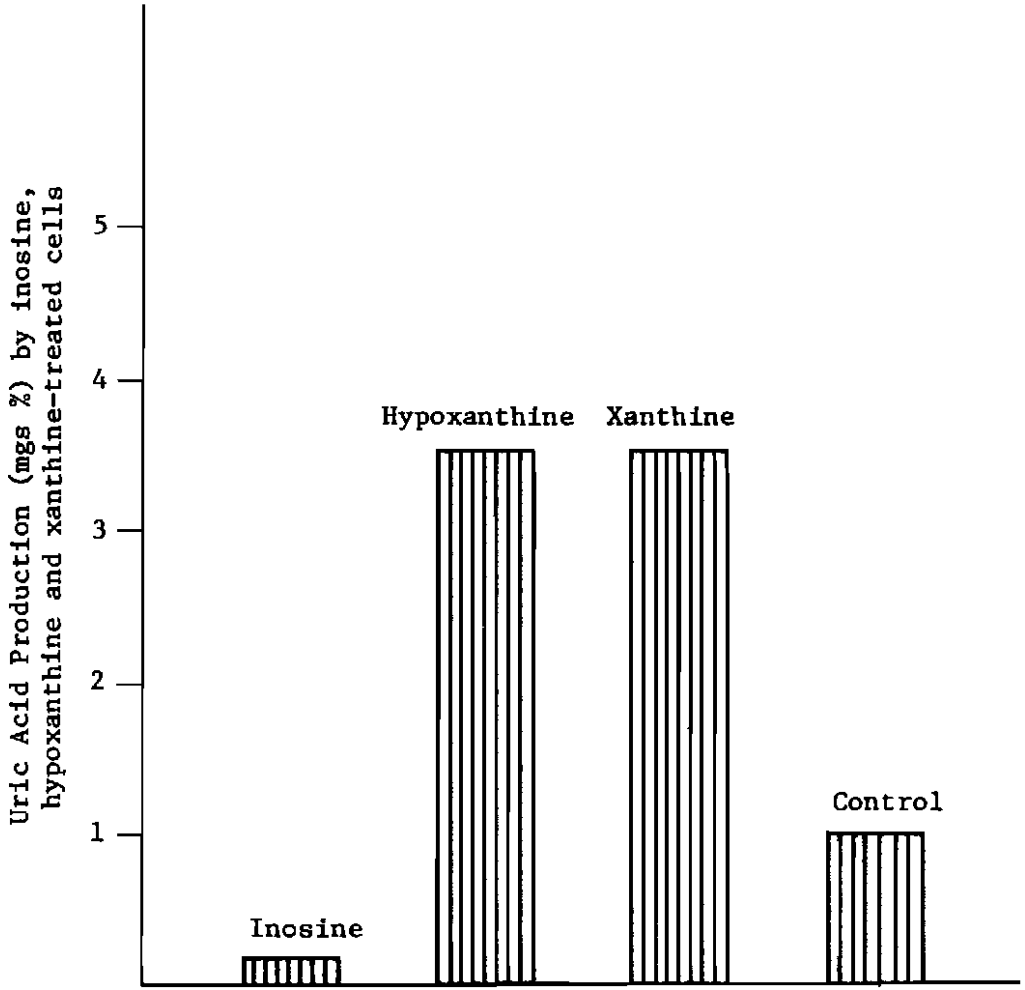

Uric Acid Production (mgs %) by inosine, hypoxanthine and xanthine-treated cells
5
4
3
2
1
Hypoxanthine
Xanthine
Control
Inosine

Fig. 5. Uric Acid Production by Drosophila cells after Purine Intermediates-Thymine Treatment.

