THE UNIVERSITY OF MANITOBA

IN VITRO INVESTIGATION OF 8-AZAGUANINE RESISTANCE IN A CHINESE HAMSTER CELL LINE

by

Phyllis Ann Gee

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ANATOMY

WINNIPEG, MANITOBA

May 1973

ABSTRACT

Spontaneous resistance to 8-azaguanine in the Chinese hamster cell line CHWC1 occurred at a rate in the order of 10^{-5}/cell/generation with selection at 10 µg aza/ml or 10^{-6} at 30 µg/ml after 42 hours mutation expression time. The rate of 10^{-6} was also obtained if selecting media were introduced without any intervening mutation expression time. Exposure to methyl methanesulfonate (MMS) increased the frequency rate twenty to fifteen hundred-fold while exposure for one hour to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) did not result in any increase in the number of resistant colonies.

Twenty-eight spontaneously derived resistant colonies were isolated and studied for stability, level of resistance, and reversion to sensitivity. Twenty-three colonies proved to be stable and five unstable in the absence of the selecting agent. All colonies except two showed levels of resistance from 45 µg to 100 µg aza/ml. One colony survived at concentrations higher than 100 µg aza/ml while the other could not form colonies above 15 µg/ml. Reversion to sensitivity as determined by single cell survival in THAG presented the only means of separating the resistant colonies; four categories were distinguished. Six

colonies had no growth in THAG medium, ten showed less than 30% survival, eight had greater than 50% survival and four were variable in response. Cell fusion between only the colonies showing no growth in THAG indicated that they were part of the same complementation group. Chromosome studies disclosed that twenty-one colonies had altered karyotypes and that fourteen colonies were tetraploid. Neither karyotype changes nor tetraploidy were related to mode of selection or growth responses. It was also shown that the tetraploids were not preferentially selected over the diploids upon selection with 8-azaguanine. Evidence presented suggests that only a small proportion of the resistant colonies can be the result of point mutations at the HGPRT locus, while others may be accounted for by regulatory mechanisms.

ACKNOWLEDGEMENTS

This study was supervised by Dr. J. L. Hamerton, Department of Anatomy, University of Manitoba. I wish to thank him for his advice and criticism during the course of this study. In addition, I wish to acknowledge the encouragement and comments on the preparation of this thesis generously given by Dr. D. M. Cox.

This research was supported by MRC operating grant MA 4061 to Dr. J. L. Hamerton.

TABLE OF CONTENTS

		Page
1.	Introduction	1
2.	Material and methods	22
	2.1 Cell line	22
	2.2 Media	22
	2.21 growth medium.	22
	2.22 selective media.	23
	2.3 Culture techniques.	24
	2.31 routine culture maintenance.	24
	2.32 plating efficiency	25
	2.33 growth curve for CHWCl	26
	2.34 selection, isolation and cloning of resistant colonies.	26
	2.35 cell fusion.	27
	2.4 Estimation of doubling time	29
	2.41 regression line method	29
	2.42 in mass culture.	29
	2.5 Estimation of mutation frequency and rate	30
	2.51 mutation frequency	30
	2.52 mutation rate.	30
	2.53 number of survivors.	32
	2.6 Chromosome studies.	32
	2.61 suspension technique	33
	2.62 in situ technique.	33
	2.63 chromosome counts.	34
3.	Characteristics of parental cell ine, CHWCl.	35
	3.1 Growth responses.	35

Page

3.11 growth curve and doubling time 35
3.12 cytotoxicity of 8-azaguanine 38
3.13 plating efficiency in THAG media . . . 38

3.2 Chromosome studies. 42

4. Mutation frequency and rate for

8-azaguanine resistance in CHWCl. 44

4.1 8-azaguanine resistance induced by MMS. . . 44

4.2 8-azaguanine resistance induced by MNNG . . 47

4.3 Spontaneous 8-azaguanine resistance in

CHWCl 51

5. Characteristics of spontaneous and chemically induced

8-azaguanine resistant colonies and clones. 54

5.1 Growth response and ploidy of

8-azaguanine resistant colonies 55

5.11 growth response. 55
5.12 chromosome ploidy. 58

5.2 Characteristics of an induced

8-azaguanine resistant clone. 58

5.21 derivation 58
5.22 stability of resistance to
8-azaguanine 61
5.23 cytotoxicity of 8-azaguanine 65
5.24 doubling time. 65
5.25 spontaneous reversion to
8-azaguanine sensitivity from
8-azaguanine resistance. 65
5.26 chromosome studies 68

Page

6. Isolation and characterisation of twenty-eight spontaneously derived 8-azaguanine resistant colonies 70

6.1 Isolation of resistant colonies 70

6.2 Growth responses of surviving colonies. . . 71

6.21 stability of resistance. 71
6.22 cytotoxicity of 8-azaguanine 75
6.23 doubling time. 78
6.24 plating efficiency in THAG 83

6.3 Complementation 87

6.4 Chromosome studies. 87

7. Comparison of the frequency of spontaneously derived 8-azaguanine resistant colonies from CHWC1 with that from a tetraploid subline. 97

7.1 Characteristics of the tetraploid CHWC1-06. 97

7.11 growth responses 97
7.12 chromosome studies 97

7.2 Estimation of mutation rates. 99

8. Discussion 102

Literature cited 128

Appendices 138

LIST OF TABLES

Table		Page
1.	Total number of cells, from consecutive counts in a three week period, from cultures initiated with 1.5×10^5 cells in MENAFC10 and THAG, after 3 days and 4 days growth.	36
2.	Plating efficiency of CHWC1 at varying concentrations of 8-azaguanine.	40
3.	Plating efficiency of CHWC1 in MENAFC10, $THAG^C$ and $THAG^L$	40
4.	Mutation frequency x 10^{-5} of azaguanine resistance in the absence of MMS and induced by 10^{-3}M MMS with selection at 10 µg and 30 µg aza/ml.	46
5.	Mutation rate for azaguanine resistance in the absence of MMS and induced by 10^{-3}M MMS with selection at 10 µg and 30 µg aza/ml; cell survivors estimated by plating efficiency	48
6.	Mutation rate for azaguanine resistance in the absence of MMS and induced by 10^{-3}M MMS with selection at 10 µg and 30 µg aza/ml; cell survivors estimated by cell counts.	48
7.	Mutation frequency x 10^{-5} of azaguanine resistance in the absence of MNNG and induced by 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4}M MNNG with selection at 10 µg aza/ml after 0 and 42 hours mutation expression time.	50

Table | Page

8. Mutation rate for azaguanine in the absence of MNNG and induced by 10^{-7}, 10^{-6}, 10^{-5}M MNNG with selection at 10 µg aza/ml after 0 and 42 hours mutation expression time; cell survivors estimated by plating efficiency. 52

9. Mutation rate for azaguanine resistance in the absence of MNNG induced by 10^{-7}, 10^{-6}, 10^{-5}M MNNG with selection at 10 µg aza/ml after 0 and 42 hours mutation expression time; cell survivors estimated by cell counts. 52

10. Mutation rate for spontaneous azaguanine resistance selected at 10 µg and 30 µg aza/ml after 0 and 42 hours mutation expression time 53

11. Plating efficiency in MENAFC10, 10 µg aza/ml and THAG of colonies selected by 10 µg aza in the absence of MMS 56

12. Plating efficiency in MENAFC10, 10 µg aza/ml and THAG of colonies selected by 30 µg aza in the absence of MMS 56

13. Plating efficiency in MENAFC10, 10 µg aza/ml and THAG of colonies selected by 10 µg aza after exposure to 10^{-3}M MMS 57

14. Plating efficiency in MENAFC10, 10 µg aza/ml and THAG of colonies selected by 30 µg aza after exposure to 10^{-3}M MMS 57

15. Doubling time of azaguanine resistant colonies and CHWC1 in MENAFC10, 10 µg and 30 µg aza/ml. 59

Table — Page

16. Per cent diploidy and tetraploidy in azaguanine resistant colonies selected by 10 µg and 30 µg aza/ml in the absence of MMS 60

17. Per cent diploidy and tetraploidy in azaguanine resistant colonies selected by 10 µg and 30 µg aza/ml after exposure to 10^{-3}M MMS 60

18. Plating efficiency in MENAFC10, 10 µg aza/ml and THAG, of clones of 65691 maintained in 10 µg and 30 µg aza/ml. 62

19. Per cent diploidy and tetraploidy, and diploid chromosome number of clones of 65691 maintained in 10 µg aza and 30 µg aza/ml. 63

20. Plating efficiency in MENAFC10, 10 µg aza/ml and THAG of 1102 maintained in MENAFC10 and in azaguanine. 64

21. Total cells and reversion frequency of 1102 in THAG 67

22. Relative plating efficiency in 10 µg aza/ml of colonies selected by 10 µg aza/ml and 0 hours mutation expression time. 72

23. Relative plating efficiency in 10 µg aza/ml of colonies selected by 10 µg aza/ml and 42 hours mutation expression time. 73

24. Relative plating efficiency in 10 µg aza/ml of colonies selected by 30 µg aza/ml and 42 hours mutation expression time. 74

Table | Page

25. Doubling time (hours) in mass culture in MENAFC10 and azaguanine of colonies selected at 10 µg aza/ml, 0 expression time 80

26. Doubling time (hours) in mass culture in MENAFC10 and azaguanine of colonies selected by 10 µg aza/ml, 42 hours expression time. 81

27. Doubling time (hours) in mass culture in MENAFC10 and azaguanine of colonies selected by 30 µg aza/ml, 42 hours expression time. 82

28. Relative plating efficiency in THAG of colonies selected at 10 µg aza/ml, 0 expression time 84

29. Relative plating efficiency in THAG of colonies selected at 10 µg aza/ml, 42 hours expression time.. 85

30. Relative plating efficiency in THAG of colonies selected at 30 µg aza/ml, 42 hours expression time 86

31. Colonies typed according to relative plating efficiency in THAG 88

32. Average number of colonies per 10^5 cells in THAG resulting from cell fusion between 8-azaguanine resistant colonies and between these colonies and a BUdR resistant mouse line, B_{82}. 89

33. Summary of chromosome studies on 8-azaguanine resistant colonies with a karyotype similar to CHWC1 91

Table | Page

34. Summary of chromosome studies on 8-azaguanine resistant colonies with altered karyotypes 91

35. Total number of resistant colonies in CHWC1-06 and CHWC1 with selection at 10 µg/ml and 30 µg aza/ml after 0 and 42 hours expression time. 101

36. Mutation rate for 8-azaguanine resistance in CHWC1 with selection at 10 µg and 30 µg aza/ml after 0 and 42 hours expression time 101

LIST OF ILLUSTRATIONS

Figure		Page
1.	Growth curve of CHWC1	37
2.	Regression lines and doubling time for CHWC1.	39
3.	Cytotoxicity of 8-azaguanine to CHWC1.	41
4.	CHWC1 karyotype	43
5.	Cytotoxicity of MMS on CHWC1 after 2 hour exposure	45
6.	Cytotoxicity of MNNG on CHWC1 after 1 hour exposure	49
7.	Cytotoxicity of 8-azaguanine on CHWC1 and 1102	66
8.	1102 karyotype.	69
9.	Plating efficiency of resistant colonies in increasing concentrations of 8-azaguanine/ml (a) Colonies selected at 10 µg aza/ml, 0 mutation expression time	76
	(b) Colonies selected at 30 µg aza/ml, 42 hours mutation expression time	77
	(c) Colonies selected at 30 µg aza/ml, 42 hours mutation expression time	77
10.	Colony size in plating efficiency of resistant colonies in MENAFC10 and 8-azaguanine.	79

Figure | Page

11. Karotypes of 8-azaguanine resistant colonies
(a) 418-02-01 ; (b) 418-28-01 92
(c) 354-17-01 ; (d) 354-13-02 93
(e) 418-16-03 ; (f) 418-16-01 94
(g) 533-02-01 ; (h) 533-15-01 96

12. Cytotoxicity of 8-azaguanine to CHWCL-06 (——) and CHWC1 (---). 98

13. CHWC1-06 karyotype. 100

1. INTRODUCTION

Study of the genetics of somatic mammalian cells in vitro is entirely dependent upon securing stable, well-characterized mutant phenotypes to serve as markers for experimentation. This requirement is evident from the significant contribution made to the development of molecular and biochemical genetics by markers in microbial systems. Genetic markers such as resistance and auxotrophy have been used successfully to elaborate upon the chemical basis of inheritance, and the induction and molecular basis of mutation. As a result many prokaryotes are better understood than most eukaryotes. This is due primarily to the fact that bacteria are more easily manipulated experimentally. Moreover interpretation of experimental results is made less difficult because microbes are haploid organisms which reproduce clonally, so that changes in genotype are readily recognized phenotypically.

The purpose of this investigation is an attempt to determine the reliability and validity of using resistance to 8-azaguanine, an anti-tumor drug (Tomizawa and Aronow, 1960), as a genetic marker in mammalian somatic cells. Resistance to 8-azaguanine was chosen as the marker because of its association with defects or deficiency of the enzyme,

hypoxanthine-guanine phosphoribosyl transferase (HGPRT, E.C. 2.4.28) which is known to be coded by an X-linked gene in man. On the assumption that this locus may be sex-linked in all mammals, a Chinese hamster cell line derived from a male animal was used as the experimental system and resistance to 8-azaguanine tested for suitability as a marker. The features required of a good genetic marker are: (i) all or none response to particular selective growth conditions, (ii) unimpaired growth rate, plating efficiency and other cultural properties under standard and selective growth conditions, and (iii) genetic stability even under non-selective growth conditions (Szybalski and Szybalska, 1962). These were the characteristics to be determined for the 8-azaguanine resistant colonies selected from a population of 8-azaguanine sensitive Chinese hamster cells. The in vitro system is preferable to in vivo methods for this type of study, because it provides a means of separating the direct effects of selection from those effects promoted or inhibited by organ-specific metabolisms of in vivo methods.

Selective methods analogous to those in microbial genetics became available for use in mammalian somatic cells through the efforts of Puck, Marcus and Cieciura (1956). They established that a single isolated mammalian cell can grow to form a colony of cells, making possible the selection of mutants in a population of cultured cells, in much the same way as that used in microorganisms. Four

selective methods have been adapted to somatic cells with success. They are: mass selection, lethal growth, thymidineless death and replica plating (Chu, 1970). Mass selection is best applied when searching for resistance to radiation and drugs. Lethal growth and thymidineless death methods are limited to the recovery of auxotrophs, while the replica plating method makes possible the selection of conditional lethal or radiation sensitive mutants.

Conditional lethals, auxotrophs, and resistant lines to a number of chemicals have been identified in somatic mammalian cells by applying these various selection methods to in vitro systems.

Temperature sensitive conditional lethal mutants have been isolated from monkey kidney cells by Naha (1969, 1970) and from mouse L-cells by Thompson et al. (1970, 1971).

Many auxotrophs, requiring amino acids and other nutrilites are cited in the literature. De Mars and Hooper (1960) selected glutamine-requiring HeLa cells by creating thymine deficiency in a medium containing aminopterin, adenine and glycine. While Kao and Puck (1968, 1972) isolated auxotrophs requiring each of glycine, inositol, thymidine and hypoxanthine from a proline-requiring Chinese hamster line, CHO (Kao and Puck, 1967). The BUdR and visible light method (Puck and Kao, 1967) was used for enhancement of selection after growing cells in a medium deficient in nine nutrilites. Similar requirements were

demonstrated for another Chinese hamster cell line, V_{79}, by Chu et al. (1969) who selected and characterized auxotrophy for L-glutamine, and Chu et al. (1972) who isolated cells requiring each of glycine, uridine, purine and a combination of glycine, hypoxanthine and thymidine. In addition to these, sixty-one mutants that could not use exogenous galactose were also isolated. Other purine-requiring mutants were isolated from the Chinese hamster line CHO by Taylor et al. (1971), and "glucose-independence" was studied in human and Chinese hamster cell lines by Shapiro et al. (1972a).

Investigations on resistance have been the most prolific, stemming from the therapeutic uses of chemicals and irradiation. In most instances resistance has been correlated with an enzyme deficiency. Resistance to bromodeoxyuridine (BUdR) and absence of thymidine kinase activity was revealed in mouse fibroblasts by Kit et al. (1963). Littlefield (1965) isolated mouse L-cells that were BUdR resistant and deficient in thymidine kinase. The same association was illustrated in a Syrian hamster line BHK, by Littlefield and Basilico (1966), and in HeLa cells by Kit et al. (1966). However, later studies by Breslow and Goldsby (1969), and Clayton and Teplitz (1972) demonstrated involvement of thymidine transport and intracellular mosaicism for thymidine kinase with BUdR resistance. These results together with the fact that BUdR is a known mutagen

(Freese 1963, Drake 1969) make BUdR resistance unreliable as a "genetic marker" for environmental mutagen screening. Resistance to some other chemicals has been also shown to involve something other than one simple enzyme deficiency. Resistance to the anti-metabolite, aminopterin, described by Biedler et al. (1963, 1965) in mouse L-cells and by Orkin and Littlefield (1971) in the Syrian hamster line BHK, may be due to defects of more than one enzyme. In other cases there may be transport barriers, as demonstrated for actinomycin D resistance in mouse L-cells by Kessel and Wodinski (1968) and in Chinese hamster cells by Biedler and Riehm (1970). Puromycin resistance fits into a similar category. This has been illustrated in mouse L-cells by Lieberman and Ove (1959), in pig kidney cells by Harris (1967, 1971) and Cass (1972), and in frog cells by Mezger-Freed (1971). In the latter two cell lines, resistance appears to be due to cell density and to membrane permeability of puromycin. Thus, it becomes apparent that the choice of a selective agent for procuring a genetic marker is of utmost importance.

Information on effects of 8-azaguanine and other purine analogues is extensive. Since the early 1950's when purine analogues were developed for treatment of certain neoplastic disorders, they have been the subject of investigation as to their mechanism of action and the biochemical basis of resistance in bacterial and mammalian cells. The

following are analogues of purines:

adenine - 8-azaadenine, 2-azaadenine, 2-fluoroadenine, 2-aminoadenine (2,6-diaminopurine)

hypoxanthine - 8-azahypoxanthine, 6-thiohypoxanthine (6-mercaptopurine)

xanthine - 8-azaxanthine

guanine - 8-azaguanine, 6-thioguanine, 6-thio-8-azaguanine, 2-aminopurine

In most there is only one atomic modification, mainly nitrogen for carbon (aza) or sulphur for oxygen (thio). Analogues with two such substitutions were found to be inactive as inhibitors.

The focal point of this investigation is 8-azaguanine resistance, and therefore a number of studies in this area will be discussed in more detail.

Law (1956) developed sublines of mouse leukemic cells resistant to 8-azaguanine, 6-mercaptopurine, 6-thioguanine as well as to amethopterin. He found the resistance to be stable, irreversible and hereditable in continued absence of the selecting analogue, and therefore, concluded that "mutation and selection appear to constitute the mechanism involved".

Lieberman and Ove (1959) studied 8-azaguanine resistance and puromycin resistance in what probably were mouse L-cells. By alternating cultures into full growth medium and then into medium with drug several times, they isolated sublines resistant to a low level of puromycin and

a high level of puromycin, and cells resistant to 8-azaguanine. They concluded that while puromycin resistance occurred by two sequential mutations, 8-azaguanine resistance involved only a "one step" mutation, because no intermediate levels of 8-azaguanine resistance were found. As in bacteria, increased resistance to toxic agents may or may not occur in relatively small steps.

Anderson and Law (1960) found resistance to 8-azaguanine to be correlated with loss of HGPRT activity in several biological systems.

Szybalski (1959), Szybalski et al. (1961) and Szybalski and Szybalska (1962) isolated three resistant sublines from the line D_{98s}, derived from human sternal bone marrow. Of the three sublines, D_{98}/AG was 8-azaguanine resistant, D_{98}/AGR was 8-azaguanosine resistant, and D_{98}/AH was 8-azahypoxanthine resistant. The HGPRT activity was similar to that in D_{98s} for D_{98}/AG, partial in D_{98}/AGR and completely absent in D_{98}/AH. D_{98}/AH reverted, regaining only partial enzyme activity. They interpreted the results as being due to single step mutations. D_{98}/AG resulted from a block in uptake of hypoxanthine and guanine prior to ribophosphorylation, D_{98}/AGR from a partial block of ribophosphorylation of purines and their analogues, and D_{98}/AH from a complete block of ribophosphorylation. D_{98}/AHR, the revertant resulted from a partial restoration of ribophosphorylation.

Differing levels of HGPRT activity were found also to occur in mouse L-cells by Littlefield (1963), who studied colonies selected at different concentrations of 8-azaguanine. At 0.3 - 0.1 µg/ml he isolated some colonies that showed an intermediate level of enzyme activity and some with levels similar to the 8-azaguanine sensitive, parental cells. At higher concentrations, 1 - 2 µg/ml the colonies isolated showed low levels of enzyme activity. Because the enzyme characteristics of the resistant colonies were similar to those of the parental type, he concluded that a decreased amount of normal enzyme was produced, which may have been the reflection of the variability in the chromosome number.

Morrow (1970) also studied 8-azaguanine resistance in mouse L-cells and obtained similar results. He isolated resistant colonies at 0.005 - 0.2 µg 8-azaguanine/ml and then subjected these colonies to a higher concentration, 0.3 - 3.0 µg/ml. The colonies isolated at the lower concentration had levels of HGPRT activity intermediate to those found in the parental line and to colonies selected at the higher concentration. The colonies selected at the higher concentration were stable but revertants from these and the colonies with intermediate enzyme levels returned to a sensitive state after about a month in nonselective medium.

Two levels of HGPRT activity have been illustrated

for 8-azaguanine resistance in human fibroblasts and in a Chinese hamster cell line. Albertini and De Mars (1970) isolated two resistant colonies from human fibroblast cultures derived from two normal males, and compared the levels of HGPRT of these cells to azaguanine sensitive and to known HGPRT deficient human fibroblast strains. The resistant colony from one individual had an enzyme level similar to the known HGPRT deficient, while the other resistant colony from the second donor showed an intermediate enzyme level. Gillin et al. (1972) isolated thirty-five spontaneous and induced azaguanine resistant mutants from the Chinese hamster line, V_{79}. These mutants were characterized as to HGPRT activity: sixteen showed no activity, five had detectable activity, and the remaining fourteen demonstrated significant activity.

Data from the biochemical assays of HGPRT in 8-azaguanine resistant cells indicates that there is not an all or none response to this drug. Moyed (1964) presented various biochemical mechanisms of drug resistance, citing examples from microbial studies. Drug resistance has been illustrated to involve enzyme inactivation (detoxification) of the drug; impermeability of the drug due to defects in membrane or carrier systems; altered sensitivity of enzymes to drugs by competitive enzyme inhibition or alteration of endproduct sensitive enzymes; increased production of sensitive enzyme; elevated production of competitive

substrate; induced phenotype resistance; and decreased conversion of the drug to an active compound. All, except perhaps the last two mentioned are more regulatory phenomena than gene mutations, a distinction which is usually difficult to make.

Induced phenotype resistance may involve either regulation or mutation. It is indicated when cells remain "resistant" as long as the analogue is present, but regain sensitivity after several generations in noninhibitory medium. This state was recognized by the effect of 2-thiazolealanine, an analogue of histidine, on Escherichia coli. Three classes of resistance were apparent. "Resistant cells", while in the analogue medium, contained elevated levels of enzymes in the histidine pathway. Thus with the resulting higher levels of these enzymes, a new steady-state rate of growth was achieved despite the presence of the drug, an example of derepression by 2-thiazolealanine. These resistant cells regained sensitivity to the drug after five to six generations in drug-free medium. A "reduced sensitive mutant" demonstrated a reduced response to the derepression by 2-thiazolealanine and a "completely insensitive mutant" showed no response whatever to 2-thiazolealanine. While the first class represented a "phenotypic shift" the other two represented selection of genetic variants.