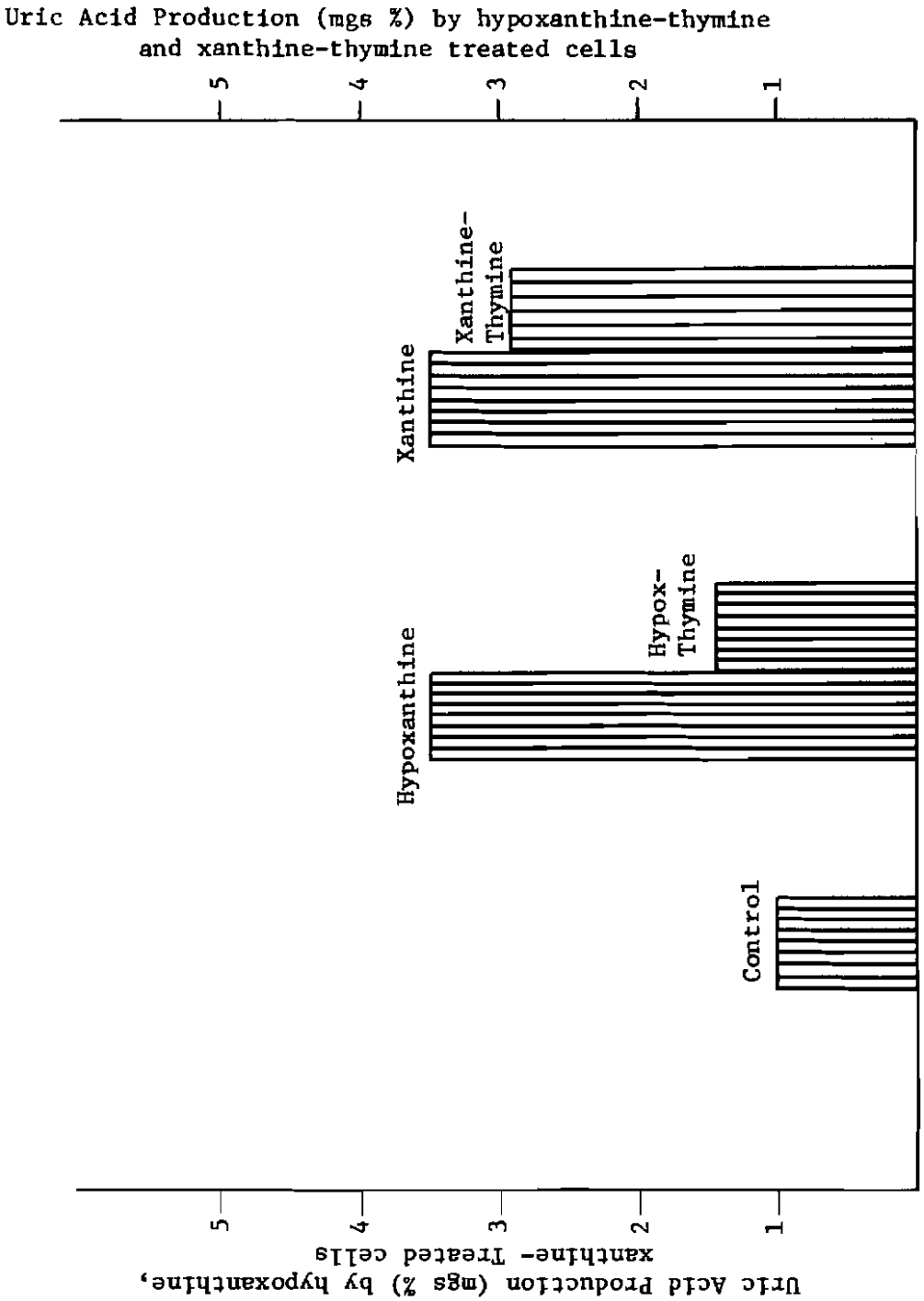

Uric Acid Production (mgs %) by hypoxanthine-thymine and xanthine-thymine treated cells
5
4
3
2
1
Xanthine-Thymine
Xanthine
Hypox-Thymine
Hypoxanthine
Control
Uric Acid Production (mgs %) by hypoxanthine, xanthine- Treated cells

Table 4. Summary of the Results from the Kc-H Drosophila treatment with certain combinations of Purine and Pyrimidine derivatives.

Treatment	Comments
Allopurinol-Adenine	Allopurinol did help the cells to partially overcome adenine toxicity.
Allopurinol-Hypoxanthine	Allopurinol did help the cells to overcome hypoxanthine toxicity.
Allopurinol-Inosine	Cells grew well and formed confluent monolayers.
Allopurinol-Xanthine	Allopurinol did help the cells to overcome xanthine toxicity.
Hypoxanthine-Thymine	Thymine did help the cells to overcome hypoxanthine toxicity.
Inosine-Thymine	Cells grew well and formed confluent monolayers.
Xanthine-Thymine	Thymine did help the cells to overcome xanthine toxicity.
Aminopterin-Adenine	Aminopterin did not help the cells to overcome adenine toxicity.
Thymidine-Adenine	Thymidine (10 μM/ml) did help the cells to overcome adenine toxicity.
Aminopterin-Thumidine-Adenine	Aminopterin + Thymidine (10 μM/ml) did not help the cells to overcome adenine toxicity.
Aminopterin-Thymidine (10 μM/ml)	Cells grew well and formed confluent monolayers.
Exogenous Uric Acid	Cells could grow and monolayered normally under this treatment.
Purine	Cells were susceptible to this treatment.
2,6 diaminopurine	Did not affect normal growth.
Thymidine (10 μM/ml)	Did not affect normal growth

at 50 μM/ml and resistant to thymidine at 10 μM/ml. Cells treated with aminopterin (0.57 to 5.7 μg/ml and 76 μg/ml) plus thymidine at 10 μM/ml grew and monolayered well during the time of exposure. It also was found that cells exposed to thymidine-adenine (10 μM/ml and 1.5×10^{-5}M respectively) could overcome adenine toxicity while cells exposed to adenine-aminopterin-thymidine (1.5×10^{-3}M, 1.7 μg/ml, and 10 μM/ml respectively) did not overcome the toxic effect (Table 4).

Kc-H Drosophila cells exposed to exogenous uric acid

Exposure of the cells to exogenous uric acid (1.44 %) showed that the cells can grow and monolayer normally under this treatment (Table 4). Uric acid determinations done in the supernatants of uric acid-treated cells for seven days (old monolayers) revealed that uric acid production was 0.75 mgs % while in the control cells the uric acid production was 1.00 mgs %. There was a 1.33 fold increase in uric acid in control cells which is not significant when compared to the 79 fold increase in uric acid production between the control and adenine (1.5×10^{-3}M)-exposed cells (Table 2). This result indicates that exogenous uric acid seems not to affect Kc-H Drosophila cells.

Hep-2 cells received the same treatment as Kc-H Drosophila cells to establish some differences between them and to gain some insights about their physiology. The results showed that Hep-2 cells were susceptible to adenine (1.5×10^{-3}M), aminopterin (.57 to 5.7 μg/ml), thymidine 50 μM/ml), and azaguanine 76 μg/ml). The cells grew and formed confluent monolayers, at the end of the incubation period, when they were treated with purine intermediates, or thymine, and allopurinol. Monolayering with guanine-treated cells was in a range of 25-30 %. Adenine's toxic effect on Hep-2 cells, as well as on Kc-H Drosophila cells was overcome by the addition of thymine (1.5×10^{-3}M) to the normal