Resistance caused by decreased conversion of a drug

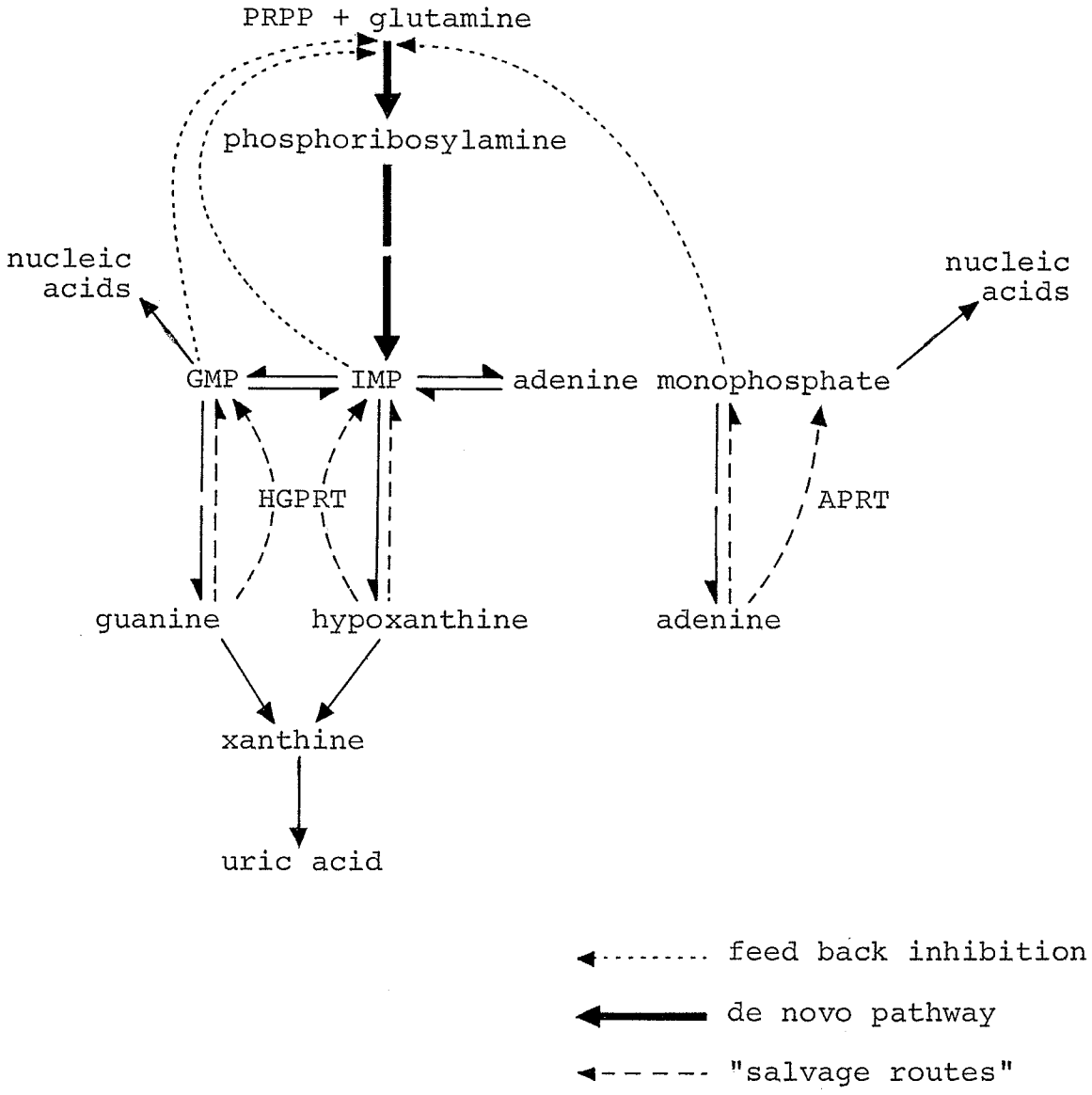
to an active compound would be demonstrated if a defect existed in the incorporating or converting enzyme. This type of resistance is genetic and resistance to purine and pyrimidine analogues have been due to this. 8-azaguanine, 8-azahypoxanthine, and 6-thioguanine show no effect on cells which have a defect of, or deficiency in HGPRT. Cells do not require preformed purines or pyrimidines because of the presence of a de novo synthetic pathway and therefore can grow without exogenous supply. However, there is an alternative pathway by which purines and pyrimidines can get incorporated, and converted into nucleosides and nucleotides under the activity of nucleotide pyrophosphorylases, or nucleoside phosphorylases and nucleoside kinases. Analogues can be picked up by these enzymes in some cell systems, producing a lethal effect.

Brockman (1965) investigated the mechanism of resistance to purine analogues in experimental leukemia systems. He established that 8-azaguanine was metabolized to azaguanine ribonucleotides in sensitive mouse cells but not in 8-azaguanine resistant cells (Brockman et al. 1961). Sensitive cells incorporated much more 8-azaguanine-2-^{14}C into nucleic acids than did resistant cells. Inhibition of azaguanine degradation with 4-amino-5-imidazolecarboxamide, did not alter the incorporation pattern of the resistant tumors but did increase incorporation of 8-azaguanine-2-^{14}C into nucleic acids of sensitive tumors. 4-amino-5-imidazole-

carboxamide inhibits deamination of azaguanine by guanase to azaxanthine (Mandel and Law, 1954). He also showed that 8-azaguanine resistant cells did not form guanylic, azaguanylic and inosinic acids, while conversion of adenine to adenylic acid was retained. A subline was developed in the presence of a low level of 6-thioguanine, which did not exhibit loss of enzyme capacity. However, a more highly resistant subline showed a greatly decreased pyrophosphorylase activity. He concluded that resistance mechanisms vary and are dependent to some extent on the selection pressure used.

It has been established that resistance to analogues of hypoxanthine and guanine is correlated with HGPRT activity. This enzyme, catalyzes transfer of the 5-phosphoribosyl moiety of 5-phosphoribosyl-1-pyrophosphate (PRPP) to hypoxanthine and guanine to form inosine monophosphate (IMP) and guanosine monophosphate (GMP). In bacterial cells there is a separate enzyme for hypoxanthine and for guanine, in mammals there is one for both these purines and a separate one, adenine phosphoribosyl transferase (APRT) for adenine (Krenitsky et al., 1969).

A schematic biosynthetic pathway for purines is presented below.



The steps from PRPP + glutamine to formation of IMP, the de novo pathway, can be blocked by aminopterin. Aminopterin is an antagonist of folic acid, disrupting single-carbon transfer reactions by inhibiting folic acid reductase. It blocks the addition of carbons 2 and 8 during

purine biosynthesis, as well as the addition of the 5-methyl group in thymidylate synthesis (De Mars, 1971). Eagle and Foley (1956) demonstrated that mammalian cells were unable to proliferate in medium containing aminopterin or amethopterin. The growth inhibiting effects however could be reversed by the addition of hypoxanthine, thymidine and glycine to the medium (Hakala, 1957; Hakala and Taylor, 1959). The aminopterin block creates a requirement for exogenous purines and a pyrimidine, therefore addition of thymidine leaves a specific requirement for adenine and hypoxanthine. By supplying one of these, cells survive and grow, incorporating the bases via "salvage routes". Therefore, in a medium containing aminopterin, thymidine and hypoxanthine cells with HGPRT will grow, but cells deficient in HGPRT will not. Thus it is possible to select cells with HGPRT in a large population of cells deficient for the enzyme. Moreover, in a medium containing azaguanine, cells with HGPRT will not grow but cells deficient for the enzyme will, making it possible to select HGPRT deficient cells. This system therefore permits selection of azaguanine resistant cells and cells which have reverted to sensitivity.

Nucleotides may be incorporated into nucleic acids or degraded to guanine, hypoxanthine and adenine. These purines may then be further degraded to uric acid for secretion or reincorporated into the nucleotide pool. There

are two enzymatic pathways by which this can occur:

(i) via HGPRT for hypoxanthine and guanine, and APRT for adenine

(ii) via nucleoside phosphorylases and nucleoside kinases.

These two pathways are referred to as the "salvage routes". The first appears to be more prominent than the second, as is suggested by the work of Kelley and Meade (1971) who could not detect guanosine kinase in fibroblast extracts. Also, the importance of the first "salvage route" is emphasized since a deficiency or defect in HGPRT in man gives rise to the Lesch-Nyhan syndrome (Lesch and Nyhan, 1964). This disorder is characterized by hyperuricemia, cerebral dysfunction, self-mutilation behaviour, and virtually total deficiency of HGPRT (Seegmiller et al., 1967). Patients with a partial deficiency of HGPRT have hyperuricemia and may have renal stone disease or gout, but do not have central nervous system abnormalities (Kelley and Meade, 1971).

The syndrome is X-linked recessive (Seegmiller et al., 1967), and therefore, because of X-inactivation, heterozygous females may be identified by the presence of two cell populations, one with normal enzyme activity and the other with enzyme deficiency (Migeon et al., 1968; Salzman et al., 1968).

Sperling et al. (1971) reports that in brain tissue there is absence of IMP and GMP synthesis from hypoxanthine

and guanine via the nucleoside phosphorylase--nucleoside kinase pathway, making HGPRT the main salvage pathway. This perhaps accounts for the neurological disorders noted in the Lesch-Nyhan syndrome in which HGPRT is deficient. Patients with a partial deficiency of HGPRT however do not demonstrate neurological disorders (Kogut et al., 1970) perhaps because a substantial incorporation of hypoxanthine and guanine could still occur in nervous tissues.

Biochemical properties have been determined for human HGPRT. Krenitsky et al. (1969) purified HGPRT 50-fold from a lysate of human erythrocytes and determined base specificity. While Arnold and Kelley (1971) purified the enzyme to homogeneity from human erythrocytes from one male donor, they determined a molecular weight of 68,000 and postulated 2 subunits having identical molecular weight and net charge. Three isoenzymes were identified using preparative isoelectric focusing. They explain that electrophoretic heterogeneity may result from a non-genetic, post-transcriptional alteration of one or both subunits. Since the enzyme is sex-linked and was prepared from a single male donor it can be concluded that these cannot be products of allelic genes. Also, the isoenzymes did not differ immunologically or catalytically and had the same molecular weight. Rubin et al. (1971) purified HGPRT 7000-fold from human erythrocytes and prepared antisera in rabbits and rats against the purified enzyme. A comparison

of the immunological result obtained from a normal erythrocyte lysate with those results from lysates of five Lesch-Nyhan patients, showed that in the patients HGPRT was synthesized in essentially normal amounts, but it lacked catalytic activity. They concluded that the defect of HGPRT in the Lesch-Nyhan syndrome is due to a mutation in a structural gene and not due to the deletion of a structural gene or defect in a regulatory gene. In the course of purification of the enzyme, they also produced evidence for isoenzymes of HGPRT in normal erythrocytes. This is expected since heterogeneity is well established for other proteins.

Recent reports have identified heterogeneity of the defective or deficient HGPRT in patients. The first such report was made by McDonald and Kelley (1971), in which they discussed the altered kinetic properties of HGPRT from a Lesch-Nyhan patient. Kelley and Meade (1971) studied fibroblast cultures from eleven patients with the enzyme deficiency; eight of these displayed different stability and kinetic properties. Three types were identified according to product inhibition and thermal stability:

(i) resistant to product inhibition; thermal labile

(ii) product inhibition normal; thermal stability normal

(iii) product inhibition normal; thermal labile.

The HGPRT locus gives rise to three phenotypic

expressions in the human population, normal, partial and deficient, each related to the level of catalytically active enzyme present. Biochemical characterization has revealed the occurrence of isoenzymes in "normal" HGPRT and altered stability and kinetic properties of some "deficient" HGPRTs. Induced mutagenesis at this locus could provide insight into the type of alterations that could produce some of the variant ("mutated") HGPRTs. Specificity of certain mutagens has been observed in the induction of reverse mutations in Escherichia coli, Neurospora crasa and rII region of T_4 phage (Freese, 1961). Each mutagen has certain chemical properties and is expected to induce only certain base pair changes to the exclusion of others. Extensive reviews on mechanisms of mutations are given by Freese (1963), Drake (1969) and Röhrborn (1970).

Freese (1963) gives the following definition of a mutation: "Mutation is any hereditary alteration in the information content or in the distribution of the hereditary material in an organism, a cell or a virus which cannot be attributed to polyploidy or to recombination". Mutations lead to changes in morphology or biochemical characteristics. From studies of microbial systems primarily, mutations have been classified into two major types; point mutations and chromosomal aberrations (Auerbach, 1967; Auerbach and Kelley, 1971).

Point mutations are intragenic "microlesions"

brought about by replacements or substitutions of bases, as follows:

transition - the replacement of a purine by another purine, or a pyrimidine by another pyrimidine;

transversion - the replacement of a purine by a pyrimidine, or pyrimidine by a purine;

frame shift - the deletion or insertion of a single base, moving the triplet code one base.

Therefore, a change of just one nucleotide pair is responsible for a mutant phenotype.

Chromosomal aberrations are intergenic "macrolesions". They arise from chromosome breakage, of which at least 90-99% rejoin in original order while the remainder may rejoin to give rise to either intra- or inter-chromosomal rearrangements.

Recombination patterns of mutants provides one way of identifying the type of mutation that occurred. For example, considering mutants of the same phenotype: if one can recombine with all others but one, it is probably a point mutation and can usually revert to the original phenotype. But, if the mutant cannot recombine with at least two other mutants, which in turn recombine with each other, it contains a large genetic alteration. Such a mutant does not revert to the original genotype and rarely to the original phenotype. However if it does revert to a phenotype similar to the original, it is usually due to a

suppressor mutation. A suppressor mutation may occur in the same functional region as the original mutation or at some other region; it counteracts the change brought about by the original mutation.

Various agents have been applied to induce 8-azaguanine resistance in Chinese hamster cells (Chu and Malling, 1968; Bridges and Huckle, 1970; Arlett and Potter, 1971; Arlett and Harcourt, 1972; Shapiro et al., 1972b; Gillin et al., 1972). Two known chemical mutagens, methyl methanesulfonate (MMS) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) were used for preliminary introduction to the selection method for obtaining resistant cells. Both are alkylating agents, and in bacteria induce transitions primarily by alkylating guanine at position 7 which in turn pairs erroneously with thymidine. The result being a G-C nucleotide pair substituted by a T-A pair (Freese 1961, 1963). However, the alkylated base may be deleted, producing a gap into which any of the four bases may be incorporated opposite the gap, resulting in a transition, transversion or reinstatement of original base pairs.

Induced mutation both forward and backward at specific gene loci, and identification of the mechanism involved in each case would be one of the prime achievements of somatic cell genetics.

If 8-azaguanine resistance is to be a genetic marker, and before proceeding with induced mutagenesis and

extensive biochemical characterization, it is necessary to establish whether distinguishable "phenotypes" in spontaneous azaguanine resistant cells occur in an in vitro system. I propose to make such an evaluation by selecting azaguanine resistant colonies from a male Chinese hamster cell line and determining for these colonies the following characteristics: stability of resistance, cytotoxicity to drug, doubling time in selective and nonselective media, plating efficiency in aminopterin medium, chromosome constitution, and complementation.

2. Materials and methods

2.1 Cell line

A Chinese hamster cell line (CHW) was established in 1968 by Lin et al. (1971) at the genetics laboratory, Children's Hospital, Winnipeg. It was derived through spontaneous transformation in culture of skin fibroblasts from a male Chinese hamster. The line is pseudodiploid, with a modal number of 22, and shows little change from the normal karyotype. In CHW, two karyotypes are distinguished, Type 1 which has two chromosome markers and Type 2 with three chromosome markers. At passage 125 the line was cloned and a line constant for cell Type 1 was isolated. A clone, designated CHWC1 was selected and has been the line used throughout this study.

2.2 Media

2.21 growth medium

The medium used in culture maintenance, MENAFC10, was prepared from Eagles Minimal Essential Medium Earle's base (Schwarz Bioresearch), nonessential amino acids, sodium pyruvate and 10% fetal bovine serum (Microbiological Associates). Antibiotics were included in the medium, 0.1 mg/ml streptomycin sulphate and 100 I.U./ml penicillin G sodium. To ensure constancy in the formulation of the

medium, only two lot numbers of each of these components were used. One lot number of fetal bovine serum was used during the period when CHWC1 was monitored, which was concurrent with the mutation frequency experiments and the characterization of isolated colonies. This effort to keep all growth factors constant was important, since Albertini and De Mars (1970) reported that fetal bovine serum contains substances that antagonize the inhibiting effect of azaguanine, and Kelley and Meade (1971) demonstrated that a low level of HGPRT (1.3 - 2.2 n moles/mg protein/hour) was consistently found to be present in fetal bovine serum.

MENAFC10 was made up in 5 or 10 litre batches, sterilized by filtration, dispensed in 200 and 500 ml aliquots and stored at -20° C. The medium was thawed as required and unused portions stored in a refrigerator at 4° C. Fresh batches were prepared at 3 to 6 week intervals, and each checked against the previous batch for growth maintenance by plating efficiency, which varied from 60 - 80%.

2.22 selective media

Selective medium containing 8-azaguanine (aza) was prepared by adding the drug at various concentrations to MENAFC10 just prior to use. 8-azaguanine (Calbiochemicals) was made up as a stock solution at 0.01 M in deionized water, sterilized by filtration, dispensed in 1 to 2 ml aliquots and stored at -20° C. To ascertain that the 8-azaguanine

medium was non-toxic for HGPRT deficient cells, fibroblasts from a Lesch-Nyhan patient were grown in it. The results are outlined in Appendix I.

Two formulations of the medium THAG were prepared by adding thymidine (Sigma), hypoxanthine, glycine (Calbiochemicals) and aminopterin (Nutritional Biochemicals) to MENAFC10 in the following concentrations:

thymidine	1.6×10^{-5}M	10^{-6}M
hypoxanthine	10^{-4}M	10^{-5}M
aminopterin	4×10^{-7}M	3.2×10^{-6}M
glycine	10^{4} M	10^{-7}M

The first THAG is similar to that used by Littlefield (1963) and the second by Chu et al. (1969). All chemicals were stored at -20° C as concentrated stock solutions made up in deionized water; thymidine at 1.6×10^{-3}M, hypoxanthine at 1.0×10^{-2}M, aminopterin at 4.0×10^{-5}M, and glycine at 1.0×10^{-2}M. For the preparation of THAG, the chemicals were added to MENAFC10 prior to filtration, after which it was dispensed in 200 and 500 ml aliquots and stored at -20° C. Each new batch of THAG was tested by plating efficiency against the previous batch.

2.3 Culture techniques

2.31 routine culture maintenance

Cultures were grown in 10 ml of medium in 8 oz. glass milk dilution bottles or in 100 mm disposable plastic

petri dishes (Falcon). Disposable plastic petri dishes were used in all experiments in this study. The cultures were incubated at 37° C in 100% humidity and 5% CO_2 in air. Subculturing was carried out twice a week. Each culture was rinsed twice with 2 ml 0.05% trypsin in phosphate buffered saline, and the cells detached and removed in 1 ml trypsin. Approximately 0.1 ml was used for reseeding a new culture.

CHWC1 was monitored in MENAFC10 and THAG for a six month period by initiating cultures with 1 - 1.5 x 10^5 cells and determining the total cell count at each subculture.

2.32 plating efficiency

Two day old cultures were used in plating efficiency experiments to ensure that cells were in the log phase of growth, and unsynchronized. Cultures were trypsinized, suspended in MENAFC10 and counted, using a hemacytometer. A cell suspension containing 2000 cells/ml was prepared and 0.1 ml inoculated into each 35 mm dish containing 1 ml medium. The dishes were incubated for 5 to 7 days, and the experiments terminated by decanting the medium and fixing the colonies for 15 minutes in 10% formalin. The dishes were stained for 15 minutes with crystal violet, rinsed with tap water and allowed to dry. The stained colonies were counted visually and the plating efficiency per cent was calculated.

For determination of cytotoxicity, cells were

initially seeded into MENAFC10 and incubated for 4 hours to allow attachment of cells. The dishes were then randomized and put into treatment blocks. MENAFC10 was suctioned off and replaced with 1 ml of test medium. The incubation was then continued for 5 to 7 days.

2.33 growth curve for CHWC1

Forty-five 100 mm dishes were seeded with 5×10^5 cells in 10 ml MENAFC10. Cell counts were done at 6 and 12 hourly intervals for 72 hours. Five replicates were selected at random for each time period. Average cell counts were plotted on semi-log graph paper.

2.34 selection, isolation and cloning of resistant colonies

selection

The method used was similar to that developed by Chu and Malling (1968). Two-day old cultures maintained in THAG were trypsinized and the cells were seeded at 1.25×10^5/100 mm dish in 5 ml MENAFC10. After 4 hours incubation, the medium was discarded and replaced in half of the dishes with 10 ml fresh MENAFC10/dish, and in the other half with 10 ml azaguanine selective medium/dish. For induction of resistance, mutagen treatment preceded this step. Serum-free medium with and without mutagen was put in the dishes and after a further incubation of 1 or 2 hours the media were discarded, each dish rinsed twice with serum-free

medium, and replaced with MENAFC10 and selective medium. Following a 42 hour incubation (mutation expression time) azaguanine medium replaced the media in all dishes. The medium was changed thereafter every 3 or 4 days for 11 to 14 days. The resulting colonies were isolated, or fixed, stained and counted.

isolation

Individual colonies were picked using stainless steel cylinders for encircling each colony. Cells were dislodged with about 5 drops of trypsin and dispersed in medium containing 10 µg aza/ml in 35 or 60 mm dishes, depending on the size of the colony. The medium was changed every 3 or 4 days and the surviving colonies were maintained routinely.

cloning

Two-day old cultures were trypsinized, the cells suspended and counted. A suspension of 10 cells/ml was prepared and 0.1 ml of this suspension was inoculated into each well of a microtest plate (Falcon). The microtest plate was incubated until colonies were large enough to pick, about 5 days. Medium was removed and replaced with trypsin. The dislodged cells were picked up with a pasteur pipet and suspended in 2 ml medium per 35 mm dish.

2.35 cell fusion

The procedure used was based on the method

described by Davidson (1969). Briefly the steps were:

Day 1: The cells were trypsinized, counted and suspended in MENAFC10, and mixed in a 1:1 ratio giving a total of $2 - 5 \times 10^5$ cells in 2 ml MENAFC10/35 mm dish. They were then incubated for 24 hours.

Day 2: The medium was discarded and each dish rinsed twice with 2 ml serum-free medium. The dishes were placed in a cold room at 4° C during the second wash. After 10 minutes the medium was discarded and 0.3 ml inactivated Sendai virus (Connaught Medical Research laboratories) diluted 1:5 in cold serum-free medium, was added to each dish. The dishes were left for an additional 15 minutes at 4° C. Each dish was then rinsed twice with serum-free medium and incubated in 0.3 ml serum-free medium for 10 minutes. Following incubation, 2 ml of MENAFC10 was added to each dish, and incubated for 24 hours.

Day 3: The cells in each dish were trypsinized and diluted in THAG medium to a density of 10^5 cell/ml. 1 ml of this suspension was added to 3 ml of THAG/60 mm dish, with 5 or 10 replicates for each original 35 mm dish.

The medium was changed every 3 or 4 days. After about 14

days when distinct colonies were visible, the dishes were fixed with 10% formalin, stained with 1% crystal violet and counted.

2.4 Estimation of doubling time

2.41 regression line method

Cell densities of 6 x 10^4/60 mm dish or 5 x 10^5/100 mm dish were set up and cell counts were carried out at specific intervals on 3 or 5 replicates selected at random at each time period. A computer program for simple linear regression was used to calculate the intercept (b) and the slope (m). These values were substituted into the formula for a straight line, $y = mx + b$, to solve for y (number of cells) when x (hours) is given. Regression lines were drawn on semi-log graph paper and the doubling time estimated.

2.42 in mass culture

During routine culture maintenance, the total cell count (N_t), the cell inoculum for each new culture (N_o) and the time of subculturing were recorded for 6 months for CHWCl and 5 weeks for resistant colonies.

This data was substituted into the formula $N_t = 2^g \times N_o$, to solve for g, the number of generations. The number of hours, t, from one trypsinization to the next, was divided by g to give the approximate doubling time in mass culture.

The working formulae were:

$$g = \frac{\log \frac{N_o}{N_t}}{\log 2}$$

$$\text{doubling time} = \frac{t}{g}$$

2.5 Estimation of mutation frequency and rate

2.51 mutation frequency

The number of resistant colonies per number of surviving cells was used to estimate mutation frequency (F).

2.52 mutation rate

Calculation of mutation rate has most frequently been made from the fluctuation test for spontaneity of resistance (Luria and Dëlbruck, 1943). The mutation rate can be calculated by predicting a Poisson distribution of the number of mutants and determining the fraction of cultures showing no mutations. This fraction, P_o, is equal to e^{-m}, from which the average number of mutations, m, may be determined, and hence the mutation rate from the equation $m = a(N_t - N_o)$; where 'a' is mutation per cell per generation, N_t the number of cells per sample at the time of observation, and N_o the number of cells per sample at initiation of experiment. The equation states that the number of mutants which occur during any finite time interval is equal to the chance of mutation per cell per time

unit multiplied by the increase in the number of cells. The cells which mutate during any time element form a random sample of the cells present at that time and will be independent from those cells which mutate in different time intervals. All mutations will show a Poisson distribution. However, this cannot be verified directly since the number of mutant cells in a culture is not just the number of mutations which have occurred, but also includes cells which have arisen by multiplication from the original mutants. The number of these depends on how far back the mutation occurred. Therefore an average number of resistant cells is obtained by noting that this number increases because of new mutations and growth of mutant cells from previous mutations. This adjustment is integrated, and provides the formula (equation 8 from the Luria Dëlbruck fluctuation test):

$$r = a\,Nt\,\log_e\,(Ca\,Nt),$$

where r = average number of mutant cells in a limited number of samples

a = mutation rate/locus/generation

Nt = number of cells per sample at the time of observation

C = number of samples.

Capizzi and Jameson (1973) have simplified the calculation by multiplying both sides of the equation by C, so that the equation becomes: $Cr = CaNt \log_e (CaNt)$ and Cr

becomes a function of CaNt. By means of a computer program they have constructed a table of values of CaNt as a function of Cr at uniform increments in Cr, for values of Cr from 1.0 to 9800. This table was used in calculation of mutation rates.

The working formula for calculating mutation rate (a) was:

$$a = \frac{CaNt}{CNt}$$

CaNt was obtained from the Capizzi Jameson table for the value of Cr, the total number of colonies, since C is the number of dishes and r the number of colonies per dish. Nt was the number of cells per dish at the time when the selective agent was introduced.

2.53 number of survivors

Two methods were used to estimate the number of surviving cells. For the first method, a plating efficiency was set up simultaneously with the selection experiment, at the beginning using the same cell suspension, or after the initial 4-hour incubation using the cells from randomly selected 100 mm dishes seeded at 1.25×10^5 cells. The second method was to determine the total cell counts for each of three randomly selected 100 mm dishes after 4-hours incubation and 42 hours mutation expression time.

2.6 Chromosome studies

Two-day old cultures were used for the preparation

of chromosome spreads. Colcemid was added to a final concentration of 0.05 µg/ml, and the cultures incubated for a further 2 to 3 hours for metaphase accumulation. Two methods were used to obtain chromosome spreading, the suspension technique (Rothfels and Siminovitch, 1958) and the in situ technique (Cox and Ray, 1971).