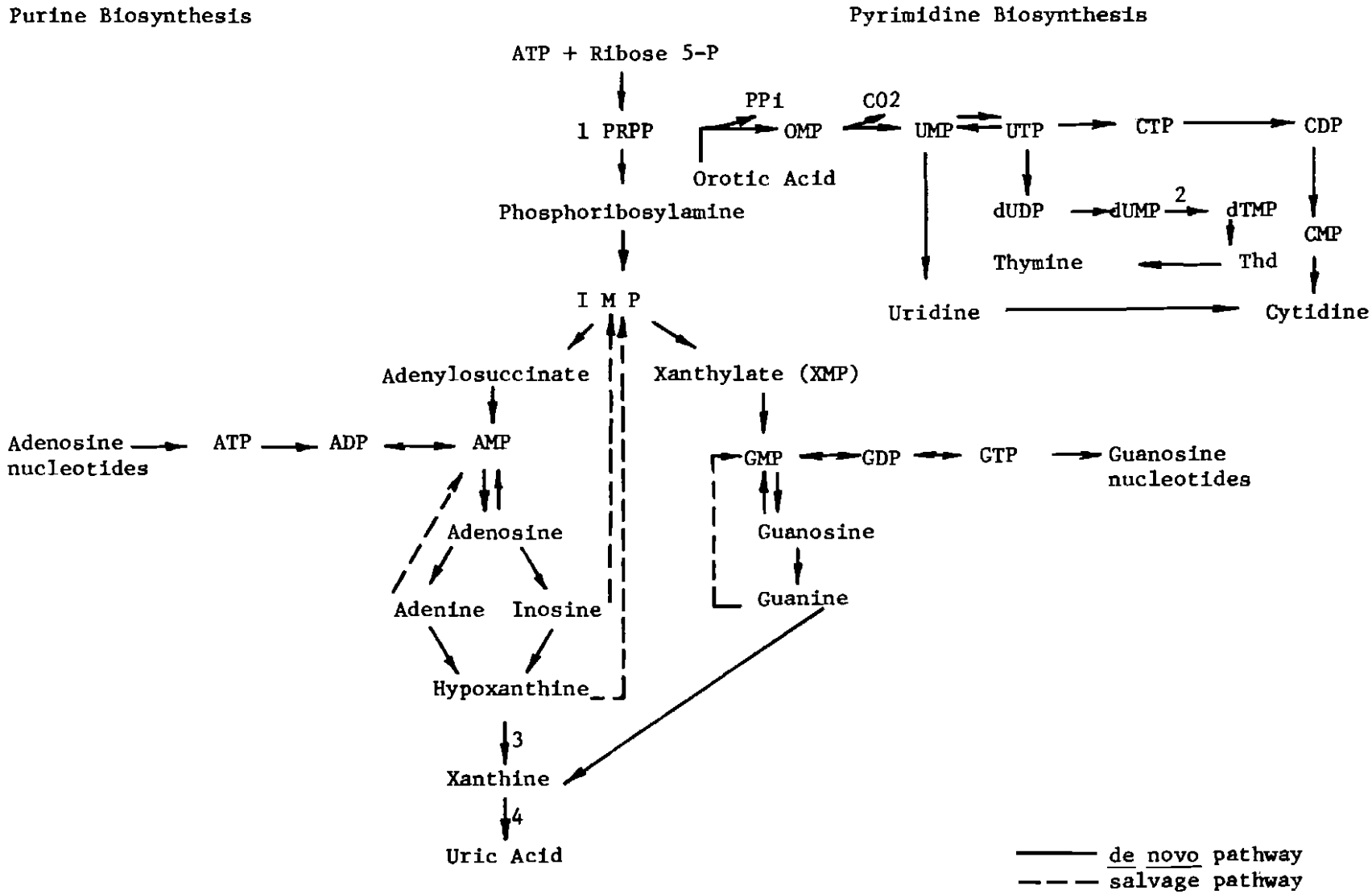
DISCUSSION

The results of the present study have shown clearly that adenine at 1.5×10^{-3}M is highly toxic to the Kc-H Drosophila cell line, confirming previous studies about adenine's toxic effects on other non-insect systems (31, 22, 45, 40, 44, 28, 6). The fact that the adenine toxic effect on Drosophila cells could be overcome by the supplementation of the other purine and pyrimidine bases (Fig. 2), leads to the assumption that there is a possible correlation between purine and pyrimidine biosynthesis. The observation that the toxic effects of purine bases are associated with a block of pyrimidine synthesis and that toxicity is completely or partially reversed by thymine and cytosine suggests that inhibition of pyrimidine synthesis may be responsible for the toxic effect of adenine to Kc-H Drosophila cells.

Previous studies have shown inhibition of pyrimidines synthesis by by purine bases (13, 37). It also is known that purine and pyrimidine pathways are linked by means of a common substrate, phosphoribosyl-pyrophosphate (PP-ribose-P), as shown in Fig. 6. This information supports the hypothesis that inhibition of pyrimidine synthesis by adenine might occur by a decrease of PP-ribose-P concentration probably by dimishing intracellular PP-ribose-P levels within the cells.

It is known that repression of specific enzyme synthesis regulates purine biosynthesis in mammalian cells (35), organisms including lower eukaryotes (9), and vertebrates (21). This suggests that a mechanism for the acute toxic effect of adenine to Kc-H Drosophila cells is a feed-back inhibition of the de novo biosynthetic pathway. Henderson's studies (21), strongly support this hypothesis. He found that adenine and hypoxanthine are potent inhibitors of de novo purine biosynthesis in Erlich ascites tumor cells, and the conversion of the added purines

Fig. 6. Purine and Pyrimidine Biosynthetic Pathways.
1. Phosphoribosyl Pirophosphate, common substrate for purine and pyrimidine biosynthesis.
2. Aminopterin inhibitory action.
3 & 4. Allopurinol inhibitory action.


Purine Biosynthesis
Pyrimidine Biosynthesis
ATP + Ribose 5-P
1 PRPP
PPi
CO2
OMP
UMP
UTP
CTP
CDP
Orotic Acid
Phosphoribosylamine
dUDP
dUMP
2
dTMP
CMP
Thymine
Thd
I M P
Uridine
Cytidine
Adenylosuccinate
Xanthylate (XMP)
Adenosine nucleotides
ATP
ADP
AMP
GMP
GDP
GTP
Guanosine nucleotides
Adenosine
Guanosine
Adenine
Inosine
Guanine
Hypoxanthine
3
Xanthine
4
Uric Acid
de novo pathway
salvage pathway

acid. Thus, adenine toxicity appears to be due both to a uric acid contribution from catabolism of adenine plus its ability to feed-back and starve the cells for pyrimidines.

Further observations from this work also support the above two conclusions about Kc-H Drosophila cells. Experiments with cells plus xanthine-thymine and hypoxanthine-thymine supplemented media, found thymine neutralizing the toxic effects of xanthine and hypoxanthine on Drosophila cells. The finding supports the hypothesis of a possible connection between purine and pyrimidine biosynthesis. The exact mechanisms by which a pyrimidine derivative, such as thymine, regulates the de novo purine biosynthesis in Drosophila cells remain to be elucidated. Nevertheless, it may be suggested that thymine allows a bypass of the levels of PP-ribose-P, the common substrate that links purine and pyrimidine biosynthesis. Further studies need to be done to measure the PP-ribose-P levels in Drosophila cells treated with adenine and the purine intermediates, hypoxanthine and xanthine, plus thymine. Double isotopes experiments (17) for assay seems to be appropriate.

Previous investigators (24) have shown that both purine and 2,6 diaminopurine (purine-base analogs) are highly toxic to egg and larval development in Drosophila flies. The present study showed a correlation with those findings in regard to purine, which was highly toxic to Drosophila cells, while 2,6 diaminopurine did not affect normal growth. This phenomenon could be explained as due to the compound's solubility and hence its capacity to get incorporated into the cells. According to the biochemistry of these compounds (38), purine is more soluble in water than 2,6 diaminopurine, which needs alkali and heat to become soluble. Consequently, it could be suggested that purine being more

soluble than 2,6 diaminopurine could easily gain incorporation into the cells and exert its actions by inhibiting purine synthesis de novo through a mechanism of enzyme feed-back inhibition. Conversely 2,6 diaminopurine, probably due to its poor solubility, could not gain entry into the cells and allowed them to follow normal metabolism, thus, no toxicity could be seen by the treatment of Drosophila cells with this compound. This hypothesis is also supported by Ho's studies (24) on the effects of these compounds on the development of Drosophila flies. The suggestion for further studies to look at the incorporation of purine and 2,6 diaminopurine by Drosophila cells is proposed to resolve this issue. Radioactive tracers would be the suggested technology.

Thymidine, a constituent of nucleic acids, also was tested for its outcome with fly cell cultures. The results of this assay found Drosophila cells resistant to thymidine at 10 μM/ml. It was also found that thymidine did help cells to overcome adenine toxic effects. However, the neutralization of thymidine protective effects was seen when the cells were co-treated with aminopterin and adenine. These results showed once again the possible regulatory connection between purine and pyrimidine biosynthesis since thymidine, which is in the pyrimidine metabolic pathway (Fig. 6) was seen exerting effects on the purine metabolic pathway of adenine. It could be hypothesized that the probable mechanisms by which thymidine exerts its protective effect toward Drosophila adenine-treated cells are similar to that already explained for thymine and cytosine. It was suggested that adenine may interfere with pyrimidine biosynthesis by lowering the intracellular levels of PP-ribose-P leading to the death of the cells by means of PP-ribose-P starvation.

potent inhibitor of dihydrofolate reductase, an enzyme crucial to both purine and thymidilate synthesis (48, 7). Surprisingly, aminopterin by itself neither caused a toxic effect on Drosophila cells nor alleviated adenine toxic effects. Conversely, aminopterin neutralized thymidine protective effects toward adenine-treated cells when aminopterin was added to adenine-thymidine supplemented media. A synergistic effect, where aminopterin and adenine combine actions to inhibit neutralizing thymidine effects, is suggested to explain the death of the cells by adenine-aminopterin-thymidine treatment. The mechanisms by which aminopterin, by itself, was not able to exert any effect on Drosophila cells are not well understood and difficult to interpret. The lack of aminopterin incorporation into the cells or an enzymatic inhibitory reaction is a possibility, but seems unlikely since aminopterin inhibited thymidine protective effects toward adenine-treated cells. Thus, further studies are suggested to study the exact mechanisms of aminopterin action on Kc-H Drosophila cells. Radioactive tracers would be a suggested technology.

8-azaguanine, a purine antimetabolite and an effective antitumor agent, although not a substrate, is a potent xanthine oxidase inhibitor. 8-azaguanine has a high degree of affinity for the xanthine oxidase active center which lowers the activity of this enzyme preventing the substrate from binding (10). On the basis of this information and as a result of this study, which found 8-azaguanine to be highly toxic to Kc-H Drosophila cells, it could be inferred that this compound chemically altered the conversion of hypoxanthine to xanthine or the conversion of xanthine to uric acid. These are the two steps where xanthine oxidase has its action on the purine catabolism, limiting the oxidation of the substrate(s) and therefore inhibiting normal purine degradation

within the cells. It is suggested that Kc-H Drosophila cell line would be a good system to explore the possible relationship between the inhibition, in vitro, of xanthine oxidase and the carcinostatic activities of certain compounds, such as pyrazolopyrimidines, which have been considered inhibitors and substrates of xanthine oxidase as well as other carcinostatic agents (10).

The lack of effects of exogenous uric acid in Drosophila cells can be explained by the high insolubility that characterizes this compound (38). It is suggested that exogenous uric acid did not get incorporated into the cells, consequently, the cells were not affected by the compound. However, it is clear that the hyperproduction of uric acid by adenine, hypoxanthine, and xanthine treatments caused a toxic effect on Drosophila cells.

The results obtained with Hep-2 cell studies were the same as those obtained with Drosophila cells with the exception that guanine and aminopterin treatments showed a partial toxicity toward Hep-2 cells (Table 3). These compounds have been found to be toxic to Erlich ascites cells (21), human carcinoma cells (43), and yeast (29). Both guanine and aminopterin exert their toxic effects by inhibiting the de novo purine and pyrimidine biosynthesis, respectively. These results imply that the observations and findings with the Kc-H Drosophila cell line are valid since these cells behaved as did the Hep-2 cells.

SUMMARY AND CONCLUSIONS

Purine derivatives, such as adenine, have been found to be highly toxic to the Kc-H Drosophila cell line. The mechanisms proposed are, the block of pyrimidine biosynthesis with a decrease of PP-ribose-P or the feed-back inhibition of the purine synthesis de novo. A hyperproduction of uric acid was found as a result of adenine, hypoxanthine, and xanthine treatments which was reduced by thymine treatment in hypoxanthine and xanthine-treated cells. It is suggested that uric acid may be considered as a factor associated with toxicity. A toxic effect also was seen with the purine-base analog, purine, which was related to the inhibition of the purine synthesis de novo by mechanisms of enzyme feed-back inhibition. The results of the experiment done with thymine, thymidine, cytosine, and guanine treatments brought about the hypothesis of a possible regulatory influence between purine and pyrimidine biosynthesis probably due to the link of these pathways by a common substrate, PP-ribose-P.