2.61 suspension technique

The cells were detached with trypsin and suspended in 12 ml hypotonic culture medium (1 part MENAFC10 : 5 parts deionized water). Cells were left in hypotonic medium in a 37° C water bath for 15 minutes, then centrifuged at 1000 rpm for 15 minutes. The supernatant was decanted and the cell button dispersed by gentle but rapid tapping against the palm of the hand. About 8 ml of fixative (1 part glacial acetic acid : 3 parts methanol) was added slowly with continuous shaking. The suspension was centrifuged at 1000 rpm for 10 minutes, the fixative decanted and fresh fixative added, the volume adjusted to obtain the desired cell density. Slides were prepared by pipetting the cell suspension dropwise on a clean, wet microscope slide. The excess suspension was drained and the slide allowed to air dry. Spreading of the chromosomes was sometimes enhanced by drying the slide with blowing or flaming.

2.62 in situ technique

Cells to be used for this technique were set up in

60 mm dishes. Two days after seeding, the cultures were treated with 0.05 µg/ml Colcemid and incubated for 2 to 3 hours. The medium was discarded and replaced with 4 ml of hypotonic culture medium. After 30 minutes of incubation, 1 ml of fixative was added dropwise to the dish. After 2 minutes, half of the hypotonic fixative mixture was replaced with fresh fixative, and after a further 2 to 5 minutes was replaced completely with fresh fixative. This was discarded after 10 minutes and the dishes were allowed to air dry, during which time chromosome spreading occurred. The preparations were hydrolyzed with 5N HCl for 10 minutes at room temperature, washed well with tap water, stained with 1% aqueous cresyl violet for 15 minutes, passed through 70%, 95% and absolute alcohol and air dried.

2.63 chromosome counts

The first 100 metaphases selected by a 40X objective, were recorded as 2n or 4n to give level of ploidy. From these 100, ten spreads were selected at random and photographed. Chromosome analyses were completed on the photographs and karyotypes prepared of representative types.

3. Characteristics of parental cell line, CHWCl.

The origin of CHWCl has been presented in section 2.1. In this section the stability as to growth rate and chromosome constitution is described. At passage 19, the modal cells composed 89% of the population, and the karyotype was that of Type 1 (Lin et al., 1971). Thirty vials, each containing cells suspended in 1 ml of 10% glycerol in MENAFC10 were stored at -70° C and in liquid nitrogen, as stock cultures.

3.1 Growth responses

The procedure used in routine culture proved to be a reliable technique for maintaining stability of cell growth in mass culture. In Table 1 are cell counts for a three week period. The counts remained within this range throughout the six months of monitoring always showing lower recovery in THAG than in MENAFC10.

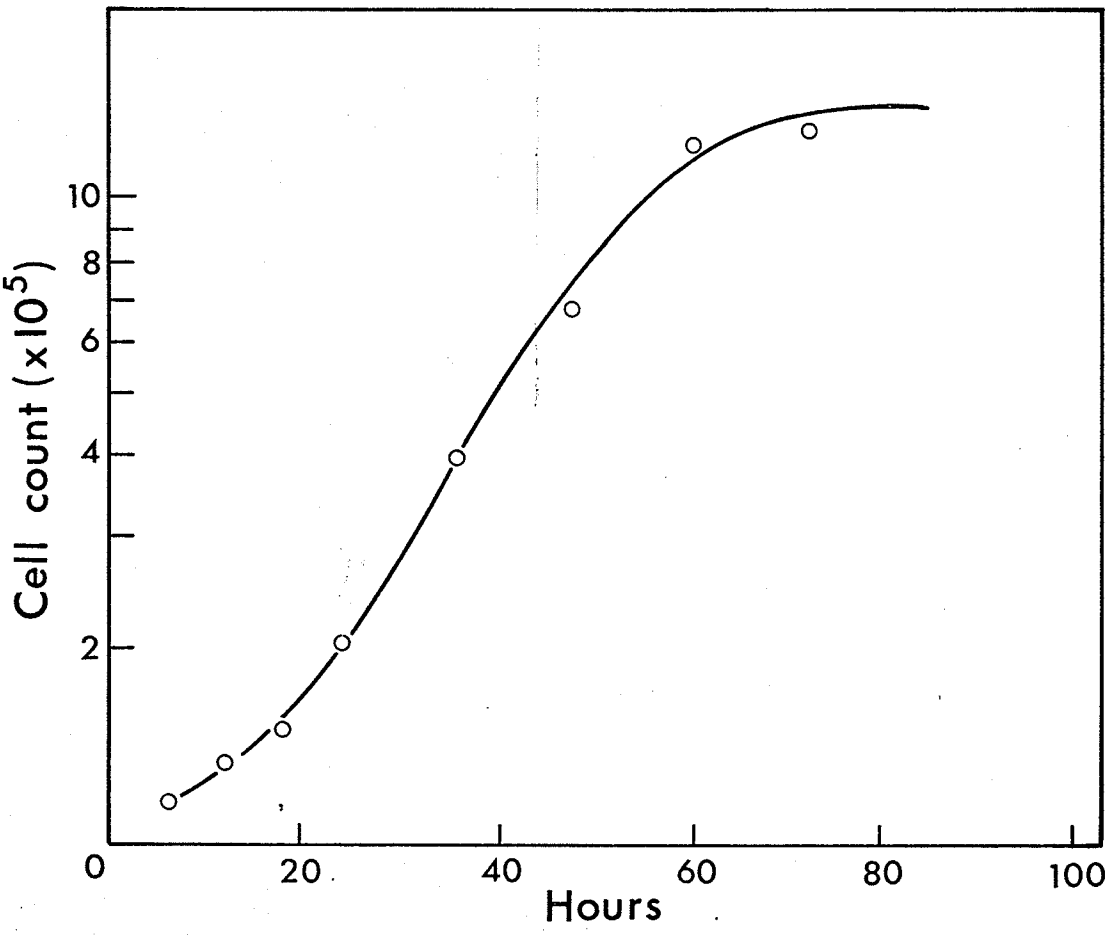
3.10 growth curve and doubling time

In cultures seeded at 5×10^5 cells/100 mm dish/10 ml MENAFC10 the end of log phase occurred about 60 hours after seeding (Fig. 1). At this time there were approximately 1.2×10^6 cells/dish representing a confluent culture in which the cells were contiguous. Thereafter additional cells rolled off into the medium. After 72 hours, the cells

TABLE 1. Total number of cells, from consecutive counts in a three week period, from cultures initiated with 1.5×10^5 cells in MENAFC10 and THAG, after 3 days and 4 days growth.

Total number of cells x 10^6			
MENAFC10		THAG	
3 days	4 days	3 days	4 days
4.0	12.4	1.6	2.0
4.6	7.3	1.5	2.7
4.2	8.0	2.1	2.2
3.4	9.0	1.3	2.6
3.0	8.2	1.6	2.6
5.2	7.0	1.6	1.8

FIGURE 1. Growth curve of CHWCl.


Cell count (x10^5)
10
8
6
4
2
0
20
40
60
80
100
Hours

became microcytic and pycnotic, reflecting depletion of the medium and perhaps accumulation of metabolites.

Regression lines (Fig. 2) were drawn from the calculations of three independent experiments (Appendix II). Estimating from these lines the doubling time was found to be 10 hours in one experiment and 12 hours in the other two.

3.12 cytotoxicity of 8-azaguanine

The mean plating efficiencies from two experiments on cytotoxicity of 8-azaguanine are given in Table 2. It appears that concentrations of the drug up to 1 µg/ml have little effect on the survival of CHWCl. But, concentrations in excess of 1 µg/ml produce a sharp decline in survival (Fig. 3).

Colony size was noted to change at 2.5 µg/ml. At this concentration the colonies were half the diameter observed in lower concentrations.

3.13 plating efficiency in THAG media

The two formulations of THAG (Sec. 2.2) were tested, and the average plating efficiencies of four experiments are given in Table 3. Since $THAG^{L}$ depressed plating efficiency by 35% or greater, $THAG^{C}$ was the medium used throughout this study.

To ascertain that aminopterin did block de novo purine synthesis, medium TAG was made up similar to $THAG^{C}$, except that the hypoxanthine was omitted. This medium

FIGURE 2. Regression lines and doubling time for $CHWCl$.

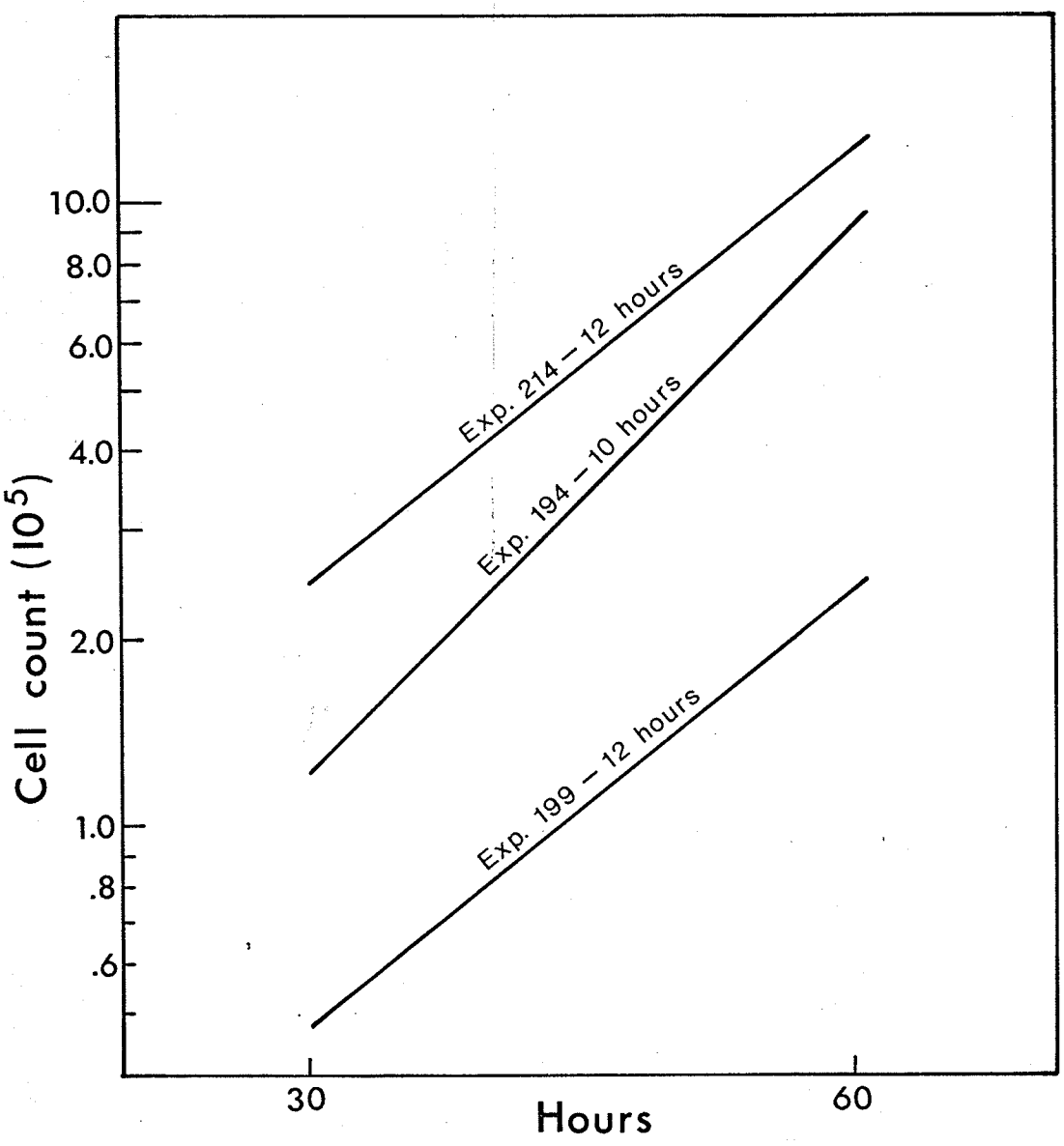

10.0
8.0
6.0
4.0
2.0
1.0
.8
.6
Cell count (10^5)
Exp. 214 – 12 hours
Exp. 194 – 10 hours
Exp. 199 – 12 hours
30
60
Hours

TABLE 2. Plating efficiency of CHWCl at varying concentrations of 8-azaguanine.

µg aza/ml							
o	0.1	0.5	1.0	2.5	5.0	7.5	10.0
72.8	76.8	76.9	72.9	49.1	0	0.1	0
84.1	81.5	72.5	76.0	59.5	0.15	0	0

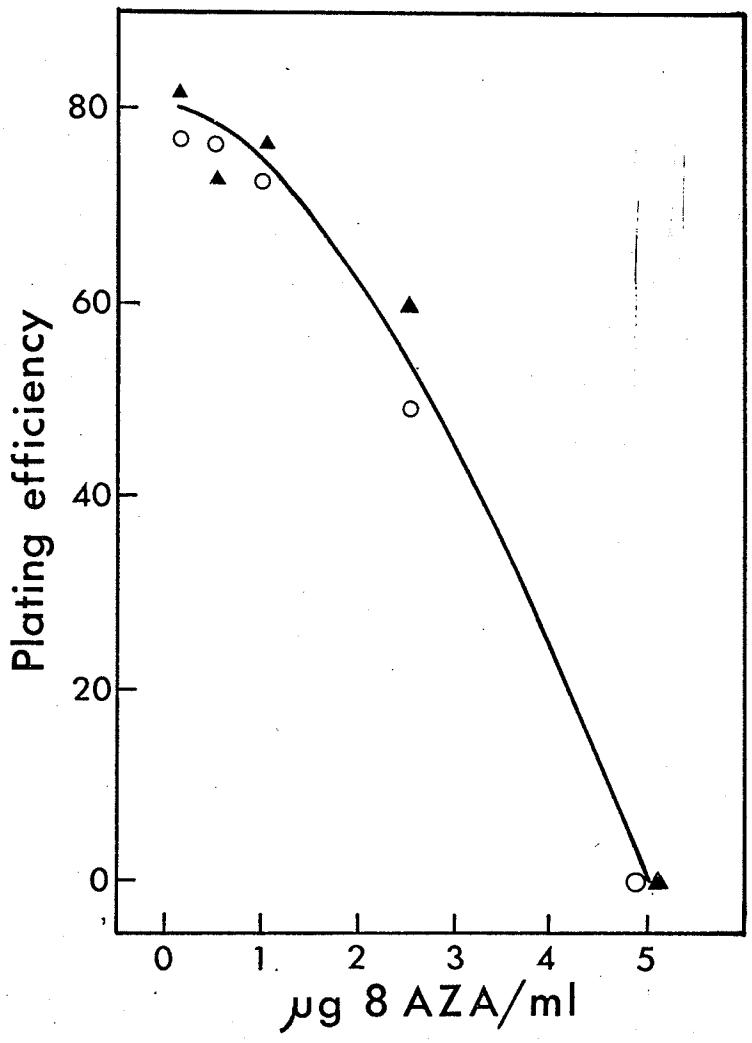
TABLE 3. Plating efficiency of CHWCl in MENAFC10, $THAG^C$ and $THAG^L$

MENAFC10	$THAG^C$	$THAG^L$
71.4	63.7	41.5
63.0	48.6	4.6
82.4	82.5	53.6
65.7	62.8	39.8

$THAG^C$ - Chu et al. (1969)

$THAG^L$ - Littlefield (1963)

FIGURE 3. Cytotoxicity of 8-azaguanine to CHWCl (two experiments).


Plating efficiency
80
60
40
20
0
0
1
2
3
4
5
µg 8 AZA/ml

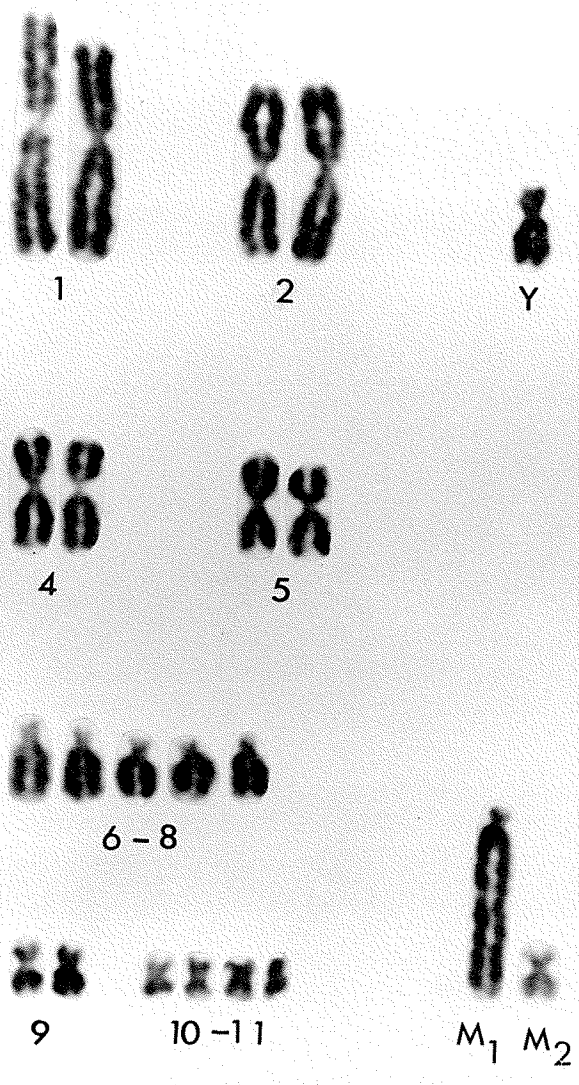
failed to support growth; the plating efficiency was:

MENAFC10	74.4%
THAG	74.3%
TAG	0 %

3.2 Chromosome studies

The diploid component was found to vary from 87-95% of the cell population. The modal karyotype (Fig. 4) remained unaltered throughout the entire study. A morphological distinction was difficult to make between M_2 and chromosomes 9 in more than only a few cells.

FIGURE 4. CHWCl karyotype. Chromosomes are numbered according to Lin et al. (1971).


1
2
Y
4
5
6 – 8
9
10 –11
M_1
M_2

4. Mutation frequency and rate for 8-azaguanine resistance in CHWC1.

The method described by Chu and Malling (1968) for obtaining azaguanine resistant colonies can be used to determine mutation frequency and rate, as well as to test any agent for mutagenicity. This was the method applied and the mutation frequency and rate determined on the basis of the number of colonies formed.

4.1 8-azaguanine resistance induced by MMS

MMS began to affect survival of CHWC1 cells at concentrations higher than 10^{-4}M, with minimal survival at 10^{-3}M (Fig. 5). The initial low plating efficiency was due to the washes and incubation in serum-free medium. 10^{-3}M MMS was the concentration chosen to induce azaguanine resistance in cultures of CHWC1 cells. Resistant cells were selected at 10 µg and 30 µg aza/ml after 42 hours mutation expression time. The details of five experiments are recorded in Appendix III and the mutation frequencies are summarized in Table 4. The mutation frequencies demonstrated clearly that the 2 hour exposure to 10^{-3}M MMS increased the number of colonies resistant to azaguanine. A larger number of colonies were observed in 10 µg/ml than in 30 µg/ml.

FIGURE 5. Cytotoxicity of MMS on CHWCl after 2 hour exposure.

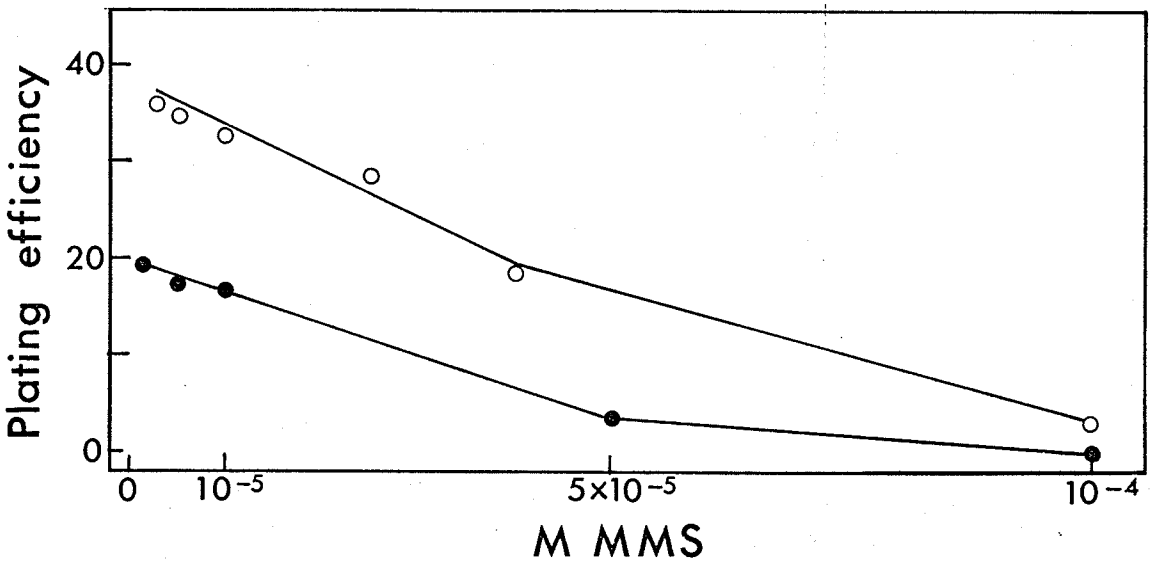

Plating efficiency
40
20
0
0
10⁻⁵
5x10⁻⁵
10⁻⁴
M MMS

TABLE 4. Mutation frequency x 10^{-5} of azaguanine resistance in the absence of MMS and induced by 10^{-3}M MMS with selection at 10 µg and 30 µg aza/ml.

0 MMS		10^{-3}M MMS	
10 µg aza	30 µg aza	10 µg aza	30 µg aza
16.6	1.79	674	280
2.05	0.92	3248.9	304
60.17	-	2381.9	-
43.12	-	867.7	-
42.04	-	22133.3	-

The mutation rates also showed an increase in the number of resistant cells following exposure to MMS (Table 5 and 6). These rates were calculated by using two methods of estimating the number of survivors, the first based on plating efficiency (Table 5) and the second on cell counts (Table 6). The rate was depressed when the number of survivors was estimated by cell counts rather than by plating efficiency.

4.2 8-azaguanine resistance induced by MNNG

The cytotoxicity of MNNG on CHWCl cells was apparent at molar concentrations 5×10^{-6} and higher, with no survival at 10^{-4} (Fig. 6). Because of the wide range at which cytotoxicity was expressed, four molar concentrations of MNNG (10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}) were used for induction of 8-azaguanine resistance. The cells were exposed to MNNG in the dark and for only one hour, as it has a half life of 90 minutes (Anderson and Burdon, 1970). Resistant cells were selected at 10 µg aza/ml after 0 and 42 hours mutation expression time.

The details of two experiments are recorded in Appendix IV and the mutation frequencies are summarized in Table 7. The frequency of resistant colonies was greater after a 42 hour mutation expression time than that after immediate selection following mutagen treatment. However, exposure to MNNG did not increase the number of resistant

TABLE 5. Mutation rate for azaguanine resistance in the absence of MMS and induced by 10^{-3}M MMS with selection at 10 µg and 30 µg aza/ml; cell survivors estimated by plating efficiency.

0 MMS		10^{-3}M MMS	
10 µg aza	30 µg aza	10 µg aza	30 µg aza
3.76×10^{-5}	7.01×10^{-6}	1.31×10^{-3}	6.17×10^{-4}
6.77×10^{-6}	3.66×10^{-6}	6.52×10^{-3}	9.58×10^{-4}
1.43×10^{-4}	-	5.46×10^{-3}	-
1.18×10^{-4}	-	1.89×10^{-3}	-
1.11×10^{-4}	-	4.90×10^{-2}	-

TABLE 6. Mutation rate for azaguanine resistance in the absence of MMS and induced by 10^{-3}M MMS with selection at 10 µg and 30 µg aza/ml; cell survivors estimated by cell counts.

0 MMS		10^{-3}M MMS	
10 µg aza	30 µg aza	10 µg aza	30 µg aza
1.11×10^{-5}	2.06×10^{-6}	3.81×10^{-4}	1.81×10^{-4}
2.28×10^{-6}	1.23×10^{-6}	2.19×10^{-3}	3.24×10^{-4}
5.68×10^{-5}	-	2.17×10^{-3}	-
3.23×10^{-5}	-	5.1×10^{-4}	-
2.89×10^{-5}	-	1.42×10^{-2}	-

FIGURE 6. Cytotoxicity of MNNG on CHWCl after 1 hour exposure (two experiments).

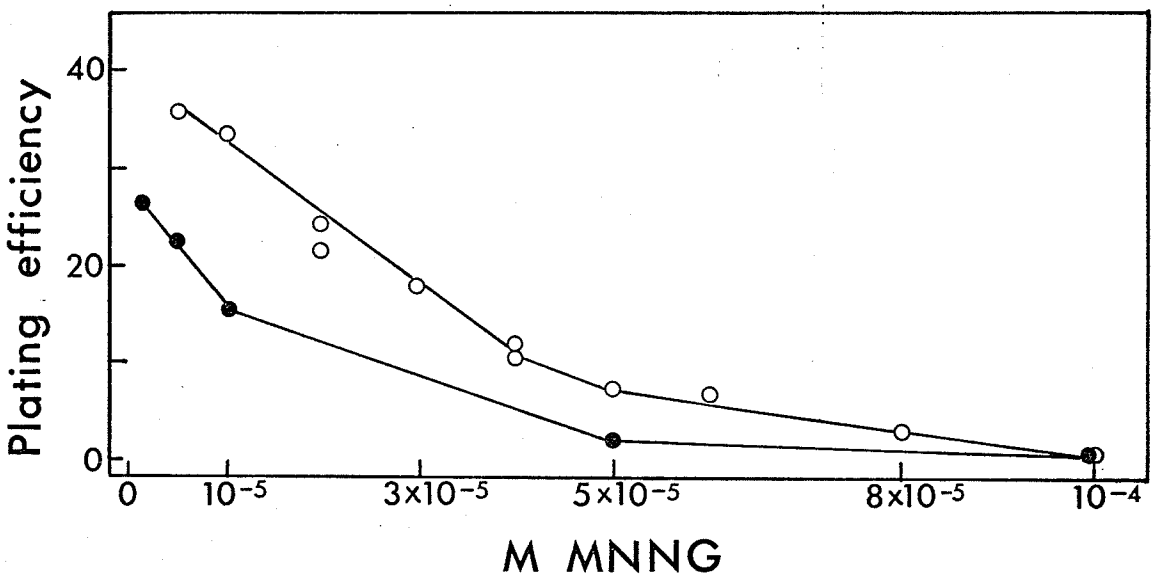

Plating efficiency
40
20
0
0
10⁻⁵
3x10⁻⁵
5x10⁻⁵
8x10⁻⁵
10⁻⁴
M MNNG

TABLE 7. Mutation frequency x 10^{-5} of azaguanine resistance in the absence of MNNG and induced by 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4}M MNNG with selection at 10 µg aza/ml after 0 and 42 hours mutation expression time.

	0	10^{-7}M	10^{-6}M	10^{-5}M	10^{-4}M
0 hours	0.52	5.50	5.27	18.75	0
	< 0.36*	0.65	< 0.38*	< 2.1*	
42 hours	55.44	33.03	90.53	20.00	0
	80.8	72.96	71.86	33.68	0

*limit set by number of replicates.

colonies.

The mutation rates were calculated by using two methods of estimating survivors (Tables 8 and 9). The rates reflected the results of the mutation frequencies. There was an increase after a 42 hour mutation expression time over immediate selection following MNNG exposure. Whereas MNNG had a severe toxic effect on the cells, it did not induce increase in the number of resistant colonies.

4.3 Spontaneous 8-azaguanine resistance in CHWCl

Details of two experiments on selection of spontaneous 8-azaguanine resistant colonies are recorded in Appendix V. The mutation rates were calculated by using cell counts for the estimation of survivors and are summarized in Table 10. The rates were slightly greater for 10 µg azaguanine selection than for 30 µg. However, there was no difference in the rates between 0 and 42 hours mutation expression time within each experiment.

The range of the mutation rates for spontaneous 8-azaguanine resistance selected at 10 µg aza/ml after 42 hours mutation expression time was 2.3×10^{-6} to 6.5×10^{-5} (Tables 6, 9 and 10). At 30 µg aza/ml the rates were in the lower region of this range. This data was not suitable for statistical analysis. However, it appears that only small differences existed from experiment to experiment, or within experiments.

TABLE 8. Mutation rate for azaguanine resistance in the absence of MNNG and induced by 10^{-7}, 10^{-6}, 10^{-5}M MNNG with selection at 10 µg aza/ml after 0 and 42 hours mutation expression time; cell survivors estimated by plating efficiency.

	0	10^{-7}M	10^{-6}M	10^{-5}M
0 hours	9.12×10^{-6}	2.95×10^{-5}	3.97×10^{-5}	1.79×10^{-4}
	--	7.65×10^{-6}	--	--
42 hours	1.61×10^{-4}	1.03×10^{-4}	2.77×10^{-4}	1.67×10^{-4}
	2.01×10^{-4}	1.81×10^{-4}	1.85×10^{-4}	1.64×10^{-5}

Table 9. Mutation rate for azaguanine resistance in the absence of MNNG induced by 10^{-7}, 10^{-6}, 10^{-5}M MNNG with selection at 10 µg aza/ml after 0 and 42 hours mutation expression time; cell survivors estimated by cell counts.

	0	10^{-7}M	10^{-6}M	10^{-5}M
0 hours	4.66×10^{-6}	1.52×10^{-5}	2.03×10^{-5}	7.28×10^{-5}
	--	5.6×10^{-6}	--	--
42 hours	3.63×10^{-5}	2.34×10^{-5}	6.25×10^{-5}	4.68×10^{-5}
	6.49×10^{-5}	5.85×10^{-5}	5.98×10^{-5}	5.30×10^{-5}

TABLE 10. Mutation rate for spontaneous azaguanine resistance selected at 10 µg and 30 µg aza/ml after 0 and 42 hours mutation expression time.

	10 µg aza	30 µg aza
0 hours	1.43×10^{-5}	NE
	6.38×10^{-6}	3.41×10^{-6}
42 hours	1.40×10^{-5}	3.46×10^{-6}
	8.26×10^{-6}	1.50×10^{-6}

NE = not possible to estimate as there must be 1 or more colonies per 2.61×10^5 surviving cells.

5. Characteristics of spontaneous and chemically induced 8-azaguanine resistant colonies and clones

This section deals with the colonies derived from one experiment, and in particular with one clone from a colony designated 65691.

A total of fifty-four colonies were picked, twenty-three did not grow and were discarded, thirty-one thrived and were stored at -70° C and in liquid nitrogen. The intention was to obtain a cell line which was resistant to 8-azaguanine and deficient in HGPRT in order to characterize its behaviour in vitro and consequently use it in cell fusion studies with human cell lines.

Many investigators using the Luria Delbrück fluctuation test have confirmed that spontaneous 8-azaguanine resistance in different cell lines occurred randomly and was independent of the selecting agent. There was no reason to assume otherwise for the occurrence of resistance to 8-azaguanine in CHWCl. To maximize the likelihood that the resistant line selected for study did involve an alteration in DNA, the cells were treated with the mutagen MMS. As was established in Section 4.1 this chemical increased the incidence of azaguanine resistant cells.

5.1 Growth response and ploidy of 8-azaguanine resistant colonies

5.11 growth response

Eleven colonies were tested for growth response by plating efficiency in MENAFC10, 10 µg aza/ml and THAG, after each had been routinely maintained in MENAFC10 for at least 6 passages (Tables 11, 12, 13 and 14).

The relative size of colonies were consistently larger in MENAFC10 than in 10 µg aza/ml. All colonies grew in MENAFC10 and in azaguanine, showing retention of resistance to the drug after three weeks or more in non-selective medium. One colony (65391, Table 13) showed marked decrease in plating efficiency in 10 µg aza/ml as compared to that in MENAFC10. Two colonies (65391, Table 13; 65171, Table 14) grew equally well in MENAFC10 and THAG. Colony 65171, in fact, grew in all three media. Two other colonies (65350, Table 11; 65371, Table 13) showed minimal plating efficiency in THAG. One of these (65350) did not initially plate out in THAG (passage 7) but subsequently did so. When, however, a frozen vial from an earlier passage was revived and tested, it showed no growth in THAG. One colony (65691, Table 14) retained resistance to azaguanine after 10 weeks of continuous maintenance in MENAFC10.

Five colonies (65350, 65171, 65281, 65274 and

TABLE 11. Plating efficiency in MENAFC10, 10 µg aza/ml and THAG of colonies selected by 10 µg aza in the absence of MMS.

Colony	Passage	MENAFC10	10 µg aza/ml	THAG
65060	7	33.2	25.8	0
65341	5	35.2	23.7	0
65350	7	78.9	57.6	0
	19	45.4	20.4	0.4
	24	127.3	93.95	1.3
	33	52.3	50.0	10.95
	36	53.6	31.9	0.8
	12*	89.4	76.1	0

*cells recovered from storage.

TABLE 12. Plating efficiency in MENAFC10, 10 µg aza/ml and THAG of colonies selected by 30 µg aza in the absence of MMS.

Colony	Passage	MENAFC10	10 µg aza/ml	THAG
65171	7	72.1	65.2	0
	20	77.5	46.2	0
65281	8	55.9	56.0	0
	22	59.4	46.9	0
	9*	58.0	44.9	0
65301	6	63.6	73.9	0

*cells recovered from storage.

TABLE 13. Plating efficiency in MENAFC10, 10 µg aza/ml and THAG of colonies selected by 10 µg aza after exposure to 10^{-3}M MMS.

Colony	Passage	MENAFC10	10 µg aza/ml	THAG
65274	7	44.7	32.8	0
	6*	41.9	42.7	+
	10	79.4	77.8	0
65371	7	58.7	54.1	0.12
65391	7	40.2	2.2	31.2

* cells recovered from storage.

+ less than 20 cells per colony.

TABLE 14. Plating efficiency in MENAFC10, 10 µg aza/ml and THAG of colonies selected by 30 µg aza after exposure to 10^{-3}M MMS.

Colony	Passage	MENAFC10	10 µg aza/ml	THAG
65691	6	66.3	55.6	0
	10	74.0	52.6	0
	20	62.7	45.4	0
	28	123.8	77.0	0
65171	7	61.9	49.4	56.1

65691) were also investigated for doubling time in MENAFC10, 10 µg and 30 µg aza/ml.

The resistant colonies all had a doubling time in the range of 12 to 18 hours, somewhat longer than the doubling time of 10 to 12 hours in the parental line CHWC1 (Table 15).

5.12 chromosome ploidy

Chromosome counts were completed on twenty-two of the surviving colonies (Tables 16 and 17). Four colonies were 100% tetraploid, seven were in excess of 70% tetraploid, six were 50% tetraploid, and only six colonies were within 70% diploid. The chromosome counts did not demonstrate any relationship to treatment with MMS and/or concentration of 8-azaguanine.

5.2 Characteristics of an induced 8-azaguanine resistant clone

5.21 derivation

The colony 65691, maintained in 10 µg and 30 µg aza/ml, was cloned six months after isolation. Fifty-one clones were picked from 10 µg aza/ml and eleven from 30 µg aza/ml. Those ten with the most vigorous growth pattern from each level of drug were chosen for plating efficiency in MENAFC10, 10 µg aza/ml and THAG, and for chromosome counts. In keeping with the parental colony

TABLE 15. Doubling time of azaguanine resistant colonies and CHWC1 in MENAFC10, 10 µg and 30 µg aza/ml*.

Colony	MENAFC10	10 µg aza	30 µg aza
65350	18	19	22
65171	17	17	33
65281	14	19	50±
65274	12	15	22
65691	15	20	46±
CHWC1	10-12	0	0

*See Appendix VI for details.

TABLE 16. Per cent diploidy and tetraploidy in azaguanine resistant colonies selected by 10 µg and 30 µg aza/ml in the absence of MMS.*

10 µg aza			30 µg aza		
colony	2n	4n	colony	2n	4n
65606	0	100	65171	57	43
65341	55	45	65221	55	45
65342	0	100	65222	71	29
65350	77	23	65281	57	43
65511	23	77	65301	20	80
65620	20	80	65652	31	69
65672	77	23	65791	51	49
65741	0	100			
65760	14	86			

*counts done at passage 3.

TABLE 17. Per cent diploidy and tetraploidy in azaguanine resistant colonies selected by 10 µg and 30 µg aza/ml after exposure to 10^{-3}M MMS.*

10 µg aza			30 µg aza		
colony	2n	4n	colony	2n	4n
65274	36	64	65691	59	41
65371	31	69	65711	77	23
65391	76	24			
65611	0	100			

*counts done at passage 3.

(Table 14) all clones showed single cell survival in MENAFC10 and in 10 µg aza/ml but not in THAG (Table 18). In both 65691 and its clones the plating efficiency in azaguanine was very near to that in MENAFC10.

Nine clones selected at 10 µg aza were diploid (60 - 88%) and one was 100% tetraploid (Table 19). Six clones isolated from 30 µg aza were diploid (78 - 100%), one was about 54% tetraploid and three were 100% tetraploid. The parental colony 65691 was 59% diploid (Table 17). However, 75% of the isolated clones were diploid with the diploid modal count in excess of 70% (Table 19). This does not necessarily represent a greater recovery of diploid cells on cloning 65691, since the clones were chosen on the basis of growth potential.

The modal chromosome number of the diploids was 22, with a minute telocentric marker, M_3, present in some clones. A clone designated 1102, arising from 10 µg aza selection was chosen for further characterization.

5.22 stability of resistance to 8-azaguanine

Cultures of 1102 were maintained in MENAFC10 and 10 µg aza/ml, and tested for stability of resistance after one month and six months in continuous culture (Table 20). The clone retained resistance to 8-azaguanine in the absence of the selective agent. The plating efficiency was not affected by the drug, 1102 grew equally in MENAFC10

TABLE 18. Plating efficiency in MENAFC10, 10 µg aza/ml and THAG, of clones of 65691 maintained in 10 µg and 30 µg aza/ml.

10 µg aza			30 µg aza		
MENAFC10	10 µg aza	THAG	MENAFC10	10 µg aza	THAG
*40.8	29.8	0	57.4	56.5	0
64.4	58.6	0	87.4	62.2	0
84.8	52.7	0	68.7	65.3	0
64.9	59.2	0	55.2	59.2	0
64.2	54.9	0	97.9	78.4	0
63.3	62.1	0	59.7	35.1	0
63.5	55.5	0	36.6	37.5	0
59.3	38.8	0	73.6	74.8	0
56.9	60.9	0	71.4	72.0	0
73.4	55.4	0	57.1	43.6	0

*clone 1102.

TABLE 19. Per cent diploidy and tetraploidy, and diploid chromosome number of clones of 65691 maintained in 10 µg aza and 30 µg aza/ml.

	10 µg aza			30 µg aza		
	2n	4n	no.	2n	4n	no.
*	85	15	22 (M_3)	85	15	21
	74	32	22	79	21	22
	88	12	22	100	0	22 (M_3)
	63	37	22/23	84	16	poor spreads
	75	29	22	82	18	22 (M_3)
	0	100	-	88	12	22
	60	40	22	0	100	-
	74	26	22/21	1	99	-
	79	21	22	0	100	-
	83	17	22	46	54	poor spreads

*clone 1102.

TABLE 20. Plating efficiency in MENAFC10, 10 µg aza/ml and THAG of 1102 maintained in MENAFC10 and in azaguanine.

Culture medium	MENAFC10	10 µg aza	THAG
MENAFC10*	40.1	33.5	0
Azaguanine	47.3	40.7	0
MENAFC10**	30.0	29.0	0
Azaguanine	36.1	37.3	0

*for 1 month.

**for 6 months.

and 10 µg aza/ml, and showed no growth in THAG. The actual plating efficiency had not altered from that when 1102 was first tested (Table 18).

5.23 cytotoxicity of 8-azaguanine

Azaguanine began to affect survival of 1102 at concentrations higher than 10 µg/ml, with minimal survival at 60 µg/ml of less than 0.5% (Fig. 7). In comparison with the plating efficiency of CHWC1, the toxic effect of the drug is markedly less severe on 1102 cells than it is on CHWC1.

5.24 doubling time

The doubling time was found to be 13 hours (Appendix VI). This is one hour more than for CHWC1 (12 hours) and two hours less than for 65691 (15 hours).

5.25 spontaneous reversion to 8-azaguanine sensitivity from 8-azaguanine resistance

Spontaneous reversion occurred at a frequency of 2×10^{-7} or less (Table 21). One colony was picked and maintained in MENAFC10 and in THAG. The cells were large and irregular. They grew very poorly and did not survive beyond 7 passages (about 5 weeks). The poor growth in mass culture was reflected in the low survival on single cell plating:

FIGURE 7. Cytotoxicity of 8-azaguanine on CHWC1 (o) and 1102 (●) (two experiments for 1102).

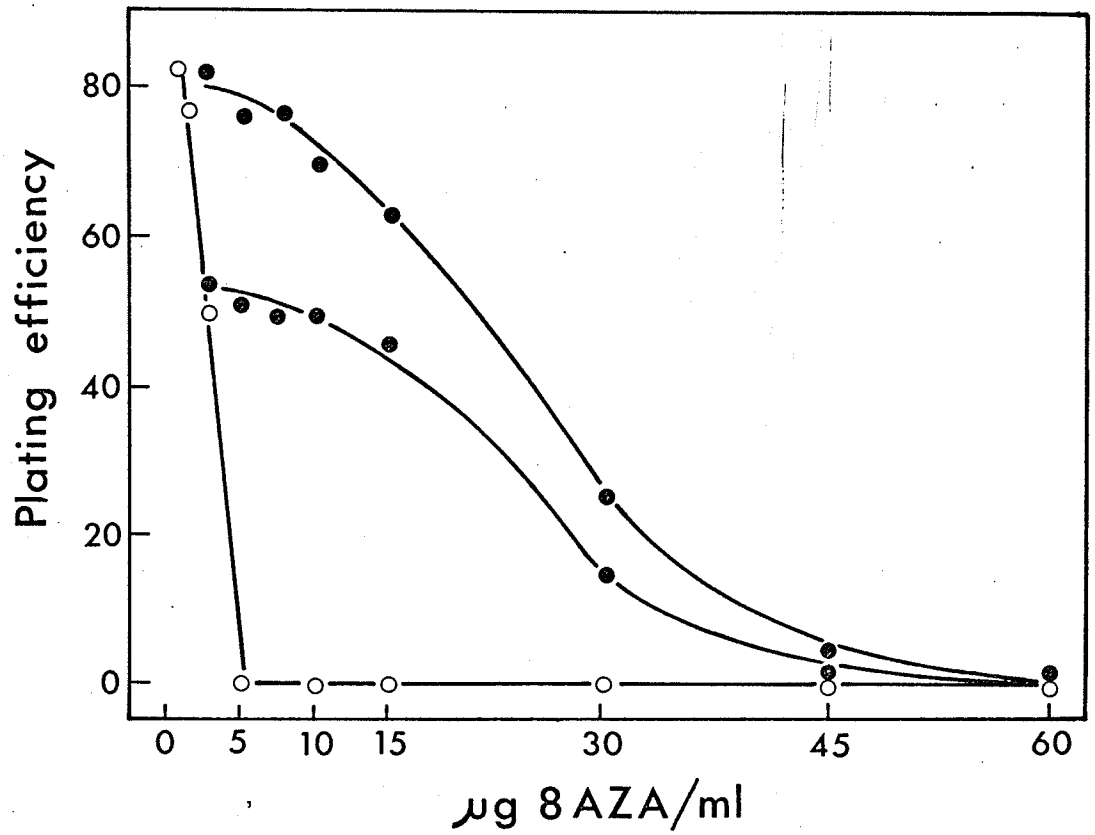

Plating efficiency
80
60
40
20
0
0
5
10
15
30
45
60
µg 8AZA/ml

TABLE 21. Total cells and reversion frequency of 1102 in THAG*

Total cells	Reversion frequency
10^6	0
6×10^6	0
10^7	0
10^7	1×10^{-7}
10^7	2×10^{-7}
10^7	0
2×10^7	0
2×10^7	5×10^{-8}
3×10^7	3×10^{-8}
6×10^7	0

*Cells were seeded at densities of 1.25×10^5 to 2×10^6/100 mm dish in THAG for 21 days, with medium changes every 3 or 4 days. The colonies observed were small and irregular in outline.

	Exp#1	Exp#2
MENAFC10	3.0	0
10 µg aza/ml	0	0
THAG	3.6	6.0

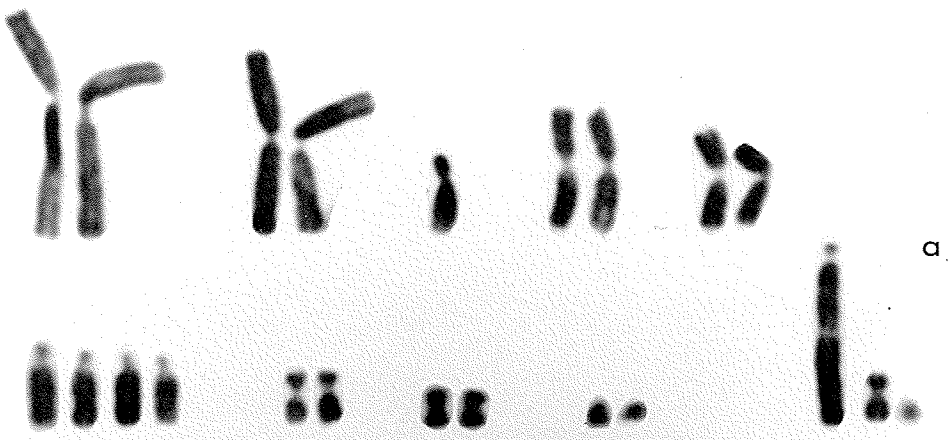
Colonies in MENAFC10 had at least 20 cells, whereas those in THAG contained 20 - 40 cells. There were large irregular cells scattered in most dishes containing each medium.

5.26 chromosome studies

The modal chromosome number for 1102 was 22. The karyotype was stable (Fig. 8) but slightly altered from that of CHWC1 (Section 3.2, Fig. 4). The variation occurred in the subtelocentrics, with one of the chromosomes missing but the additional marker (M_3) made up the modal number of 22. The frequency of diploid cells was 85% (Table 19).

Five metaphase spreads of 1102 "revertant" were analysed. Three had 22 chromosomes with a karyotype similar to 1102, the remaining two had 20 and 38 chromosomes.

FIGURE 8. 1102 karyotype.



a

6. Isolation and characterisation of twenty-eight spontaneously derived 8-azaguanine resistant colonies

The purpose of this section was to characterize spontaneous 8-azaguanine resistant colonies in an attempt to answer the following questions:

(a) is there more than one resistant phenotype,

(b) if so, do they form categories related to expression time and/or concentration of selecting agent, and

(c) can variants be distinguished from true mutants?

Parameters set out for measurement were: stability of resistance to 8-azaguanine after a number of generations in the absence of the selecting agent, cytoxicity of 8-azaguanine, doubling time in mass cultures maintained in MENAFC10 and 10 µg aza/ml, plating efficiency in THAG, complementation by cell fusion, and chromosome studies.

6.1 Isolation of resistant colonies

Twenty-eight colonies were picked from four independent mutation frequency experiments. Ten colonies were isolated after 10 µg aza/ml selection and 0 hours mutation expression time, fourteen after 10 µg/ml and 42 hours expression time and four after 30 µg/ml and 42 hours

hours expression time.

6.2 Growth responses of surviving colonies

6.21 stability of resistance

Each colony was maintained in parallel culture in MENAFC10 and 10 µg aza/ml for a number of generations. For each culture, plating efficiency in MENAFC10 and 10 µg aza/ml was determined on at least two occasions. Tables 22, 23 and 24 give the plating efficiency in 10 µg aza/ml relative to that in MENAFC10 at the beginning (P_1) and end (P_{10}) of a ten passage interval for each culture of each colony.

With the exception of four colonies, resistance to 8-azaguanine was retained in the absence of the selecting agent; twenty-one colonies grew as well in the drug as in MENAFC10, regardless whether the culture was maintained in MENAFC10 or azaguanine. The four colonies 518-17-01, 354-13-01, 354-17-01 and 418-06-01 showed reduced survival in 10 µg aza/ml after growing in nonselective medium. In 354-13-01 (Table 23) relative plating efficiency did not alter from one plating to the next, but was always lower in MENAFC10 cultures than in azaguanine cultures. 518-17-01 (Table 22), 354-17-01 and 418-06-01 (Table 23) demonstrated higher relative plating efficiency in MENAFC10 cultures at the first plating (P_1) than in the final plating (P_{10}), suggesting a gradual loss of resistance. 354-17-01 also illustrated decreased single cell plating for the azaguanine

TABLE 22. Relative plating efficiency in 10 µg aza/ml of colonies selected by 10 µg aza/ml and 0 hours mutation expression time.

Colony	Culture in MENAFC10		Culture in 10 µg aza	
	P_1	P_{10}	P_1	P_{10}
354-08-01	119	103	120	121
354-08-02	202	106	151	105
354-08-03	109	110	107	112
354-08-04	108	105	100	100
354-08-05	95	93	108	100
354-08-08	108	75	112	62
518-04-01	73	100	80	103
518-12-01	77	96	83	87
518-14-01	86	87	82	100
518-17-01	119	67	120	106

P_1 beginning of parallel cultures in MENAFC10 and 10 µg aza

P_{10} after at least 10 passages in MENAFC10 and 10 µg aza

TABLE 23. Relative plating efficiency in 10 µg aza/ml of colonies selected by 10 µg aza/ml and 42 hours mutation expression time.

Colony	Culture in MENAFC10		Culture in 10 µg aza	
	P_1	P_{10}	P_1	P_{10}
354-02-01	118	93	100	94
354-02-02	99	75	113	105
354-02-03	83	98	77	108
354-04-01	65	94	65	96
354-12-01	53	115	70	128
354-13-01	62	68	100	109
354-13-02	133	105	117	117
354-17-01	96	20	98	51
354-17-04	67	98	83	141
418-02-01	106	90	107	101
418-06-01	108	4	47	97
418-16-01	0	95	41	0
418-16-02	107	89	95	85
418-16-03	101	99	105	94

P_1 beginning of parallel cultures in MENAFC10 and 10 µg aza

P_{10} after at least 10 passages in MENAFC10 and 10 µg aza

TABLE 24. Relative plating efficiency in 10 µg aza/ml of colonies selected by 30 µg aza/ml and 42 hours mutation expression time.

Colony	Culture in MENAFC10		Culture in 10 µg aza	
	P_1	P_{10}	P_1	P_{10}
418-28-01	116	115	108	111
533-02-01	131	84	98	108
533-13-01	110	92	114	107
533-15-01	85	82	70	69

P_1 beginning of parallel cultures in MENAFC10 and 10 µg aza

P_{10} after at least 10 passages in MENAFC10 and 10 µg aza

culture, whereas 418-06-01 demonstrated increased plating for the azaguanine culture.

Colony 418-16-01 (Table 23) could not be maintained in the drug for more than one passage. However, when plated into 10 µg aza/ml from MENAFC10, the relative plating efficiency was 0 at the first plating, but 95 at the final plating.