Aminopterin was found to be related with the inhibition of thymidine protection of Kc-H Drosophila adenine-treated cells. The death of the cells was suggested to be due to a synergistic effect exerted by adenine and aminopterin, which inhibited thymidine effects. 8-azaguanine was found toxic to Drosophila cells. It was suggested that its toxic action was due to the inhibition of xanthine oxidase, which limited the oxidation of the substrates, inhibiting the normal degradation of purines within the cells. Hep-2 cells were considered as a control that served to compare the results of this study with those already found in human cell lines as well as to verify the quality of the reagents used.

In conclusion, the goals of this study were accomplished since the

results correlated with the Ho, et al. findings on Drosophila flies with only two exceptions. Firstly, Drosophila cells in culture, seem to be a better system to study bio-sensitivity since the present work showed that concentrations of adenine, 10 fold less than those used by Ho, et al. were able to produce a high toxicity on Drosophila cells. Second, 2,6 diaminopurine did not have the toxic effect observed by the Ho, et al. studies. It is concluded that the exact mechanisms by which certain purine derivatives and intermediates caused toxicity to the Kc-H Drosophila cell line remain to be defined. However, these findings suggest that toxicity could be due to an alteration of pyrimidine biosynthesis at the PP-ribose-P levels. Further studies are suggested to gain insight into the mechanisms that make adenine, hypoxanthine, and xanthine toxic for Drosophila cells.

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APPENDIX

APPENDIX I

Medium D-22 Minus Antibiotics (based on Echalier, 1976). Reagents and procedure for 1,760 ml.

1) 7.35 g glutamic acid

3.74 g glycine

50 ml deionized water

Mix and adjust to pH 7.0 with 10N KOH (28.5 g/50 ml) freshly filterated (does not dissolve until near this pH). Adjust to 100 ml with water.

2) 11.76 g glutamic acid

5.98 g glycine

80 ml deionized water

Mix and adjust to pH 7.0 with 10N NaOH (20.5 g/50 ml) freshly filtered. Adjust to 160 ml with water.

3) 1.6 g $MgCl_2.6H_2O$

5.92 g $MgSO_4.7H_2O$

0.66 g $NaH_2PO_4.H_2O$

2.4 g yeastolate (Difco)

1.07 g malic acid

0.0052 g succinic acid

0.024 g sodium acetate

3.2 g glucose

Dissolve in approximately 200 ml of water. Add 86.4 ml of (1) and 150 ml of (2).

4) 24 g lactalbumin hydrolysate, dissolved in approximately 300 ml of water.

5) 1.88 g $CaCl_2$ in 40 ml of water.

6) Add (3) plus (4). Adjust to approximately 1,600 ml.

7) Add (5) plus (6) plus 3.2 ml of vitamins mix.

8) Stir all together using 10N KOH to adjust to pH 6.7 exactly. Add water for final volume of 1,760 ml.

9) Let sit in the refrigerator overnight to allow some precipitation.

10) Next day, run through Whatman No. 1 filter paper with suction apparatus. Filter through 0.22 μm Millipore with suction.

11) Store in refrigerator.

Vitamins mix (taken from Grace, 1962) in 1 L. final volume.

10 mg thamine HCl

10 mg riboflavin

10 mg Ca pantothenate

10 mg folic acid

10 mg niacin

10 mg inositol

5 mg biotin

100 mg choline chloride.

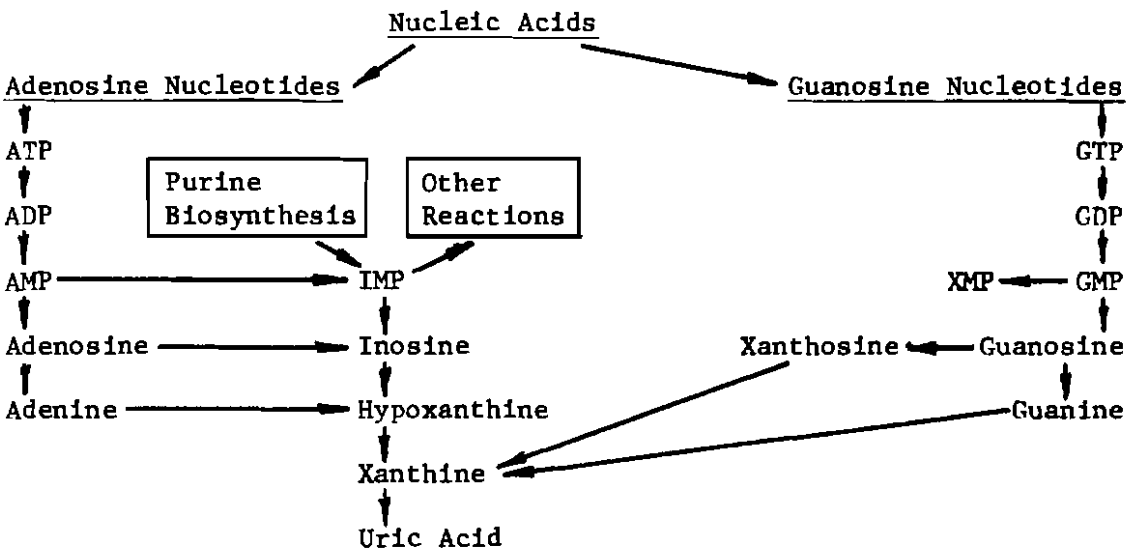
Store frozen and in dark.