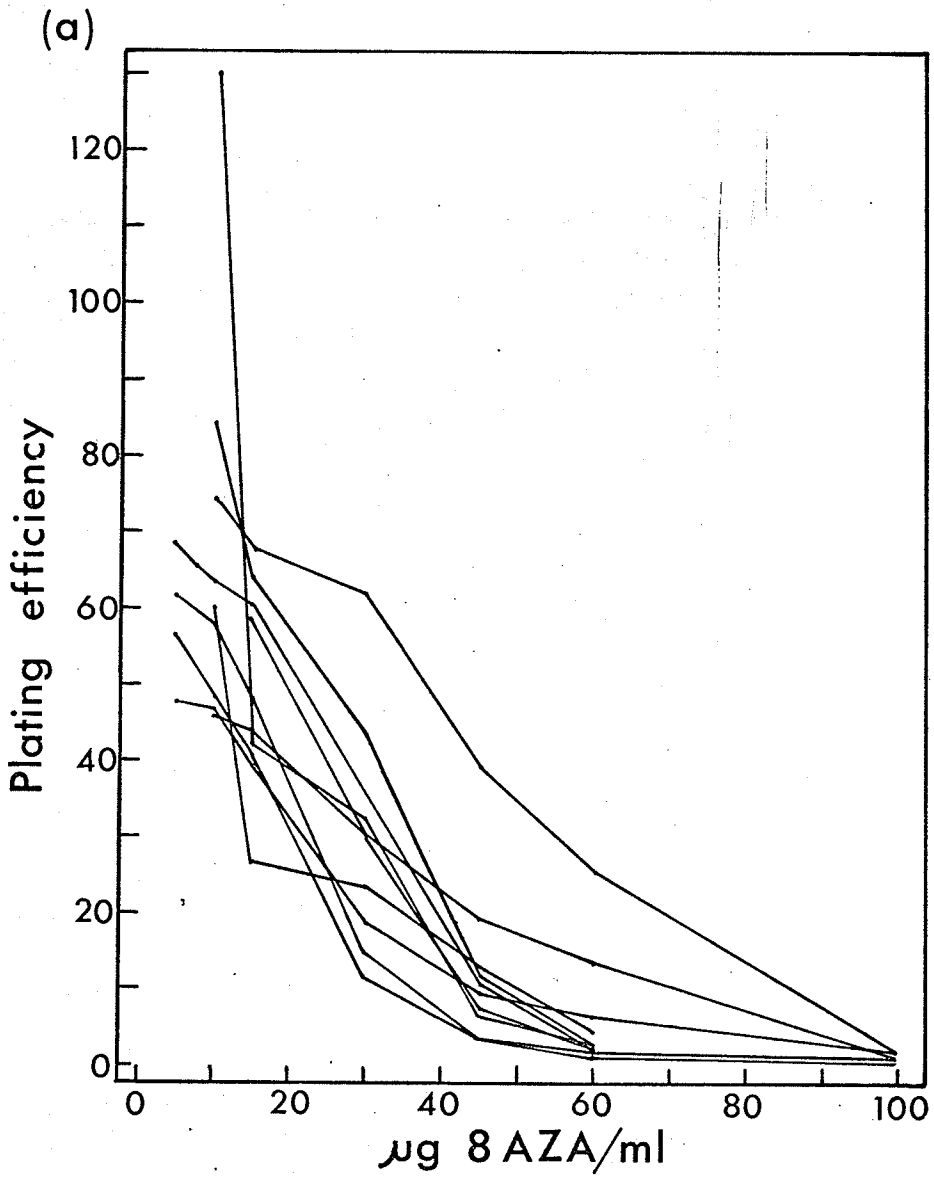
6.22 cytotoxicity of 8-azaguanine

All colonies were exposed to a range of azaguanine concentrations up to 100 µg/ml in plating efficiency experiments. Figure 9 depicts a typical survival curve for each colony, carried out on cultures as near as possible to the 8th-10th passage in 10 µg aza/ml.

With the exception of four colonies 354-02-01, 418-02-01, 418-16-02 (Fig. 9(b)) and 418-28-01 (Fig. 9(c)), all showed similar survival curves, a decrease in survival occurring between 5-10 µg aza/ml, and with minimal survival (less than 2%) at 100 µg aza/ml. These four colonies on initial testing showed extended shoulder regions. The plating efficiencies did not begin to decrease until after 45 µg aza/ml, and 12 to 34% of the cells survived in 100 µg aza/ml. However on further study of these four, only one, 354-02-01, retained the extended shoulder effect with high survival in 100 µg aza/ml. The three others, 418-16-02, 418-02-01 and 418-28-01, assumed a response, characteristic of the other

FIGURE 9. Plating efficiency of resistant colonies in increasing concentrations of 8-azaguanine/ml.

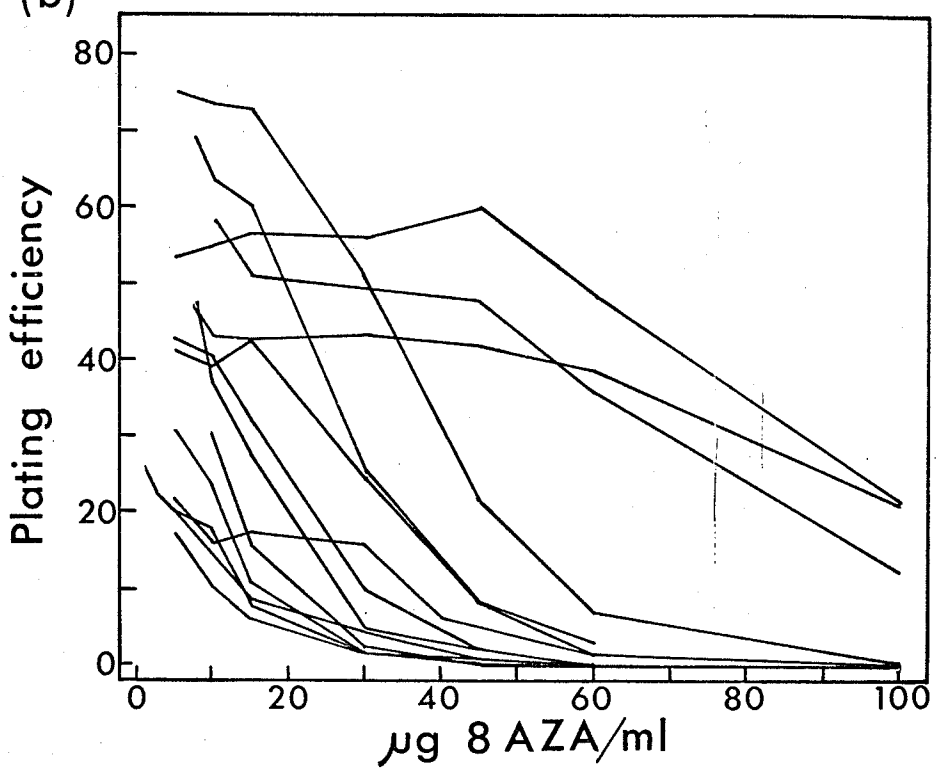
(a) Colonies selected at 10 μg aza/ml, 0 mutation expression time.


(a)
Plating efficiency
120
100
80
60
40
20
0
0
20
40
60
80
100
µg 8AZA/ml

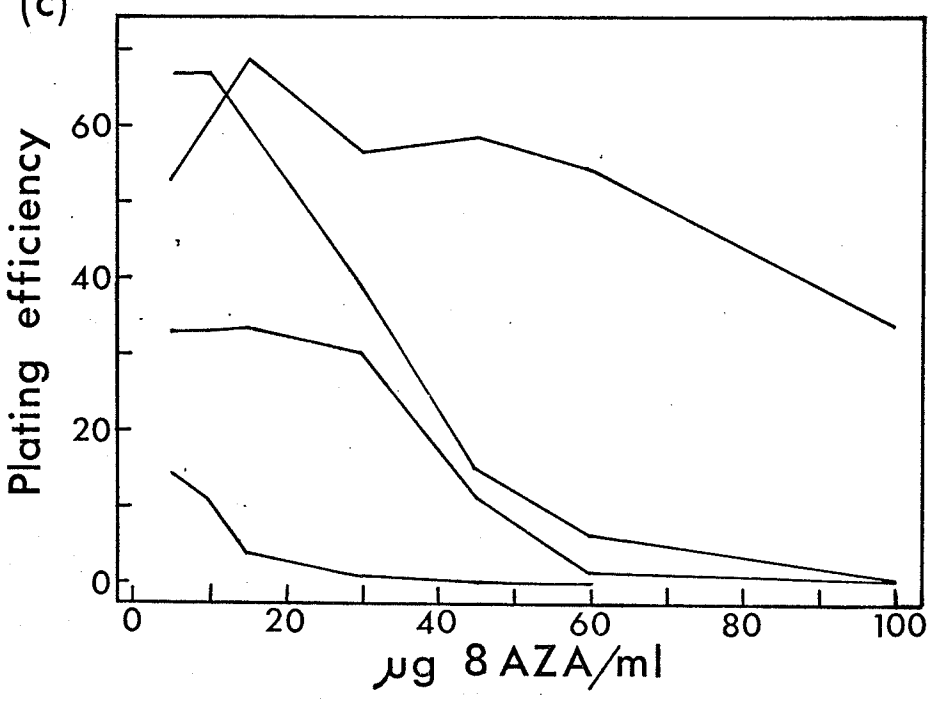
(b) Colonies selected at 10 µg aza/ml, 42 hours mutation expression time.

(c) Colonies selected at 30 µg aza/ml, 42 hours mutation expression time.

(b)


Plating efficiency
80
60
40
20
0
0
20
40
60
80
100
µg 8 AZA/ml

(c)


Plating efficiency
60
40
20
0
0
20
40
60
80
100
µg 8 AZA/ml

colonies.

One other colony deserves mention, 418-16-01, selected by 10 µg aza/ml after 42 hours. At first the cells grew in azaguanine, and at passage 9 the cytotoxicity of the drug was similar to the response of the majority of colonies. However, six passages later the colony could no longer be maintained in 10 µg aza/ml. A cytotoxicity experiment was carried out with the MENAFC10 culture, and the plating efficiency was:

MENAFC10	54.0
THAG	61.3
5 µg aza/ml	9.5
10 µg aza/ml	58.5
15 µg aza/ml	0.2

In all experiments there was a noticeable decrease in the diameter of the colonies with increasing azaguanine concentration, beginning at about 10 µg/ml (Fig. 10). Colonies with less than 20 cells were not counted.

6.23 doubling time

Doubling time in mass culture was calculated at each subculture, for a number of passages, for cells maintained in MENAFC10 and 10 µg aza/ml. The average doubling time for each colony is given in Tables 25, 26 and 27. The overall average doubling time was 17.1 hours in MENAFC10 and 20.4 hours in azaguanine for colonies selected at

FIGURE 10. Colony size in plating efficiency of resistant colonies in MENAFC10 and 8-azaguanine (from top to bottom, MENAFC10, 5 µg aza/ml, 10 µg aza/ml, 15 µg aza/ml).

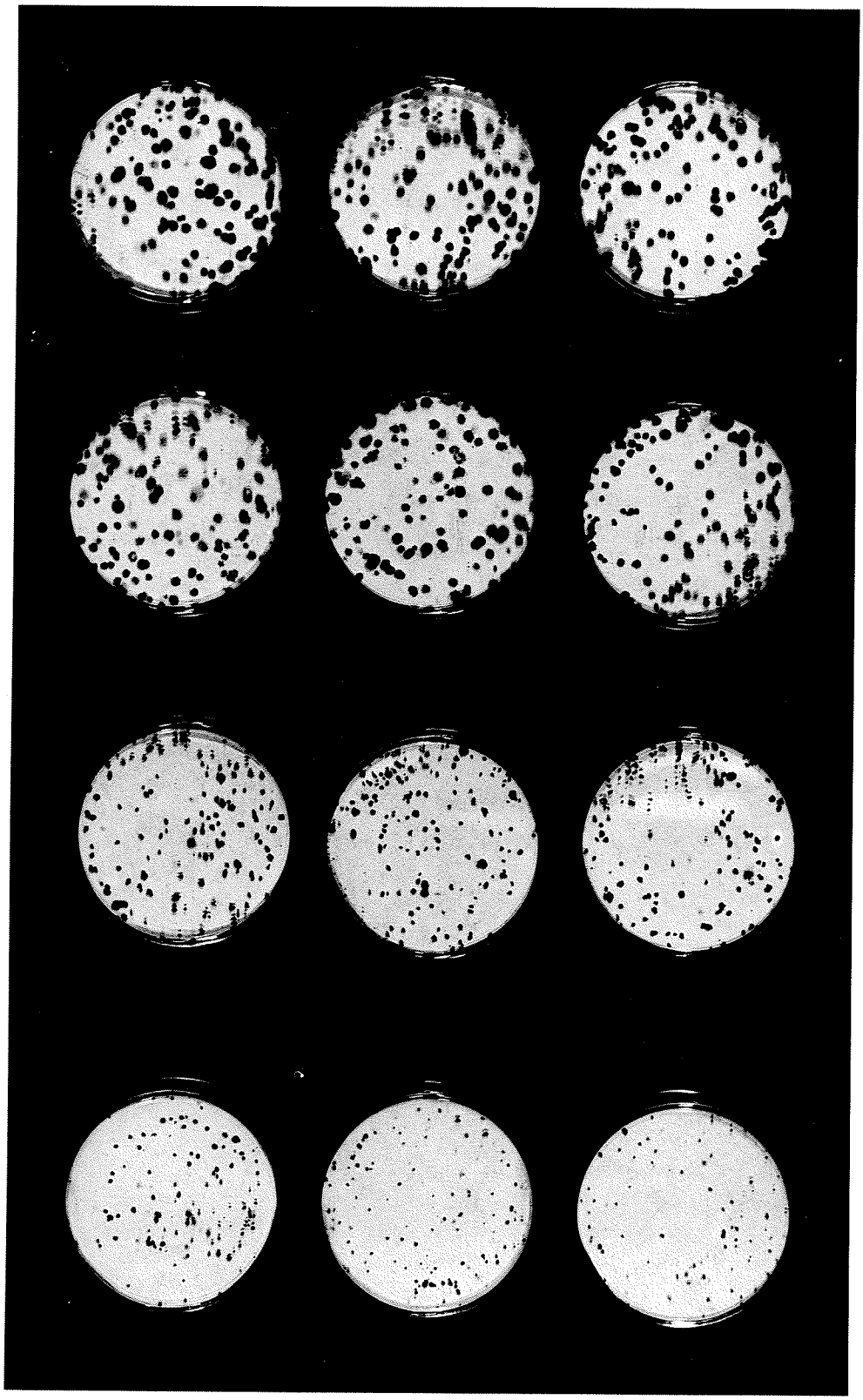


TABLE 25. Doubling time (hours) in mass culture in MENAFC10 and azaguanine of colonies selected at 10 µg aza/ml, 0 expression time.*

Colony	MENAFC10	Azaguanine
354-08-01	16.4	16.2
354-08-02	16.5	18.3
354-08-03	14.9	17.1
354-08-04	18.5	21.3
354-08-05	18.5	20.3
354-08-08	16.2	19.7
518-04-01	17.7	21.4
518-12-01	17.8	23.3
518-14-01	17.6	22.2
517-17-01	17.4	23.5

*See Appendix VII for calculations.

TABLE 26. Doubling time (hours) in mass culture in MENAFC10 and azaguanine of colonies selected by 10 µg aza/ml, 42 hours expression time.*

Colony	MENAFC10	Azaguanine
354-02-01	16.4	18.8
354-02-02	19.8	23.7
354-02-03	30.2	38.3
354-04-01	20.4	25.3
354-12-01	17.3	20.7
354-13-01	29.7	31.0
354-13-02	32.5	29.5
354-17-01	15.5	17.3
354-17-04	20.8	23.9
418-02-01	16.6	19.9
418-06-01	20.0	31.1
418-16-01	17.9	--
418-16-02	16.1	18.6
418-16-03	18.3	20.4

*See Appendix VIII for calculations.

TABLE 27. Doubling time (hours) in mass culture in MENAFC10 and azaguanine of colonies selected by 30 µg aza/ml, 42 hours expression time.*

Colony	MENAFC10	Azaguanine
418-28-01	17.4	20.9
533-02-01	21.9	21.9
533-13-01	15.9	18.9
533-15-01	25.0	26.6

*See Appendix IX for calculations.

10 µg aza/ml and 0 hours mutation time (Table 25), 20.8 and 24.5 for colonies selected at 10 µg aza/ml, 42 hours mutation time (Table 26) and, 20.1 and 22.1 for colonies selected at 30 µg aza/ml, 42 hours mutation time (Table 27). Colonies selected at 10 µg aza/ml and 0 hours mutation expression time demonstrated slightly shorter doubling time in both media when compared to that of colonies selected after 42 hours mutation expression time.

6.24 plating efficiency in THAG

Cells resistant to 8-azaguanine and concomitantly defective in HGPRT are not expected to grow in THAG unless reversion occurs, i.e., loss of resistance and recovery of active HGPRT. Thus, THAG is a selective medium for revertants in which single cell survival determination gives a measure of the revertibility of a cell population. In Tables 28, 29 and 30, plating efficiency in THAG relative to that in MENAFC10 is listed for each colony cultured in MENAFC10 and 10 µg aza/ml. Plating efficiencies were determined at least twice; once soon after isolation (P_1) and again at the termination of the study on each colony (P_{10}).

Three obvious categories of response to THAG were apparent: no survival (0), minimal growth (< 30) and maximal growth (> 50). A small fourth category existed of colonies that demonstrated irregular growth response. The

TABLE 28. Relative plating efficiency in THAG of colonies selected at 10 µg aza/ml, 0 expression time.

Colony	Cultured in MENAFC10		Cultured in 10µg aza	
	P_1	P_{10}	P_1	P_{10}
354-08-01	27	0.5	28	21
354-08-02	5	+	21	1
354-08-03	3	3	6	8
354-08-04	1	2	3	3
354-08-05	2	2	3	5
354-08-08	13	+	14	3
518-04-01	+	1	4	1
518-12-01	3	1	1	1
518-14-01	5	4	3	2
518-17-01	8	0.2	14	4

P_1 beginning of parallel cultures in MENAFC10 and 10 µg aza

P_{10} after at least 10 passages in MENAFC10 and 10 µg aza

\+ scattered cells

TABLE 29. Relative plating efficiency in THAG of colonies selected at 10 µg aza/ml, 42 hours expression time.

Colony	Cultured in MENAFC10		Cultured in 10 µg aza	
	P_1	P_{10}	P_1	P_{10}
354-02-01	0	0	0	0
354-02-02	114	116	104	100
354-02-03	52	108	91	113
354-04-01	101	147	102	115
354-12-01	79	110	99	122
354-13-01	112	110	30	74
354-13-02	79	100	62	131
354-17-01	106	153	105	15
354-17-04	115	135	112	248
418-02-01	0	0	0	0
418-06-01	30	129	0	91
418-16-01	109	114	132	–
418-16-02	0	+	0	+
418-16-03	62	8	31	0

P_1 beginning of parallel cultures in MENAFC10 and 10 µg aza

P_{10} after at least 10 passages in MENAFC10 and 10 µg aza

\+ scattered cells

TABLE 30. Relative plating efficiency in THAG of colonies selected at 30 µg aza/ml, 42 hours expression time.

Colony	Cultured in MENAFC10		Cultured in 10 µg aza	
	P_1	P_{10}	P_1	P_{10}
418-28-01	0	0	0	0
533-02-01	0	0	0	0
533-13-01	0	0	0	0
533-15-01	79	88	103	80

P_1 beginning of parallel cultures in MENAFC10 and 10 µg aza

P_{10} after at least 10 passages in MENAFC10 and 10 µg aza

colonies are grouped according to these categories in Table 31.

6.3 Complementation

Of the twenty-eight colonies resistant to 8-azaguanine, only six were suitable for complementation because of the limitation of the selection system THAG for isolating complementary heterokaryons. The results of two separate experiments are summarized in Table 32. In the first experiment, colonies 354-02-01, 418-02-01, 418-16-02, 418-28-01, 1102 and B_{82} were used for making crosses. The second experiment had in addition colonies 533-02-01 and 533-13-01 but excluded 418-16-02.

All Chinese hamster colonies formed complementary heterokaryons with B_{82}, as expected. Results of the first experiment are lower than those of the second. A variable pattern of results was also observed in other B_{82} x 1102 fusion experiments (Gee, unpublished data). Variability has been noticed to occur from batch to batch of virus, and also among vials of virus from the same lot number.

Colonies were observed in some crosses between Chinese hamster resistant lines, but the frequency never exceeded 6×10^{-6} and it is unlikely that these colonies represented heterokaryons.

6.4 Chromosome studies

The estimated percentage of diploidy and

TABLE 31. Colonies typed according to relative plating efficiency in THAG.

0	< 30	> 50	Variable
354-02-01	354-08-01	354-02-02	354-13-01
418-02-01	354-08-02	354-02-03	354-17-01
418-16-02	354-02-03	354-04-01	418-06-01
418-28-01	354-08-04	354-12-01	418-16-03
533-02-01	354-08-05	354-13-02	
533-13-01	354-08-08	354-17-04	
	518-04-01	418-16-01	
	518-12-01	533-15-01	
	518-14-01		
	518-17-01		

TABLE 32. Average number of colonies per 10^5 cells in THAG resulting from cell fusion between 8-azaguanine resistant colonies and between these colonies and a BUdR resistant mouse line, B_{82}.

	354-02-01	418-02-01	418-16-02	418-28-01	533-02-01	533-13-01	1102	B_{82}
354-02-01	0 0.2	0 0	0.1 -	0 0	- 0.2	- 0.2	0.1 0	5.9 65.2
418-02-01		0 0	0 -	0 0	- 0.4	- 0	0 0	2.2 55.8
418-16-02			0.2	0.1	-	-	0.2	5.5
418-28-01				0 0	- 0	- 0	0 0	5.4 63.5
533-02-01					0	0	0.6	93.5
533-13-01						0	0.2	4.2
1102							0 0	3.5 13.4
B_{82}								0 0

tetraploidy and karyotypic analyses have been summarized in Tables 33 and 34.

The twenty-eight colonies are grouped according to relative plating efficiency in THAG. Percentage ploidy has been narrowed into three divisions: 70-100% diploid as 100-2n, 69-30% diploid as 50-2n, and 8-0% diploid as 100-4n. With the exception of three colonies in the last group, all the remainder were 100% tetraploid. Two karyotypes were distinguished, those unchanged from CHWC1 and the remainder showing chromosome alterations.

Fourteen colonies were tetraploid, of which five showed exact duplications of the modal karyotype of CHWC1 and nine demonstrated chromosome changes. Eight colonies were approximately 50% diploid and only one of these had a karyotype similar to CHWC1. The remaining six colonies were diploid with the mode in excess of 70%, one had the same chromosome constitution as CHWC1. In total, seven colonies showed no chromosome change from the karyotype of CHWC1, while the remaining twenty-one did so, although in most of the diploid colonies the number of chromosomes was 22.

There were two commonly occurring altered karyotypes. One was similar to that of 1102 in which one subtelocentric chromosome was absent but an additional marker (M_3) was present (Figs. 11(a) and (b)). The other altered karyotype showed a deletion in a chromosome number 4 or 5 (Figs. 11(c) and (d)). 418-16-03 (Fig. 11(e)) had both alterations, while

TABLE 33. Summary of chromosome studies on 8-azaguanine resistant colonies with a karyotype similar to CHWC1.

PE in THAG	100-2n	50-2n	100-4n
0	418-16-02		354-02-01
< 30			354-08-01 354-08-03 354-08-05
> 50		354-02-02	354-12-01

TABLE 34. Summary of chromosome studies on 8-azaguanine resistant colonies with altered karyotypes.

PE in THAG	100-2n	50-2n	100-4n
0	533-02-01	418-02-01* 533-13-01*	418-28-01*
< 30	518-04-01* 518-12-01* 518-14-01*		354-08-02** 354-08-04** 354-08-08* 518-17-01*
> 50		354-02-03* 354-13-02* 533-15-01	354-04-01* 354-17-04** 418-16-01**
"irregular"	354-17-01**	418-06-01* 418-16-03*,**	354-13-01**

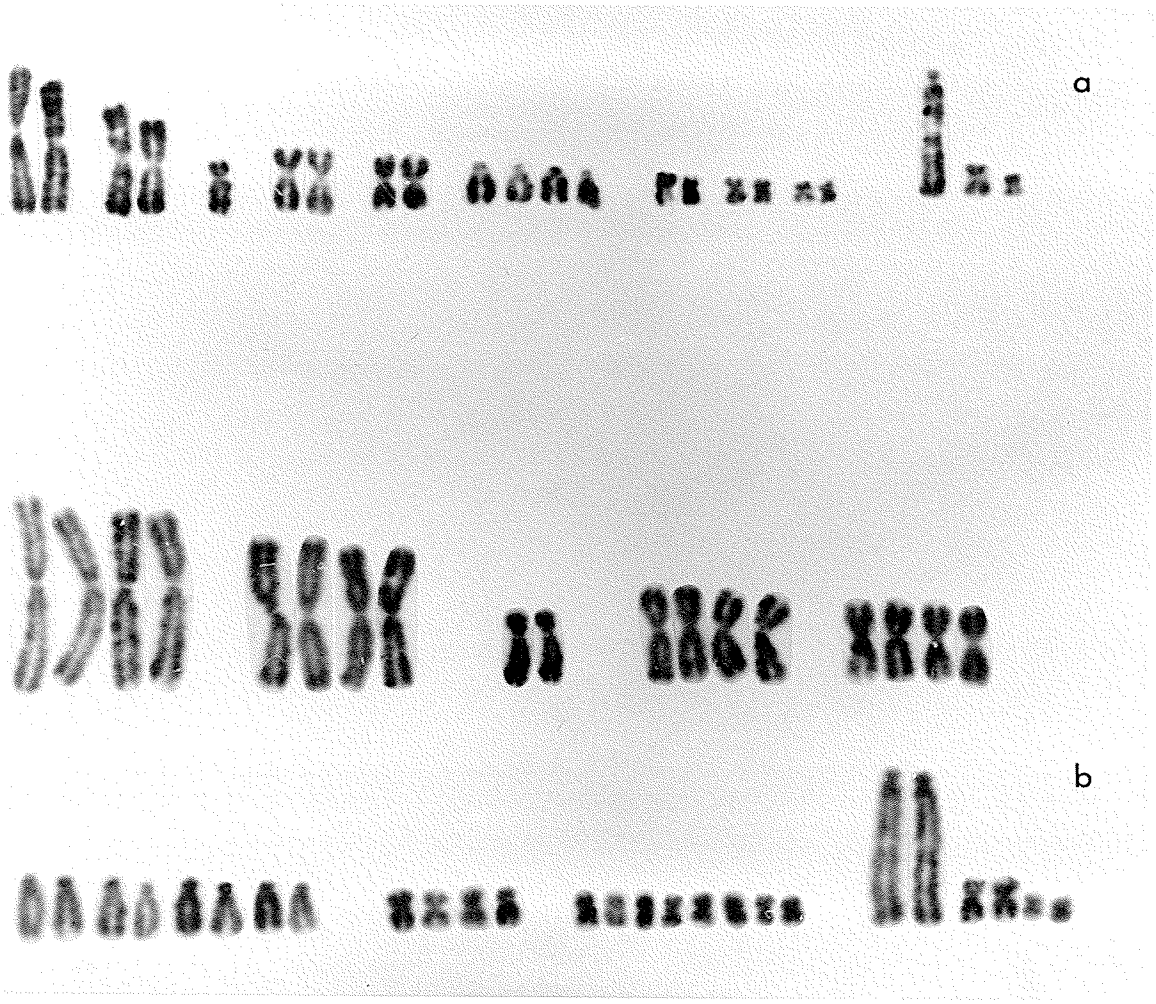
* similar to 1102

** deletion or absence of no. 4 or 5 submetacentric chromosome.

FIGURE 11. Karyotypes of 8-azaguanine resistant colonies.

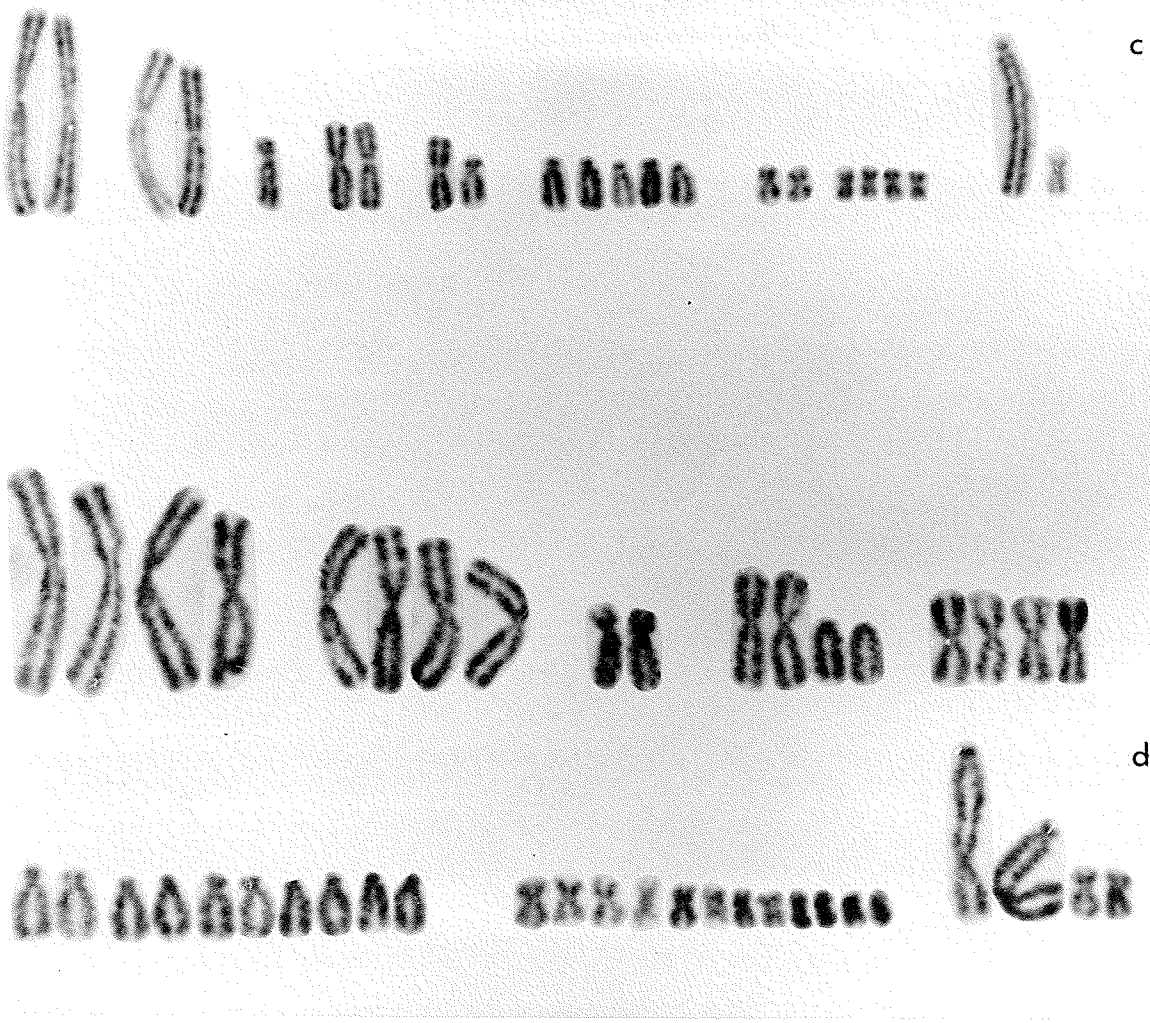
(a) 418-02-01

(b) 418-28-01



a

b



c

d

(e) 418-16-03

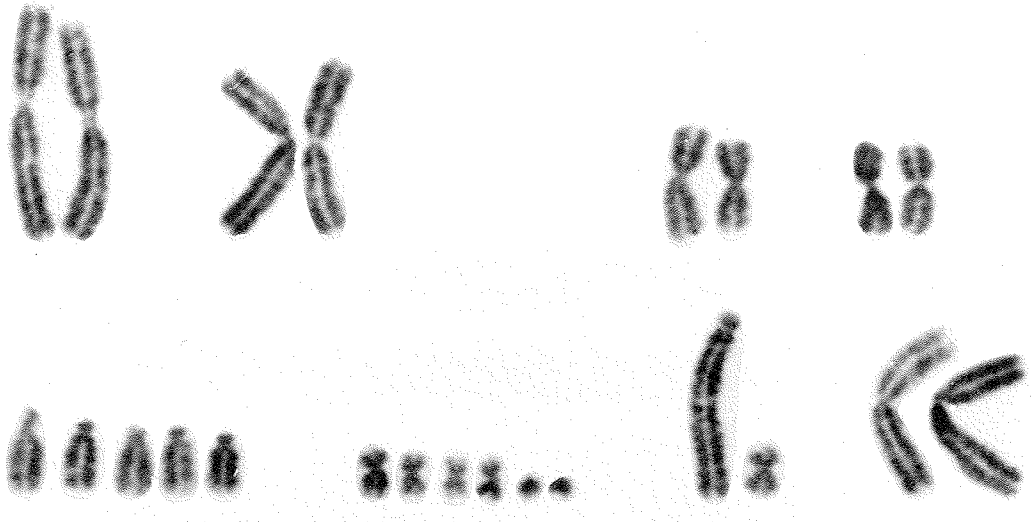
(f) 418-16-01

e

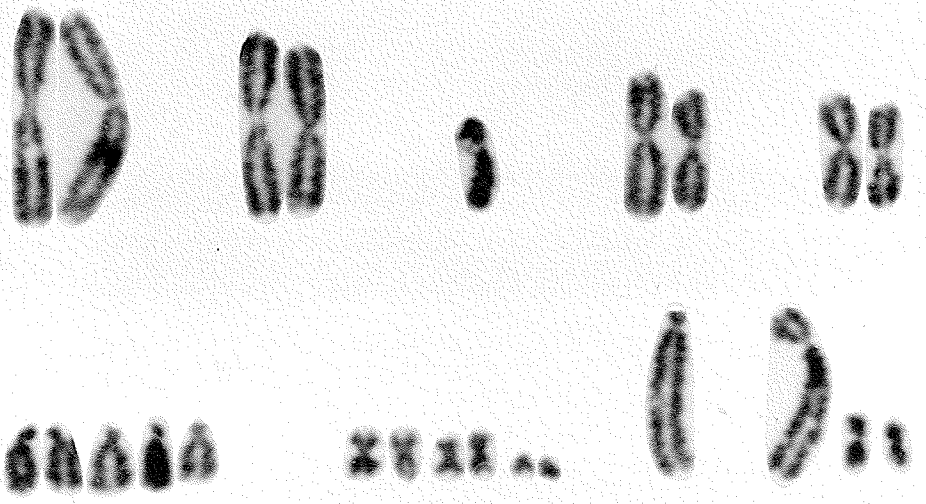
f

(g) 533-02-01

(h) 533-15-01



g



h

7. Comparison of the frequency of spontaneously derived 8-azaguanine resistant colonies from CHWC1 with that from a tetraploid subline.

A high proportion of the spontaneously derived 8-azaguanine resistant colonies were tetraploid. Fourteen of the twenty-eight were 100% tetraploid while eight others had a higher tetraploid complement than CHWC1.

The objective was to obtain a tetraploid cell line from CHWC1 and to determine if the frequency of resistant colonies from it differed from that from CHWC1.

The tetraploid subline CHWC1-06 was obtained from CHWC1 by colcemid treatment (Cox and Puck, 1969).

7.1 Characteristics of the tetraploid CHWC1-06

7.11 growth responses

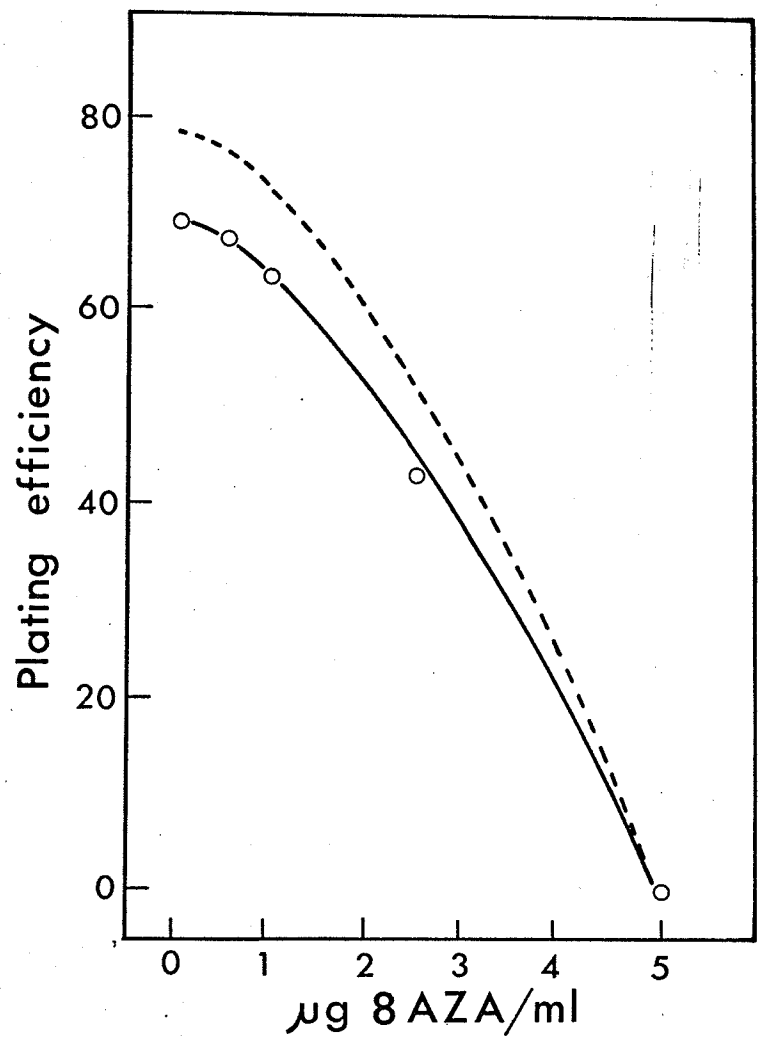
The cytotoxicity of 8-azaguanine to CHWC1-06 was not different from that to CHWC1, in fact the survival curves overlapped (Figure 12).

The plating efficiency of CHWC1-06 in MENAFC10 and in THAG was 83.7% and 87.5% respectively, which was comparable to that observed for CHWC1.

7.12 chromosome studies

Immediately after isolation the chromosome count was 44 ± 1. 83% were exactly 44 and had a duplication of

FIGURE 12. Cytotoxicity of 8-azaguanine to CHWC1-06 (——) and CHWC1 (---).


Plating efficiency
80
60
40
20
0
0
1
2
3
4
5
µg 8AZA/ml

the CHWC1 diploid karyotype (Figure 13). On the fourth passage, ten cells were analyzed, all were of the same karyotype as seen in Figure 13.

The chromosome counts of both cell types used in the azaguanine selection experiments remained unchanged from their initial descriptions.

7.2 Estimation of mutation rates

Two experiments were set up using both the diploid and tetraploid cell lines. The total number of colonies and mutation rates are given in Tables 35 and 36, respectively. No resistant colonies were recorded from CHWC1-06 (Table 35).

The mutation rates calculated for CHWC1 in these two experiments (Table 36) were comparable to those recorded for other experiments (Sec. 4.3, Table 10).

FIGURE 13. CHWC1-06 karyotype.

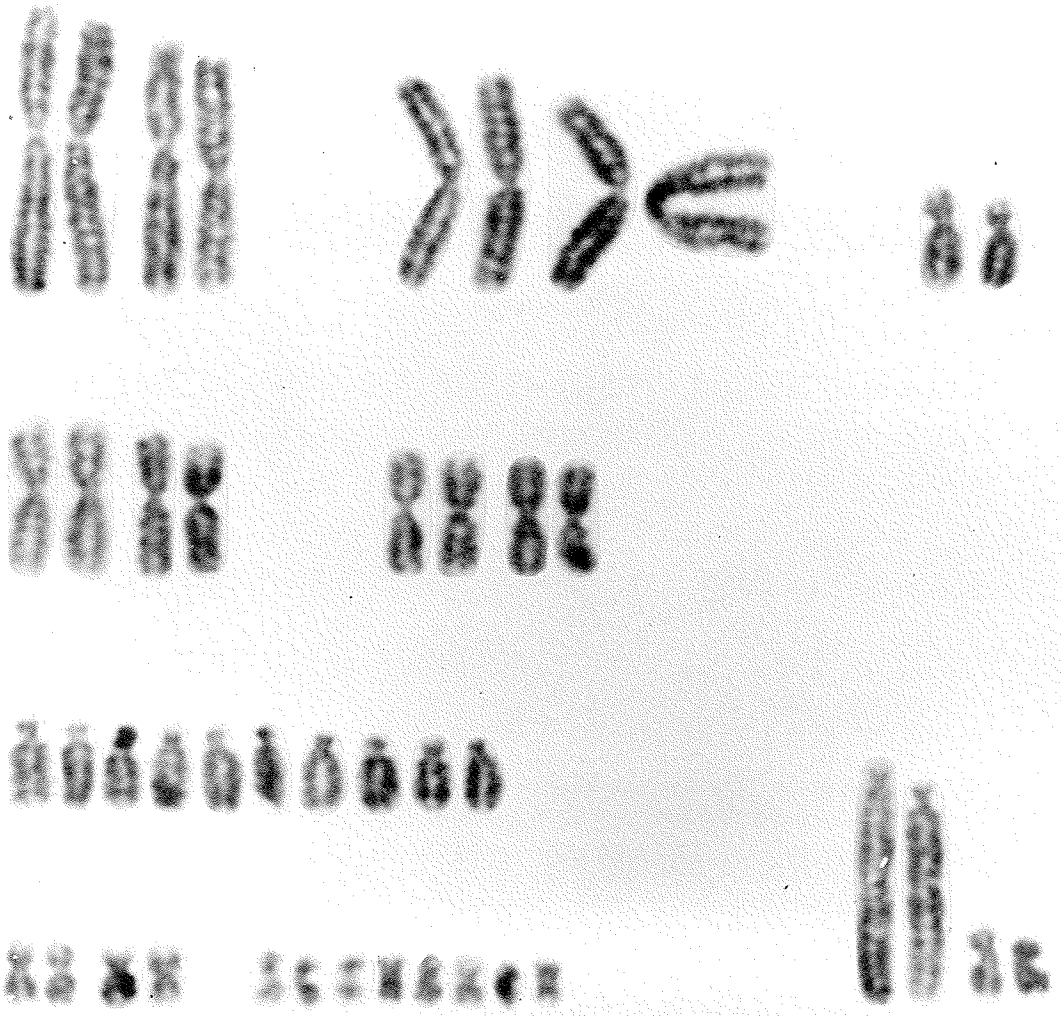


TABLE 35. Total number of resistant colonies in CHWC1-06 and CHWC1 with selection at 10 µg/ml and 30 µg aza/ml after 0 and 42 hours expression time.

Cells	10 µg aza		30 µg aza	
	0 hrs.	42 hrs.	0 hrs.	42 hrs.
CHWC1-06	0	0	0	0
CHWC1	51	107	0	0
CHWC1-06	0	0*	0	0
CHWC1	1	137	0	5

*Two small foci of cells were noticed before fixation but were less than the minimal size requirement for scoring. They were picked and dispersed in 10 µg aza/ml. One colony did not show any attached cells, the other responded but did not survive beyond the fourth passage (25 days in culture). Ten metaphase spreads were counted, the modal chromosome number was 41, with a range of 37 to 43.

TABLE 36. Mutation rate for 8-azaguanine resistance in CHWC1 with selection at 10 µg and 30 µg aza/ml after 0 and 42 hours expression time.

10 µg aza		30 µg aza	
0 hrs.	42 hrs.	0 hrs.	42 hrs.
1.58×10^{-5}	1.26×10^{-5}	-	-
1.41×10^{-6}	2.55×10^{-5}	-	2.48×10^{-6}

8. Discussion

characteristics of CHWC1

The Chinese hamster cell line CHWC1 proved to possess those qualities desired for quantitative and qualitative experiments in vitro. It demonstrated a consistent and vigorous growth capacity in mass culture, with a generation time of less than twelve hours. This is very similar to the times (ten and twelve hours) reported for Chinese hamster cell lines CHO and V_{79} (Kao and Puck, 1967; Chu and Malling, 1968). The high plating efficiency in standard medium MENAFC10 was also observed in THAG medium. However, there was a lower recovery of cells in mass cultures maintained in THAG to that in MENAFC10. This implies that cell survival was not impaired by THAG but rather that the cell cycle was extended. It is probable that aminopterin, since it is an antimetabolite, retarded cell growth by blocking several metabolic pathways. CHWC1 was intolerant to 8-azaguanine at concentrations higher than 1 µg/ml, a similar observation to that made by Chu and Malling (1968) on the cell line V_{79}.

CHWC1 is pseudodiploid with only little alteration from the normal karyotype of the Chinese hamster (Hsu and Zenzes, 1964). There are two chromosomes missing which are

replaced by two marker chromosomes. The cell lines CHO and V_{79} exhibit even greater changes from the normal karyotype. CHO has a modal number of 21 with ten chromosomes missing but having nine others not present in the normal karyotype (Kao and Puck, 1967). V_{79} has a modal number of 23 with seven missing and eight new chromosomes (Yu and Sinclair, 1964). The level of diploidy in CHWC1 cultures remained in excess of 80% throughout prolonged standardized routine culturing. The stable karyotype, only marginally changed from the normal, and the consistent growth responses make CHWC1 a line of considerable value for somatic cell genetics.

selection of azaguanine-resistant colonies

The method used to select azaguanine-resistant cells was described by Chu and Malling (1968). They observed that maximum mutation frequencies were obtained with cell densities of 1.25×10^5 per 100 mm dish, and with a mutation expression time of forty-two hours. They used the line V_{79}, and since CHWC1 demonstrated some similarity in characteristics, essentially the same experimental conditions were used.

induced mutation frequency and rate

The mutagens methyl methanesulfonate (MMS) and N-methyl-N'-nitro-N-nitrosoguanidine were used to induce resistance in CHWC1 cells to 8-azaguanine. The yield of resistant colonies was seen to increase after exposure to

MMS but not to MNNG. Exposure to 10^{-3}M MMS increased the frequency of cells resistant to 10 µg aza/ml from twenty to fifteen hundred-fold over that for no mutagen treatment. This wide range of the estimated frequencies could in part be accounted by the variability of cell survival after MMS treatment. The highest mutation frequency was observed when the plating efficiency after 10^{-3}M MMS exposure was 0.25%, whereas the lowest frequency occurred when the plating efficiency was 6.05%. By assuming a 0.25% plating efficiency for the latter, the calculated increase would be five hundred-fold over the control rather than the twenty-fold observed. Thus, variability of mutation frequencies from experiment to experiment may have arisen from the variability of cell survival following mutagen treatment. This variation in cell survival may have resulted from the instability of the mutagen, since the frequencies recorded for no mutagen treatment with selection at 10 µg aza/ml, were very much less variable, 2.1×10^{-5} to 6.0×10^{-6} as compared to 6.7×10^{-3} to 0.2 for 10^{-3}M MMS.

Another factor affecting the mutation frequency of azaguanine-resistant colonies included the concentration of the selecting agent. The number of resistant colonies selected at 30 µg/ml was observed to be lower than that with selection at 10 µg/ml. It is possible that some colonies would have been resistant at 10 µg but were not resistant at 30 µg. A lower frequency of resistant colonies

was similarily reported by Chu and Malling (1968) when they increased the concentration of the selecting agent.

The calculated mutation rate for azaguanine resistant cells indicated the same overall trend as did the frequency of resistant colonies. There was an increase in the mutation rate after mutagen treatment and the concentration of the selecting agent also affected the rate which was slightly higher for 10 µg/ml than for 30 µg/ml.

MNNG at four different concentrations failed to increase the incidence of resistant colonies. Chu and Malling (1968) reported a frequency of 132.9×10^{-5} after 10^{-5}M MNNG in contrast to 7.0×10^{-5} in the absence of the mutagen. Whereas under comparable conditions here, there was no appreciable difference in the frequencies, which were 20×10^{-5} and 55.4×10^{-5} respectively. It has been suggested by Orkin and Littlefield (1971) that it is the breakdown products of MNNG that are responsible for mutagenesis and not MNNG itself. Similarily, Cerdá-Olmedo and Hanawalt (1968) recorded a breakdown product, diazomethane to be the active agent in nitrosoguanidine mutagenesis and lethality. In the experiments of this study, the cells were exposed to MNNG for only one hour, with light and pH conditions controlled during preparation of dilutions and during the incubation period of one hour. All procedures were completed within the 90 minute half life of the drug. Therefore MNNG exerted only a cytotoxic

effect and did not have a remarkable influence on increasing the number of resistant colonies. This is not to say that MNNG is not mutagenic. Freese (1963) and Drake (1969) point out that different mutagens have different specific sites within the region of a gene, bringing about different mutations. It is not known whether the 8-azaguanine resistant colonies observed, with and without MNNG treatment formed a homogeneous population. Cerdá-Olmedo et al. (1968) showed that MNNG induces mutations in bacteria only during DNA replication and then mainly at the replicating fork, resulting in transitions and transversions but no frameshifts. Barranco and Humphrey (1971) using the Chinese hamster line CHO, demonstrated at least two populations of cells with different sensitivities to MNNG; cells entering into S phase being the more sensitive. On the other hand Orkin and Littlefield (1971) using the Syrian hamster line BHK, showed MNNG mutagenesis to be independent of the position of the cell in the cell cycle.

It is apparent then, that until there is more information on the characteristics and "phenotypes" of 8-azaguanine resistant cells derived spontaneously, experiments investigating the induction of 8-azaguanine resistance lacks a standard.

spontaneous mutation rate

The mutation rate for spontaneous resistance in

CHWC1 to 8-azaguanine was in the range of 2.3×10^{-6} to 6.5×10^{-5} with selection at 10 µg aza/ml, which is within the extremes recorded for other Chinese hamster cell lines. Chu et al. (1969) reported a mutation rate of 1.8×10^{-8} for V_{79}, with selection at 30 µg aza/ml, while Arlett and Harcourt (1972) using the same cell line reported rates of 6×10^{-5} to 3×10^{-3} with selection at 7.5 µg/ml. Shapiro et al. (1972a) reported a rate of 1.5×10^{-5} for a hypoploid Chinese hamster cell line with selection at 30 µg/ml. Direct comparison of the rate for CHWC1 with those recorded for other cell lines is difficult because of the variability of experimental conditions such as concentration of selecting agent and cell densities. Supporting this contention, Arlett and Harcourt (1972) found that mutation rates were not random and that rates from groups of experiments with common media and serum batches fell into similar ranges. They concluded that the variability to some extent, was due to the failure of a proportion of mutants to form colonies under certain circumstances. The variability of mutation rates recorded here is about one-third less than that reported by Arlett and Harcourt, accountable perhaps by the restrictions placed on the source of materials used in media preparation.