APPENDIX II

Biochemistry of uric acid in insects.

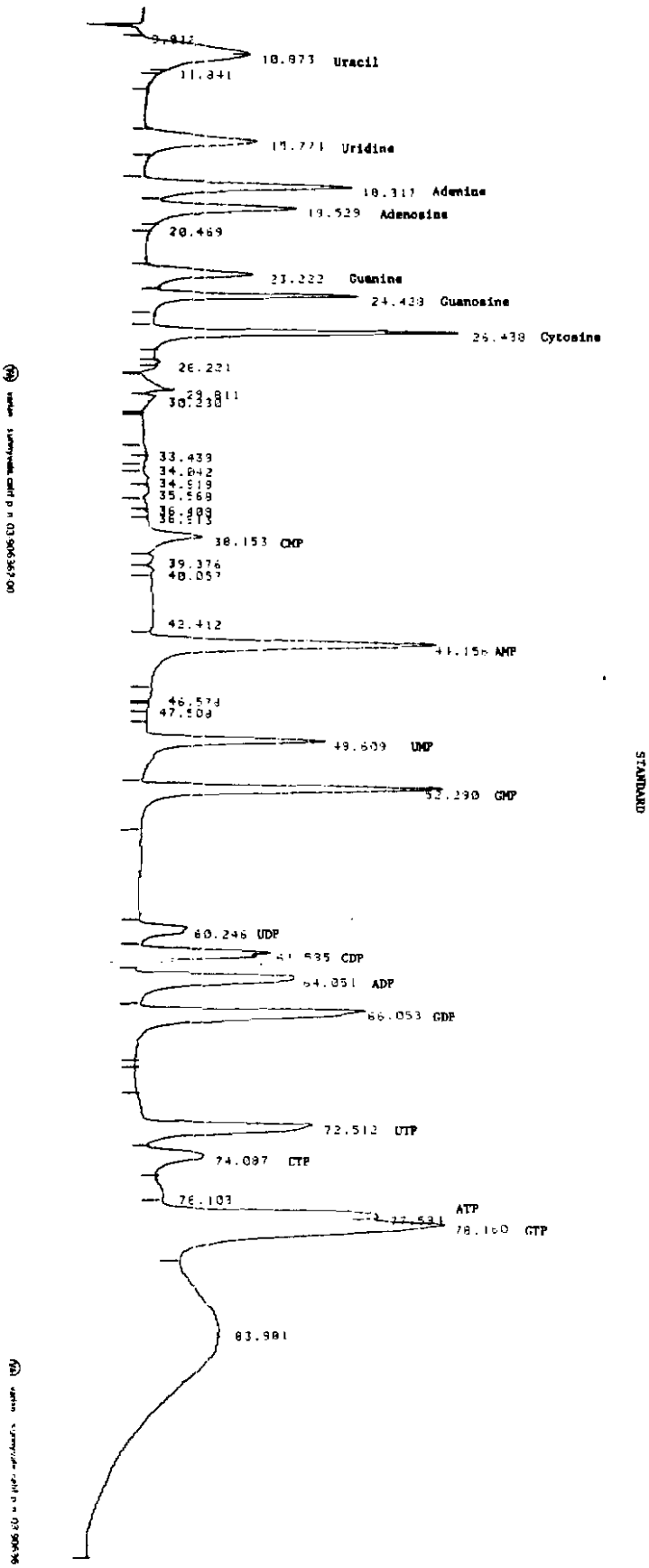
Three generalized pathways have been considered to be involved in the uric acid production in insects. First, there is the de novo synthetic process utilizing protein nitrogen and this is usually called the uricotelic pathway. Second, the degradative pathway which nucleic acids or their components are the starting material, this pathway has been referred to as the urocolytic or nucleicolytic pathway. The third, is the pathway whereby uric acid is degraded in insect tissues, and for which the term uricolytic pathway should be reserved.

Nucleicolytic uric acid production mechanisms in insects.