The Luria Dëlbruck fluctuation test has been used to confirm spontaneous 8-azaguanine resistance for human cells (Szybalki, 1959), mouse cells (Morrow, 1970) and

Chinese hamster cells (Chu et al. 1969; Shapiro et al., 1972a). Also, the mutation rates were calculated from prediction of a Poisson distribution of resistant cells. Mutation rates in somatic cells in vitro do not appear to differ from those in mouse, about 10^{-6}, or man, 10^{-4} to 10^{-6} per fertilized gamete (Vogel, 1970).

Chu (1970) identified certain factors affecting mutation rate to be cell density, concentration of selecting agent and time of addition of selecting agent (mutation expression time). Cell density was kept constant in this study in order to minimize frequency variability. An increase in the concentration of azaguanine from 10 µg/ml to 30 µg/ml was observed to decrease the number of resistant colonies. The mutation frequency appeared to increase with intervening expression time, but the mutation rate based on cell counts did not. The difference was accountable by the method used to estimate surviving cells.

Both frequency and rate were over-estimations when the number of surviving cells was based on the plating efficiency of two hundred cells multiplied by the total number of cells at beginning of experiment. Plating efficiency provides the percentage of single cells that attach and divide to form macroscopic colonies. It does not indicate the number of surviving cells available for selection. In a given experiment, an average of 202 colonies per dish was counted, whereas the average total

cell count for a comparable dish was 4.3×10^3 (Gee, unpublished data). If in that dish, there was one resistant colony, then using plating efficiency the mutation rate would be 8.7×10^{-3}; but, using cell count, the mutation rate would be 4.09×10^{-4}. Plating efficiency over-estimates mutation rate because it does not give an accurate enumeration of total surviving cells.

The concentration of the selecting agent but not its time of addition affected the mutation rate. Variability from experiment to experiment was reduced by using cell counts to estimate survivors at the time at which the selecting agent was introduced.

characteristics of spontaneous and chemically induced resistant colonies

Eleven azaguanine resistant colonies were chosen for characterization. Five colonies were isolated after exposure to 10^{-3}M MMS, three with selection at 10 µg aza/ml and two at 30 µg/ml. The other six colonies were isolated in the absence of the mutagen, three at 10 µg/ml and three at 30 µg/ml. All colonies grew in 10 µg azaguanine/ml as well as in MENAFC10. Two showed survival in THAG equal to that in MENAFC10, while one had minimal survival in THAG. Seven others did not show single cell growth in THAG. However, one colony, 65350, which did not plate out in THAG initially, did so in subsequent experiments, and moreover

the growth in MENAFC10 and azaguanine remained equal. This variable response to THAG suggests that a mechanism other than a primary gene mutation may give rise to azaguanine resistance. The ability to survive in THAG did not appear to be related to the concentration of the selecting agent or the mutagen treatment.

The doubling time was found to be longer for these colonies than for CHWC1. Littlefield (1963) also observed that 8-azaguanine resistant cells had a longer generation time than the sensitive cells. The increase in the doubling time in azaguanine from that in MENAFC10, explains the difference in size noted in the resistant colonies. Colonies in azaguanine were smaller than those in MENAFC10. Since it would take longer for the cells to divide in the drug, there would be fewer cells and hence, smaller colonies.

Chromosome ploidy did not reveal any pattern as to mode of selection, response to THAG, or doubling time. Seventeen of the twenty-three isolated azaguanine resistant colonies showed more than 50% tetraploidy. This is in contrast to the parental line CHWC1, which always showed above 80% diploidy, the proportion being maintained amongst the clones upon single cell plating (Cox and Ray, 1971).

There is little reference to chromosome ploidy in reports on 8-azaguanine resistance in mammalian cells. Morrow (1970) used a mouse line which was subject to a good deal of chromosomal variation thus being unfavorable for

karyotype studies. He carried out chromosome counts on six sensitive clones and five resistant clones and found no significant difference in chromosome number. Shapiro et al. (1972a) used a Chinese hamster line with a chromosome modal number of 18. They studied ten 8-azaguanine resistant clones of which nine maintained a modal number of 18 and one clone was found to have a modal number of 33.

characteristics of a chemically induced resistant clone, 1102

To ascertain in vitro characteristics of an 8-azaguanine resistant mutant, the clone 1102 was obtained from a MMS induced azaguanine resistant colony. 1102 demonstrated properties expected of a mutant. These are stability of resistance in the absence of the selecting agent, an unaltered growth rate, and a low frequency of reversion to sensitivity. The frequency of revertants was 3×10^{-8} to 2×10^{-7}. Chu et al (1969) reported spontaneous reversion frequencies for two azaguanine resistant lines derived from V_{79}, to be 5.5×10^{-7} and 2.5×10^{-7}, using an inoculum of 10^6 cells. The frequency found here was slightly lower. Using the Capizzi and Jameson table (1973) the reverse mutation rate would be between 5.6×10^{-8} to 2.35×10^{-7}.

Although the rare appearance of revertants of 1102 falls within the frequency expected, 1102 did not revert to

the "wild type" CHWC1 phenotype. It is not known whether the growth response demonstrated by the 1102 'revertant' was shared by the other colonies observed in the reversion experiments. The microscopic appearance of the cells in culture and their poor response in both MENAFC10 and THAG suggests some gross disturbance in cell metabolism, since regardless of whether it was a revertant or not, growth in MENAFC10 would not be expected to change.

Freese (1963) proposed that practically all mutants produced by single base changes do revert, but reversions of deletions and insertions may be too small to be seen in a genetic system as yet. However, lack of reversion does not prove that more than one nucleotide pair has been altered, neither is it established as a point mutation merely because it reverts. He claims that it must be known that the mutant had been induced by a chemical, which induces nothing but point mutations in other genetically well-studied systems, because revertants may be the result of suppressor mutations. 1102 was a clone picked from a MMS induced resistant colony. Although MMS does produce point mutations in microorganisms, this has not been conclusively demonstrated in mammalian systems.

From these preliminary investigations into azaguanine resistance in the Chinese hamster cell line CHWC1, it appeared that considerable variability occurred in response to mutagens, survival in THAG and in the level

of tetraploidy. To understand better the basis of this variability it became necessary to

(1) extend the investigation of spontaneous 8-azaguanine resistant cells selected at 10 µg and 30 µg aza/ml after 0 and 42 hours mutation expression time, and

(2) apply the selecting method to a tetraploid subline of CHWC1.

characteristics of spontaneously derived resistant colonies

Twenty-eight spontaneous azaguanine resistant colonies were isolated. Of these, ten were selected at 10 µg aza/ml after 0 hours mutation expression time. The remaining colonies were isolated after 42 hours mutation expression time, fourteen selected at 10 µg/ml and four at 30 µg/ml.

Twenty-three of these colonies showed a similar stability of resistance to the drug as 1102. The five remaining colonies had decreased plating efficiencies in azaguanine after eight to ten passages in MENAFC10, suggesting a nonhereditable resistance. One colony, 418-16-01 showed remarkable response to the drug. A culture of these cells could not be maintained in azaguanine, but when plated into 10 µg aza/ml from a MENAFC10 culture, the relative plating efficiency was 95%. There is no obvious explanation for this behaviour.

The toxic effect of azaguanine on the resistant colonies was much less severe than on CHWCl. This is the expected response for cells deficient in HGPRT, which cannot incorporate 8-azaguanine, thus providing resistance up to that concentration of drug which interferes with other aspects of cell metabolism. CHWCl cells with normal HGPRT activity, incorporate 8-azaguanine into the nucleic acids with lethal consequences. The minimal resistance offered by normal cells would be due to detoxification and the concentration effect whereby normal substrate in excess of drug competes for the enzyme. There may also be a small effect due to the presence of an antagonist to 8-azaguanine in fetal bovine serum.

Definite levels of resistance to the drug were not apparent. In all but two colonies, cell survival decreased to a minimum at 45 µg to 100 µg aza/ml. One colony retained about 20% survival in 100 µg/ml while another could not support growth beyond 15 µg/ml. In general, the colonies selected at 10 µg and 30 µg aza/ml after 42 hours expression time illustrated a broader spectrum of cytotoxic effects and resistance than did those selected by 10 µg without the intervening expression time. The latter had a plating efficiency in 10 µg aza/ml in excess of 45% whereas the colonies selected after 42 hours mutation expression time had a plating efficiency in 10 µg aza/ml as low as 10%. It is possible that the colonies selected without the intervening

expression time were more homogeneous in the underlying cause of resistance.

Stability of resistance and level of resistance were not interrelated, nor was there any clear relationship apparent with concentration of selecting agent or expression time. The level of resistance has been reported to increase with increase in the concentration of the selecting agent (Littlefield 1963; Morrow 1970). However, this could have easily resulted from the method of consecutive selection and was not the property of a number of colonies, each isolated only once at a specific concentration of the drug.

The estimation of doubling time in mass culture is a simple technique to quantify what may be apparent in culture maintenance, i.e., cells maintained in 10 µg aza/ml take longer to reach confluency than in its absence. With few exceptions there was a longer doubling time in azaguanine than in MENAFC10. This would explain the size difference of colonies in the cytotoxicity experiments. At 10 µg aza/ml the effect of the drug seemed to be that of retarding the cell cycle rather than that of toxicity, since survival in 10 µg aza/ml was comparable to that in MENAFC10, and only colony size was different. However in azaguanine concentrations greater than 10 µg/ml, the plating efficiency decreased and a toxic effect can be assumed to have been operative.

The selective medium, THAG, made it possible to

separate the colonies into four categories, each demonstrating different relative plating efficiencies. Six colonies did not show single cell survival in THAG. Ten colonies demonstrated less than 30% survival in THAG relative to that in MENAFC10, and eight colonies showed greater than 50% survival. The four remaining colonies showed variable response in THAG, and were the same colonies that illustrated instability of resistance. Two other associations could be noted; the colony with the highest level of resistance had 0% growth in THAG, and the colonies selected without mutation expression time formed the group that had less than 30% survival in THAG. Although there appeared to be variability of response in some individual colonies, this occurred at such a low level that most could be ascribed to technical variation.

Seven of the colonies that demonstrated greater than 50% survival in THAG, also showed equally high plating efficiency in 10 µg aza/ml, a criterion for stability of resistance. If resistance to azaguanine is a forward mutation, and growth in THAG indicates reversion, the number of revertants in these colonies would be equal to the total number of mutant cells. However since the cells are plated into the respective media at the same time, same density and from the same dilution; it appears to be an "off-on" situation with 100% response in each medium, rather than selection of resistant mutants in azaguanine

and selection of an equal number of revertants in THAG. In the four cultures with irregular response to THAG, the cells maintained in azaguanine showed a relative plating efficiency which was high in 10 μg aza/ml but low in THAG. Whereas the parallel cultures maintained in MENAFC10 showed a relative plating efficiency which was low in 10 μg aza/ml but high in THAG. In cultures maintained in nonselective medium the capacity of resistance to azaguanine decreased considerably concomitant with increased survival in THAG. This type of resistance is suggestive of adaptation, representing a "phenotypic shift", since cells remained resistant to the drug only when constantly exposed to it (Moyed, 1964; Harris, 1971).

Complementation did not disclose any additional information since only those resistant colonies that did not show single cell survival in THAG could be used effectively for cell fusion. A few colonies were observed to arise after crosses between certain azaguanine resistant cells, but these were never as numerous as when the resistant cells were crossed with the mouse cell line, B_{82}. It is highly unlikely that any of the colonies resulting from crosses between the azaguanine resistant cells were due to complementation. More probably the colonies so formed represented reversion to azaguanine sensitivity even though the resistant cells did not plate in THAG. It has been demonstrated that 1102 showed no plating efficiency

in THAG, but did have a very low frequency of revertants. Thus it is an acceptable proposition that the spurious colonies formed may be "revertants" since far fewer cells are tested in a plating efficiency than are used for cell fusion. Therefore, these results demonstrated that the azaguanine resistant colonies with a plating efficiency of 0% in THAG were not complementary. Resistance in these colonies may be due to the same defect, perhaps in HGPRT. All have been shown to retain resistance in the absence of the selecting agent and to have a very low frequency of "revertants" in THAG.

Chu et al. (1969) suggested that azaguanine resistance in the Chinese hamster line V_{79} is recessive. They obtained a possible hybrid between a glutamine-requiring cell and an azaguanine resistant cell, as indicated by karyotypic analysis and growth response in THAG medium without glutamine. Functional tests showed that both glutamine auxotrophy and azaguanine resistance behaved as recessive characters in the hybrid cell. Nadler et al. (1970) were successful in showing intra-allelic complementation in fused human diploid cells. The cells used were from different patients with galactosemia, a rare autosomal recessive disorder caused by defects in galactose-1-phosphate uridyl transferase. They speculated that the hybrid enzyme in the fused cells was formed through the association of altered subunits, although they

did not rule out the possibility of an intergenic rather than intra-allelic complementation. Kao and Puck (1972) were able to demonstrate complementation for auxotrophs in the Chinese hamster line CHO. Four separate complementation groups of glycine-requiring mutants and two groups of adenine or hypoxanthine-requiring mutants were distinguished. They interpreted all the auxotrophs to be recessive. These reports have shown that it is possible to make complementary intraspecific hybrids for either intergenic or intra-allelic mutations. This strengthens the probability that the seven azaguanine resistant colonies tested here, represent a homogeneous group of mutants.

The chromosomes of some resistant cell lines have been reported to be the same for the mutants and the parental lines (Harris and Ruddle, 1960; Albertini and De Mars, 1970; Morrow, 1970; Huberman et al., 1971; Sato et al., 1972), while some variation has been reported in other resistant lines (Chu et al., 1969; Chu and Ho, 1970; Shapiro et al., 1972a). Biedler et al. (1965) reported the association of a specific chromosome with aminopterin resistance in mouse cells, and more recently Biedler and Riehm (1970) documented association between actinomycin D resistance in a Chinese hamster line with specific chromosome abnormalities. Also, Balacco and colleagues quoted by Morrow (1970), recorded that in an established human line, increasing levels of azaguanine resistance were due to the

loss of a chromosome in the size range of the X chromosome.

In this study, an altered karyotype was observed in 75% of the azaguanine resistant colonies. This was an unexpected result since the parental line, CHWC1, had demonstrated a very stable karyotype. The modal chromosome number of 22 was retained by the majority of colonies. Some of the altered karyotypes were similar to that seen in 1102, with four subtelocentrics and three marker chromosomes, while other karyotypes showed deletions of a number four or five chromosome. In CHWC1 less than 20% of the cells were tetraploid, whereas after selection by 8-azaguanine, fourteen of the twenty-eight colonies were 100% tetraploid. Chromosomal variation is not necessarily responsible for the phenotypic change and, neither karyotypic alteration nor chromosome ploidy could be associated with the pattern of growth response or selection. The chromosome variation itself may be induced by the altered internal physiological state brought about by the process of cloning and pressure of the selecting agent.

There are two possible reasons why a disproportionate number of tetraploids were recovered. Firstly, the tetraploid colonies may all have been derived from the tetraploid cells already present in the CHWC1 population. Or, secondly, there may have been some physiological instability in the selected resistant diploid cells, which in some became counterbalanced by increased ploidy. Thus,

the tetraploid cells could have either been preferentially selected or could have arisen as a consequence of the selecting process.

azaguanine resistance in CHWC1-06

To determine if tetraploids were selected in preference to diploids, a tetraploid subline CHWC1-06 was produced and processed through the azaguanine selection system. This tetraploid did not yield resistant colonies. In contrast, Harris (1971) and Mezger-Freed (1971, 1972), reported that mutation rates were unchanged in cells of different ploidy levels.

Harris (1971) prepared a matched set of polyploid sublines, 2n, 4n and 8n, from the Chinese hamster line V_{79}. He found no significant increase or decrease in mutation rates for cells at different ploidy levels and all were in the order of 10^{-5} for 8-azaguanine resistance and 10^{-6} for heat resistance. Mezger-Freed (1971, 1972) studied puromycin resistance in haploid and heteroploid frog cells and BUdR resistance in haploid and diploid frog cells. She also, did not observe a difference in the mutation frequencies for cells at different levels of ploidy. However, Mezger-Freed went on to demonstrate that resistance to puromycin and BUdR was due to membrane impermeability to these drugs. Harris suggested that resistance may not necessarily be the product of mutation, because in

established cell lines and tumor cell populations de novo variation is both characteristic and common. Many changes that resemble mutations could be the result of impaired control of phenotypic expression. He considered that the stable shifts in phenotype, which are a familiar part of embryonic development, could possibly produce the resistance which is maintained by culture conditions.

On the basis of the mutation rates for CHWC1 and for CHWC1-06, it is apparent that the tetraploids were not preferentially selected over the diploids, despite the fact that over half of the spontaneously derived 8-azaguanine resistant colonies were tetraploid or had a high proportion of tetraploidy. This leaves the other alternative, that the tetraploidy was a consequence of selection.

The absence of resistant cells in CHWC1-06 might be accountable if 8-azaguanine resistance is due simply to a recessive mutation, then the mutation rate in a tetraploid would be expected to be the square of the mutation rate in the diploid. This would apply to mutation from the heterozygous to the homozygous or hemizygous condition. In this case, the expected mutation rate would be in the order of 10^{-10} which is beyond the limits of discrimination and moreover exceeds the technical capacity, of the method used.

mechanisms for resistance

In this investigation phenotypic variants could only be distinguished by the level of response to THAG. However, an over-estimation of the growth of azaguanine resistant cells in THAG can occur as a result of metabolic cooperation if normal cells are also present in the cell population. Metabolic cooperation allows mutant cells to behave in a way similar to wild-type cells. This phenomenon has been studied in mixed populations of $HGPRT^-$ and HGPRT+ cells. Subak-Sharpe et al. (1966, 1969) used the term to describe the autoradiographic results where $HGPRT^-$ cells incorporated ^{3}H-hypoxanthine into their nucleic acids when in contact with $HGPRT^+$ cells. In addition there has been some evidence of transfer of a protein from a normal cell to a deficient one when the two types are in contact with each other (Fujimoto and Seegmiller, 1970; Ashkenazi and Gartler, 1971; Cox et al., 1970, 1972). The prerequisite for metabolic cooperation is cell contact. De Mars (1971) found that in population densities above 50 cells/mm^2, $HGPRT^-$ cells often became significantly labelled by ^{3}H-hypoxanthine in the presence of normal cells. In this study a culture from each colony was maintained in azaguanine and two hundred cells only were plated in each 35 mm dish. Normal cells can not survive in 10 µg aza/ml and at the cell density used for plating efficiency, cell to cell contact would be extremely rare. Thus, the method of determining

plating efficiency excludes metabolic cooperation as the cause of the observed growth in THAG.

Four groups of azaguanine resistant colonies were distinguished according to the plating efficiency in THAG. One group of six colonies showed no survival in THAG, a second group of ten colonies had less than 30% survival in THAG while a third group of eight had greater than 50% survival and the fourth group of four colonies showed a variable response to THAG. Can these levels of response to THAG be related to proposed mechanisms for resistance? Resistance of a cell to a drug may be the result of a defect in the incorporating enzyme, impermeability of the drug, or an adaptive mechanism under the control of regulatory genes.

The activity of the incorporating enzyme may be altered in a number of ways. Deletion of a chromosome region can result in the loss of the structural gene for the enzyme, thus creating a deficiency. Also, a defect in the gene can lead to loss of enzyme activity, but final evidence that the defect was a point mutation, is the demonstration of an altered gene product. Reversion rate has been used as an indirect indication of a point mutation however a suppressor mutation may restore growth partially or even completely. Resistance to 8-azaguanine in mammalian somatic cells has been ascribed to mutation in the structural gene for HGPRT (Rubin et al., 1971). Intermediate levels of resistance have been attributed to the number of copies of

the HGPRT locus (Szybalski et al., 1963; Littlefield, 1963; Morrow, 1970). Whereas Chu and Malling (1968) suggested that "leaky" mutations, those resistant to different levels of 8-azaguanine, each represent "a different isoallele with a different level of enzymatic activity controlled by the azg locus".

Mezger-Freed (1971, 1972) reported that membrane impermeability resulted in resistance while Harris (1971) and Cass (1972) demonstrated that impermeability of a drug could be a function of cell density.

Regulation of enzyme activity in higher organisms has been demonstrated. The studies of Conrad and Ruddle (1972) and Tiemeier and Milman (1972) indicate that normal maintenance of high enzyme activity and subsequent disappearance of enzyme activity may involve post-transcriptional events which operate at the level of RNA translation or subsequent protein turnover. Riccardi and Littlefield (1972) described a strain of Lesch-Nyhan fibroblasts that showed full simultaneous resistance to aminopterin and 6-thioguanine. By radioautographic studies they showed that when the cells were kept in HAT, there was a gradual and uniform incorporation of ^{3}H-hypoxanthine, suggesting a generalized adaptation rather than selection of variant cells. This aminopterin resistance was associated with a 2-fold increase in HGPRT activity. Once out of HAT medium, uptake of ^{3}H-hypoxanthine and HGPRT activity was

re-established to that commonly found in Lesch-Nyhan fibroblasts.

It is attractive to speculate on the possible mechanisms which may have been operative in the production of the azaguanine resistant colonies isolated here. Although changes in chromosome ploidy and karyotype were observed, these did not relate to any definite growth response. It is unlikely that the chromosome changes per se are implicated as mechanisms of resistance. There is the possibility that those colonies which did not demonstrate single cell survival in THAG were true "mutants" in that they may have possessed a defect in the structural gene for HGPRT. And perhaps the colonies which demonstrated a low plating efficiency in THAG may have had a partial deficiency of the enzyme as a result of post-transcriptional modification. The colonies with the irregular response to THAG as well as to azaguanine suggest some other regulatory mechanism, a type of adaptive phenotypic shift. Perhaps a phenomenom similar to that described by Riccardi and Littlefield (1972) was responsible for some of the resistant colonies which were stable to resistance and also maintained a plating efficiency in THAG equal to that in 10 µg aza/ml and MENAFC10. No conclusive statements can be made in regard to the mechanisms of resistance that may have been responsible for the variation observed in the isolated 8-azaguanine resistant colonies until a more detailed and

comprehensive study is made on each colony.

The twenty-eight spontaneously derived resistant colonies do not represent a uniform mode of resistance and it is doubtful whether even half of them represent point mutations. Until the time when normal variation can be distinguished from true mutation, the use of 8-azaguanine resistance in vitro as a genetic marker, for example in environmental mutagen screening, lacks credibility.

LITERATURE CITED

Albertini, R. J. and R. DeMars (1970). Diploid azaguanine-resistant mutants of cultured human fibroblasts. Science 169: 482-485.

Anderson, T. J. and R. H. Burdon (1970). N-methyl-N'-nitro-N-nitrosoguanidine: reactions of possible significance to biological activity with mammalian cells. Cancer Res. 30: 1773-1781.

Anderson, E. P. and L. W. Law (1960). Biochemistry of cancer. Ann. Rev. Biochem 29: 577-608.

Arlett, C. F. and S. A. Harcourt (1972). Expression time and spontaneous mutability in the estimation of induced mutation frequency following treatment of Chinese hamster cells by ultraviolet light. Mutation Res. 16: 301-306.

Arlett, C. F. and J. Potter (1971). Mutation to 8-azaguanine resistance induced by γ-radiation in a Chinese hamster cell line. Mutation Res. 13: 59-65.

Arnold, W. J. and W. N. Kelley (1971). Human hypoxanthine-guanine phosphoribosyl transferase. J. Biol. Chem. 246: 7398-7404.

Ashkenazi, Y. E. and S. M. Gartler (1971). A study of metabolic cooperation utilizing human mutant fibroblasts. Exp. Cell Res. 64: 9-16.

Auerbach, C. (1967). The chemical production of mutations. Science 158: 1141-1147.

Auerbach, C. and W. N. Kelley (1971). Mutation in eukaryotes. Ann. Rev. Genet. 5: 163-218.

Barranco, S. C. and R. M. Humphrey (1971). The response of Chinese hamster to N-methyl-N'-nitro-N-nitrosoguanidine. Mutation Res. 11: 421-429.

Biedler, J. L., A. W. Schrecker, and D. J. Hutchison (1963). Selection of chromosomal variant in amethopterin-resistant sublines of leukemia L1210 with increased levels of dihydrofolate reductase. J. Nat. Cancer Inst. 31: 575-601.

Biedler, J. L., A. M. Albrecht, and D. J. Hutchison (1965). Cytogenetics of mouse leukemia L1210. 1. Association of a specific chromosome with dihydrofolate reductase activity in amethopterin-treated sublines. Cancer Res. 25: 246-257.

Biedler, June L. and H. Riehm (1970). Cellular resistance to actinomycin D in Chinese hamster cells in vitro: cross-resistance, radioautographic, and cytogenetic studies. Cancer Res. 30: 1174-1184.

Breslow, R. E. and R. A. Goldsby (1969). Isolution and characterization of thymidine transport mutants of Chinese hamster cells. Exp. Cell Res. 55: 339-346.

Bridges, B. A. and J. Huckle (1970). Mutagenesis of cultured mammalian cells by X-radiation and ultraviolet light. Mutation Res. 10: 141-151.

Brockman, R. W. (1965). Resistance to purine antagonists in experimental leukemia systems. Cancer Res. 25: 1596-1605.

Brockman, R. W., G. G. Kelley, P. Stutts, and V. Copeland (1961). Biochemical aspects of resistance to 6-mercaptopurine in human epidermoid carcinoma cells in culture. Nature 191: 469-471.

Capizzi, R. L. and J. W. Jameson (1973). A table for estimation of the spontaneous mutation rate of cells in culture. Mutation Res. 17: 147-148.

Cass, C. E. (1972). Density-dependent resistance to puromycin in cell cultures. J. Cell. Physiol. 79: 139-146.

Cerdá-Olmedo, E. and P. C. Hanawalt (1968). Diazomethane as the active agent in nitrosoguanidine mutagenesis and lethality. Molec. Gen. Genetics 101: 191-202.