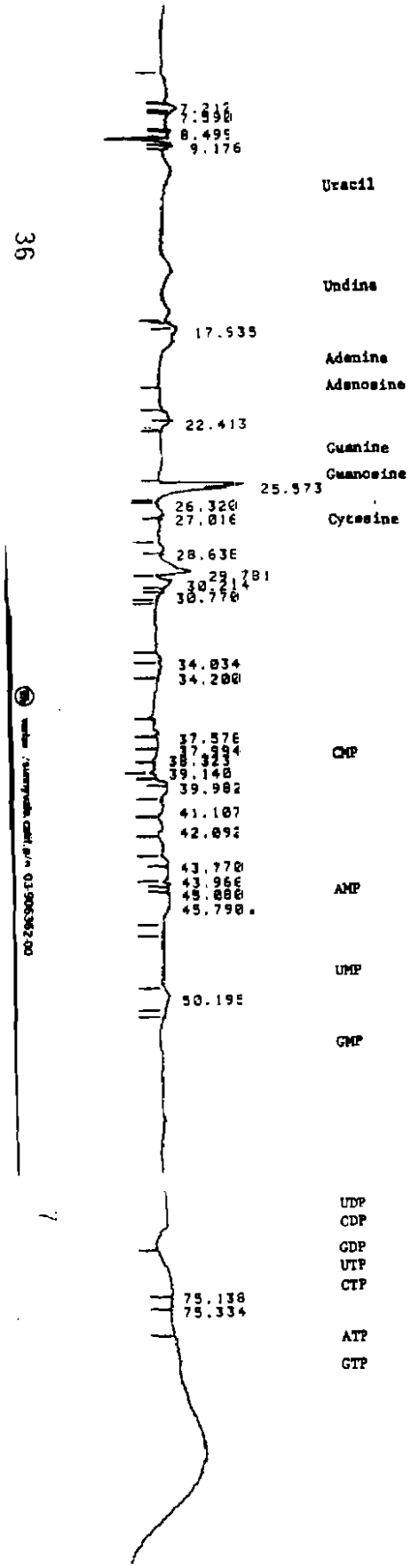


Adopted from Insect Biochemistry and Function (London: Chapman and Hall, 1975), p. 198.

APPENDIX III. Purine and Pyrimidine Standard Levels


10.873 Uracil
11.341
15.771 Uridine
18.317 Adenine
19.529 Adenosine
20.469
23.222 Guanine
24.438 Guanosine
26.438 Cytosine
26.231
30.230
33.439
34.042
34.619
35.568
38.153 CMP
39.376
40.057
42.412
46.574
47.509
49.609 UMP
52.290 GMP
60.246 UDP
64.051 ADP
66.053 GDP
72.512 UTP
74.087 CTP
76.103
ATP
78.160 GTP
83.981
STANDARD

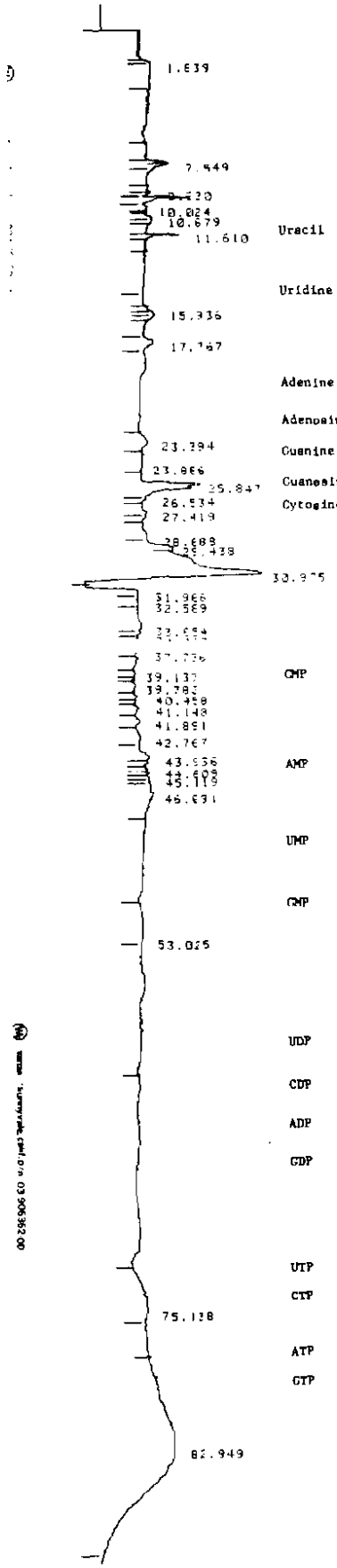
APPENDIX IV. Data from the analysis of D-22 medium without 10 % Calf Serum to detect the levels of purine and pyrimidine bases.



D-22 MEDIUM WITHOUT CALF SERUM

36

APPENDIX V. Data from the analysis of D-22 medium plus 10 % Calf Serum to detect the levels of purine and pyrimidine bases.



D-22 MEDIUM PLUS 10 % CALF SERUM