Cerdá-Olmedo, E., P. C. Hanawalt and N. Guerola (1968). Mutagenesis of the replication point by nitroguanidine: map and pattern of replication of the Escherichia coli chromosome. J. Mol. Biol. 33: 705-719.

Chu, E. H. Y. (1970). Point mutations in mammalian cell cultures as measures for mutagenicity testing. In Chemical Mutagenesis in Mammals and Man (eds. Vogel and Röhrborn). Springer-Verlag, New York. 241-250.

Chu, E. H. Y., P. Brimer, K. B. Jacobson, and E. V. Merriam (1969). Mammalian cell genetics I. Selection and characterization of mutations auxotrophic for L-glutamine or resistant to 8-azaguanine in Chinese hamster cells in vitro. Genetics 62: 359-377.

Chu, E. H. Y., P. Brimer, and H. V. Malling (1969b). Reversion studies of chemically induced 8-azaguanine-resistant mutants in Chinese hamster cells in culture. Genetics 61: S10-S11.

Chu, E. H. Y. and T. Ho (1970). Foreward and reverse mutations in the resistance of Chinese hamster cells to 5-bromodeoxyuridine. Mammalian Chromosomes Newsletter 11: 58-59.

Chu, E., and V. Malling (1968). Mammalian cell genetics II: Chemical induction of specific locus mutations in Chinese hamster cells in vitro. Proc. Nat. Acad. Sc. 61: 1306-1312.

Chu, E. H. Y., N. C. Sun, and C. C. Chang (1972). Induction of auxotrophic mutations by treatment of Chinese hamster cells with 5-BUdR and black light. Proc. Nat. Acad. Sc. 69: 3459-3463.

Clayton, D. A., and R. L. Teplitz (1972). Intracellular mosaicism (nuclear^{-}/mitochondrial^{+}) for thymidine kinase in mouse L cells. J. Cell Sc. 10: 487-493.

Conrad, A. H., and F. H. Ruddle (1972). Regulation of thymidylate synthetase activity in cultured mammalian cells. J. Cell Sc. 10: 471-486.

Cox, D. M., and T. T. Puck (1969). Chromosomal nondisjunction: the action of colcemid on Chinese hamster cells in vitro. Cytogenetics 8: 158-169.

Cox, D.M., and M. Ray (1971). Evaluation of cell viability in chromosomally heterogeneous cell populations. Can. J. Genet. Cytol. 13: 484-488.

Cox, R. P., M. R. Krauss, M. E. Balis, and J. Dancis (1970). Evidence for transfer of enzyme product as the basis of metabolic cooperation between tissue culture fibroblasts of Lesch-Nyhan disease and normal cells. Proc. Nat. Acad. Sci. 67: 1573-1579.

Cox, R. P., M. R. Krauss, M. E. Balis, and J. Dancis (1972). Communication between normal and enzyme deficient cells in tissue culture. Exp. Cell Res. 74: 251-268.

Davidson, R. (1969). Regulation of melanin as studied by somatic hybridization. III. A method of increasing the frequency of cell fusion. Exp. Cell Res. 55: 424-426.

De Mars, R. (1971). Genetic studies of HG-PRT deficiency and the Lesch-Nyhan syndrome with cultured human cells. Fed. Proc. 30: 944-955.

De Mars, R., and J. L. Hooper (1960). A method of selecting for auxotrophic mutants of HeLa cells. Exp. Med. 111: 559-573.

Drake, J. (1969). Mutagenic mechanisms. Ann. Rev. Genet. 3: 247-268.

Eagle, H., and G. E. Foley (1956). The cytotoxic action of carcinolytic agents in tissue culture. Amer. J. Med. 21: 739-749.

Freese, E. B. (1961). Transitions and transversions induced by depurinating agents. Proc. Nat. Acad. Sc. 47: 540-545.

Freese, E. (1963). Molecular mechanism of mutations. In Molecular Genetics (H. Taylor ed.). Academic Press Inc., New York, 207-269.

Fujimoto, W. Y., and J. E. Seegmiller (1970). Hypoxanthine-guanine phosphoribosyl transferase deficiency: activity in normal, mutant and heterozygote-cultured human skin fibroblasts. Proc. Nat. Acad. Sci. 65: 577-584.

Gillin, F. D., D. J. Roufa, A. L. Beaudet and C. T. Caskey (1972). 8-azaguanine resistance in mammalian cells 1. Hypoxanthineguanine phosphoribosyl transferase, Genetics 72: 239-252.

Hakala, M. T. (1957). Prevention of toxicity of amethopterin for sarcoma-180 cells in tissue culture. Science 126: 255.

Hakala, M. T., and E. Taylor (1959). The ability of purine and thymine derivatives and of glycine to support the growth of mammalian cells in culture. J. Biol. Chem. 234: 126-128.

Harris, M. J. (1967). Phenotypic expression of drug resistance in cell cultures. Nat. Cancer Inst. 38: 185-192.

Harris, M. J. (1971). Mutation rates in cells at different ploidy levels. J. of Cell Physiol. 78: 177-184.

Harris, M. and F. H. Ruddle (1960). Growth and chromosome studies on drug resistant lines of cells in tissue culture. In Cell Physiology of Neoplasia. University of Texas Press, Austin. pp. 524-547.

Huberman, E., L. Aspiras, C. Heidelberger, P. L. Grover and P. Sims (1971). Mutagenicity to mammalian cells of epoxides and other derivatives of polycyclic hydrocarbons. Proc. Nat. Acad. Sci. 68: 3195-3199.

Hsu, T. C., and M. T. Zenzes (1964). Mammalian chromosomes in vitro (XVII). Idiogram of the Chinese hamster. J. Nat. Cancer Inst. 32: 857-869.

Kao, F., and T. T. Puck (1967). Genetics of somatic mammalian cells, IV. Properties of Chinese hamster cell mutants with respect to the requirement for proline. Genetics 55: 513-521.

Kao, F., and T. T. Puck (1968). Genetics of somatic mammalian cells VII. Induction and isolation of nutritional mutants in Chinese hamster cells. Proc. Nat. Acad. Sci. 60: 1275-1281.

Kao, F., and T. T. Puck (1969). Genetics of somatic mammalian cells IX. Quantitation of mutagenesis by physical and chemical agents. J. Cell. Physiol. 74: 245-258.

Kao, F. T., and T. T. Puck (1972). Genetics of somatic mammalian cells XIV. Genetic analysis in vitro of auxotrophic mutants. J. Cell. Physiol. 80: 41-50.

Kelley, W. N., and Jean C. Meade (1971). Studies on hypoxanthine-guanine phosphoribosyl transferase in fibroblasts from patients with the Lesch-Nyhan syndrome. J. Biol. Chem. 246: 2953-2958.

Kessel, D., and I. Wodinski (1968). Uptake in vivo and in vitro of actinomycin D by mouse leukemia cells as factors in survival. Biochem. Pharmacol. 17: 161-164.

Kit, S., D. R. Dubbs, and P. M. Frearson (1966). HeLa cells resistant to bromodeoxyuridine and deficient in thymidine kinase activity. Internat. J. Cancer 1: 19-30.

Kit, S., D. R. Dubbs, L. J. Piekarski, and T. C. Hsu (1963). Deletion of thymidine kinase activity from L cells resistant to bromodioxyuridine. Exp. Cell Res. 31: 297-312.

Kogut, M. D., G. N. Donnell, W. L. Nyhan and L. Sweetman (1970). Disorders of purine metabolism due to partial deficiency of hypoxanthine-guanine phosphoribosyl transferase. Amer. J. Med. 48: 148-161.

Krenitsky, T. A., R. Papioannou, and G. B. Elion (1969). Human hypoxanthine phosphoribosyl transferase I. Purification, properties and specificity. Biol. Chem. 244: 1263-1270.

Law, L. W. (1956). Differences between cancers in terms of evaluation of drug resistance. Cancer Res. 16: 639-724.

Lesch, M., and W. L. Nyhan (1964). A familial disorder in uric acid metabolism and central nervous system function. Amer. J. Med. 36: 561-570.

Lieberman, I., and P. Ove (1965). Isolation and study of mutants from mammalian cells in culture. Proc. Nat. Acad. Sci. 45: 867-872.

Lin, C. C., T. D. Chang, and V. Niewczas-Late (1971). The establishment and chromosome analysis of a new cell line of Chinese hamster from spontaneous transformation in vitro. Can. J. Genet. Cytol. 13: 9-13.

Littlefield, J. W. (1963). The inosinic acid pyrophosphorylase activity of mouse fibroblasts partially resistant to 8-azaguaine. Proc. Nat. Acad. Sci. 50: 560-576.

Littlefield, J. W. (1965). Studies on thymidine kinase in cultured mouse fibroblasts. Bioch. Bioph. Acta 95: 14-22.

Littlefield, J. W., and C. Basilico (1966). Infection of thymidine kinase-deficient BHK Cells with polyoma virus. Nature 211: 250-252.

Luria, S. E., and M. Delbrück (1943). Mutations of bacteria from virus sensitivity to virus resistance. Genetics 28: 491-511.

McDonald, J. A., and W. N. Kelley (1971). Lesch-Nyhan Syndrome: Altered kinetic properties of mutant enzyme. Science 171: 689-772.

Mandel, H. G., and L. W. Law (1954). The effect of 4-amino-5 imidazolecarboxamide on carcinostatic action of 8-azaguanine. Cancer Res. 14: 808-811.

Mezger-Freed, L. (1971). Puromycin resistance in haploid and heteroploid frog cells: gene or membrane determined? J. Cell Biol. 51: 742-751.

Mezger-Freed, L. (1972). Effect of ploidy and mutagens on bromodeoxyuridine resistance in haploid and diploid frog cells. Nature New Biol. 235: 245-246.

Migeon, B. R., V. M. Kaloustian, W. L. Nyhan, W. J. Young and C. Barton (1968). X-linked hypoxanthine-guanine phosphoribosyl transferase deficiency: heterozygote has two clonal populations. Science 160: 425-427.

Morrow, J. (1970). Genetic analysis of azaguanine resistance in an established mouse cell line. Genetics 65: 279-287.

Moyed, H. S. (1964). Biochemical mechanisms of drug resistance. Ann. Rev. Microbiol. 18: 347-364.

Nadler, H. L., C. M. Chacko, and M. Rachmeler (1970). Interallelic complementation in hybrid cells derived from human diploid strains deficient in galactose-1-phosphate uridyl transferase activity. Proc. Nat. Acad. Sci. 67: 976-982.

Naha, P. M. (1969). Temperature sensitive conditional mutants of monkey kidney cells. Nature 223: 1380-1381.

Naha, P. M. (1970). Initial characterization of a temperature sensitive mutant of monkey kidney cell. Nature 228: 166-168.

Orkin, S. H., and J. W. Littlefield (1971). Mutagenesis to aminopterin resistance in cultured hamster cells. Exp. Cell Res. 69: 174-180.

Puck, T. T. and F. T. Kao (1967). Genetics of somatic mammalian cells V. Treatment with 5-bromodeoxyuridine and visible light for isolation of nutritionally deficient mutants. Pro. Nat. Acad. Sci. 58: 1227-1234.

Puck, T. T., P. I. Marcus, and S. J. Cieciura (1956). Clonal growth of mammalian cells in vitro growth characteristics of colonies from single HeLa cells with and without a "feeder" layer. J. Exp. Med. 103: 273-284.

Riccardi, V. M., and J. W. Littlefield (1972). Adaptation of Lesch-Nyhan cells to aminopterin. Exp. Cell Res. 74: 417-422.

Röhrborn, G. (1970). Biochemical mechanisms of mutation. In Chemical Mutagenesis in Mammals and Man (ed. Vogel and Röhrborn). Springer-Verlag, New York. 1-15.

Rothfels, K. H. and L. Siminovitch (1958). An air-drying technique for flattening chromosomes in mammalian cells grown in vitro. Stain Technol. 33: 73-77.

Rubin, C. S., J. Dancis, L. Yip, R. C. Nowinski, and M. E. Balis (1971). Purification of IMP: pyrophosphate phosphoribosyl transferase. Catalytically incompetent enzymes in Lesch-Nyhan disease. Proc. Nat. Acad. Sci. 68: 1461-1464.

Salzman, J., R. De Mars, and P. Benke (1968). Single allele expression at an X-linked hyperuricemia locus in heterozygous human cells. Proc. Nat. Acad. Sci. 60: 545-552.

Sato, K., R. S. Slesinski and J. W. Littlefield (1972). Chemical mutagenesis at the phosphoribosyl transferase locus in cultured human lymphoblasts. Proc. Natl. Acad. Sci. 69: 1244-1248.

Seegmiller, J. E., F. M. Rosenbloom, and W. N. Kelley (1967). Enzyme defect associated with a sex-linked human neurological disorder and excessive purine synthesis. Science 155: 1682-1684.

Shapiro, N. I., A. E. Khalizev, E. V. Luss, M. I. Marshak, O. N. Petrova, and N. B. Varshaver (1972a). Mutagenesis in cultured mammalian cells I. Spontaneous gene mutations in human and Chinese hamster cells. Mutation Res. 15: 203-214.

Shapiro, N. I., A. E. Khalizev, E. V. Luss, E. S. Manuilova, O. N. Petrova, and N. B. Varshaver (1972b). Mutagenesis in cultured mammalian cells II. Induction of gene mutations in CH cells. Mutation Res. 16: 89-101.

Sperling, O., G. Eilam, R. Schmidt, G. Mundel, and A. DeVries (1971). Purine base incorporation into erythrocyte nucleotides in hypoxanthine-guanine phosphoribosyl transferase deficiency. Biochem. Med. 5: 173-176.

Subak-Sharpe, H., R. R. Burk, and J. D. Pitts (1966). Metabolic cooperation by cell to cell transfer between genetically different mammalian cells in tissue culture. Heredity 21: 342-343.

Subak-Sharpe, H., R. R. Burk, and J. D. Pitts (1969). Metabolic cooperation between biochemically marked mammalian cells in tissue culture. J. Cell Sci. 4: 353-367.

Szybalski, W. (1959). Genetics of human cell lines. II. Method for determination of mutation rates to drug resistance. Exp. Cell Res. 18: 588-591.

Szybalski, W., and E. H. Szybalska (1962). Approaches to the genetic analysis of mammalian cells. Drug sensitivity as a genetic marker. Univ. Mich. Med. Bull. 28: 277-293.

Szybalaski, W., E. H. Szybalsak, and R. W. Brockman (1961). Biochemical basis of sequential mutations toward resistance to purine analogs in human cell lines. Proc. Amer. Assn. Cancer Res. 3: 272.

Szybalski, W., E. H. Szybalska and G. Ragni (1963). Genetic studies with human cell lines. Natl. Cancer Inst. Monograph 7: 75-89.

Taylor, M. W., M. Souhrada, and J. McCall (1971). New class of purine mutants of Chinese hamster ovary cells. Science 172: 162-163.

Thompson, L. H., R. Mankovitz, R. M. Baker, J. E. Till, L. Siminovitch and G. F. Whitmore (1970). Isolation of temperature-sensitive mutants of L-Cells. Proc. Nat. Acad. Sci. 66: 377-384.

Thompson, L. H., R. Mankovitz, R. M. Baker, J. A. Wright, J. E. Till, L. Siminovitch and G. F. Whitmore (1971). Selective and nonselective isolation of temperature-sensitive mutants of mouse L-cells and their characterization. J. Cell Physiol. 78: 431-440.

Tiemeier, D. C. and G. Milman (1972). Regulation of glutamine synthetase in cultured Chinese hamster cells. J. Biol. Chem. 247: 5722-5727.

Tomizawa, S. and L. Aronow (1960). Studies on drug resistance in mammalian cells. II. 6-mercaptopurine resistance in mouse fibroblasts. J. Pharm. Exp. Ther. 128: 107-114.

Vogel, F. (1970). Spontaneous mutation in man, 16-68, in Chemical Mutagenesis in Mammals and Man, (ed. by F. Vogel and G. Röhrborn). Springer-Verlag, New York.

Yu, C. K. and W. K. Sinclair (1964). Homogeneity and stability of chromosomes of Chinese hamster cells in vitro. Can. J. Genet. and Cytol. 6: 109-116.

APPENDICES

APPENDIX		PAGE
I.	Plating efficiency of diploid human fibroblasts in selective media, plated as 200 cells per 60 mm dish and grown for 9 to 14 days	139
II.	Cell counts and calculations for regression lines to estimate doubling time for CHWC1.	140
III.	8-azaguanine resistance in CHWC1 following two hours exposure to methyl methanesulfate (MMS).	141
IV.	8-azaguanine resistance in CHWC1 following 1 hour exposure to M-methyl-N'-nitro-N-nitroso-guanidine (MNNG)	146
V.	Selection of 8-azaguanine resistance colonies, spontaneously derived in two concentrations, 10 µg and 30 µg aza/ml with 0 and 42 hours expression time.	149
VI.	Cell counts and regression lines for estimation of doubling time in MENAFC10, 10 µg and 30 µg aza/ml for colonies 65350, 65171, 65281, 65274 and 65691.	151
VII.	Colonies selected at 10 µg aza/ml, 0 hours expression time.	162
VIII.	Colonies selected at 10 µg aza/ml, 42 hours mutation expression time.	172
IX.	Colonies selected at 30 µg aza/ml, 42 hours mutation expression time.	186

APPENDIX I

Plating efficiency of diploid human fibroblasts in selective media, plated as 200 cells per 60 mm dish and grown for 9 to 14 days.

Plating efficiency (average of 5 replicates)

	Media			
	MENAFC10	1 µg aza/ml	10 µg aza/ml	HAT[1]
SK4490[2]	39.6	37.3	33.8	0
	19.3	22.6	20.9	0
SK4489[3]	18.9	2.4	0	+
	10.2	9.7	0	+

[1]HAT = hypoxanthine 1.0 x 10^{-4}M, aminopterin 4.0 x 10^{-7}M and thymidine 1.6 x 10^{-5}M added to MENAFC10

[2]SK4490 = culture of skin from Lesch-Nyhan patient, HGPRT deficient

[3]SK4489 = culture of skin from father of 4490, HGPRT normal

0 = no cells

+ = scattered cells, but in groups less than 20 cells.

APPENDIX II

Cell counts and calculations for regression lines to estimate doubling time for CHWCl.

Cell counts x 10^3/60 mm dish, seeded at 6 x 10^4 cells/dish.

Hours	Exp. 194	Exp. 199	Exp. 214
18	31	20	76
	7	12	70
	22	18	78
24	48	14	64
	32	20	76
	83	18	80
42	213	82	352
	192	58	382
	227	84	414
48	294	94	515
	422	68	468
	277	112	504
66	1070	332	1640
	1036	294	1700
	1036	266	1540
72	1710	410	1786
	1728	380	1840
	1590	360	1930
Intercept	735.794	155.321	793.957
Slope	28.508	6.713	34.329
Values for y, given x:			
30	1.19 x 10^5	4.6 x 10^4	2.4 x 10^5
60	9.75 x 10^5	2.5 x 10^5	1.3 x 10^6

APPENDIX III

8-azaguanine resistance in CHWC1 following two hours exposure to methyl methanesulfate (MMS).

EXPERIMENT 60

Method: 8-azaguanine resistance

Cells: CHWC1/42 at 1.25 x 10^6/100 mm dish in 5 ml medium for 4 hours

Mutagen: 10^{-3}M MMS made up in serum-free medium from .2M MMS stock in distilled water, exposure 2 hours

Selection: 10 µg and 30 µg aza/ml in medium, 10 ml selective medium per dish, medium change every 3 or 4 days

Replicates: 10/treatment

plating efficiency (for estimation of cell survival)

Cells: CHWC1/42 at 200/35 mm dish in 1 ml medium

Mutagen: 10^{-3}M MMS (from above)

Replicates: 10/treatment

Results: Calculation of mutation frequency (F) and rate (a)

MMS	0		10^{-3}M	
PE	29.45		1.75	
µg aza/ml	10	30	10	30
total colonies (Cr)	367	33	883	429
CaNt*	83.06	12.9	171.595	94.32
Survivors (CNt) ^{1} (x 10^5)	22.1	18.4	1.31	1.53
^{2}	75	62.5	4.5	5.2

F ($\times 10^{-5}$)	16.6	1.79	674	280
a ($\times 10^{-5}$) [1]	3.758	.701	130.99	61.65
[2]	1.107	.206	38.132	18.138

*values from Capizzi and Jameson (1973)

[1]estimated by plating efficiency

[2]estimated by cell counts.

EXPERIMENT 65

Method: 8-azaguanine resistance

Cells: CHWCl/35 at 10^6/100 mm dish in 5 ml medium for 4 hours

Mutagen: MMS at 10^{-3}M (made up as in #60)

Selection: azaguanine at 10 µg/ml and 30 µg/ml in medium

Replicates: 10/treatment

plating efficiency

Cells: CHWCl/35 at 200/35 mm dish in 1 ml medium

Mutagen: 10^{-3}M MMS (from above)

Replicates: 10/treatment

RESULTS: Calculation of mutation frequency and rate

MMS	0		10^{-3}M	
PE	33.65		0.25	
µg aza/ml	10	30	10	30
total colonies	62	31	731	76
CaNt	20.52	12.335	146.65	23.94

survivors ($\times 10^5$)	1	30.3	33.7	.225	.25
	2	90	100	.669	.74
F ($\times 10^{-5}$)		2.05	0.92	3248.9	304
a ($\times 10^{-5}$)	1	0.677	0.366	651.8	95.8
	2	0.228	0.123	219.2	32.4

EXPERIMENT 69

Method: 8-azaguanine resistance

Cells: CHWCl/41 at 1.25 x 10^5/100 mm dish in 5 ml medium for 4 hours

Mutagen: MMS at 10^{-3}M (made up as for #60)

Selection: 10 µg aza/ml medium

Replicates: 10/treatment

plating efficiency

Cells: CHWCl/41 at 200/35 mm dish in 1 ml medium

Mutagen: 10^{-3}M MMS (from above)

Replicates: 10/treatment

Results: Calculation of mutation frequency and rate

MMS		0	10^{-3}M
PE		54.5	1.65
total colonies		287	343
CaNt		68.03	78.57
survivors ($\times 10^5$)	1	4.77	0.144
	2	11.97	0.362
F ($\times 10^{-5}$)		60.17	2381.9
a ($\times 10^{-5}$)	1	14.26	545.6
	2	5.68	217.04

EXPERIMENT 97

Method: 8-azaguanine resistance

Cells: CHWCl/63 at 1.25 x 10^5/100 mm dish in 5 ml medium for 4 hours

Mutagen: 10^{-3}M MMS (made up as for #60)

Selection: 10 µg aza/ml medium

Replicates: 10/treatment

plating efficiency

Cells: CHWCl/63 at 200/35 mm dish in 1 ml medium

Mutagen: 10^{-3}M MMS (from above)

Replicates: 10/treatment

Results: Calculation of mutation frequency and rate

MMS		0	10^{-3}M
PE		37.35	6.05
total colonies		141	459
CaNt		38.61	99.72
survivors ($\times 10^5$)	1	3.27	0.529
	2	11.97	1.94
F ($\times 10^{-5}$)		43.12	867.7
a ($\times 10^{-5}$)	1	11.81	188.5
	2	3.23	51.4

EXPERIMENT 104

Method: 8-azaguanine resistance

Cells: CHWCl/65 at 1.25 x 10^5/100 mm dish in 5 ml medium for 4 hours

Mutagen: 10^{-3}M MMS (made up as for #60)

Selection: 10 µg aza/ml medium

Replicates: 10/treatment

plating efficiency

Cells: CHWCl/65 at 200/35 mm dish in 1 ml medium

Mutagen: 10^{-3}M MMS (from above)

Replicates: 10/treatment

Results: Calculation of mutation frequency and rate

MMS		0	10^{-3}M
PE		35.75	0.15
total colonies		169	415
CaNt		44.49	91.8
survivors (x 10^5)	1	4.02	0.0188
	2	15.39	0.0646
F (x 10^{-5})		42.04	22133.3
a(x 10^{-5})	1	11.07	4896
	2	2.89	1421

APPENDIX IV

8-azaguanine resistance in CHWC1 following 1 hour exposure to M-methyl-N'-nitro-N-nitrosoguanidine (MNNG).

EXPERIMENTS 300 and 314

Method: 8-azaguanine resistance

Cells: CHWC1/27 at 1.25 x 10^5/100 mm dish in 5 ml medium for 4 hours

Mutagen: 10^{-7}M, 10^{-6}M, 10^{-5}M, 10^{-4}M as serial dilutions from stock MNNG 10^{-2}M, made up in serum-free medium

Selection: 10 µg aza/ml with no mutation expression time (0 hours) and 42 hours mutation expression time.

Replicates: 5 dishes/mutagen conc/time for mutation expression.

plating efficiency (for estimation of cell survival)

Cells: one 100 mm dish from each of the five MNNG concentrations and one without mutagen treatment trypsinized after mutagen quenching and seeded at 200 cells/35 mm dish in 1 ml medium

Replicates: 5/treatment

Results: #300 Calculation of mutation frequency (F) and rate (a)

MNNG	0	10^{-7}M	10^{-6}M	10^{-5}M	10^{-5}M
PE	30.9	34.8	15.2	3.2	0
	0 hours mutation expression time				
total colonies	1	12	5	3	0
CaNt	1.76	6.44	3.77	2.86	-
survivors (x 10^5) 1	1.93	2.18	0.95	0.2	-
2	3.775	4.25	1.86	0.393	-
F (x 10^{-5})	0.52	5.50	5.27	18.75	
a (x 10^{-5}) 1	0.912	2.95	3.97	17.88	
2	0.466	1.52	2.03	7.28	
	42 hours mutation expression time				
total colonies	107	72	86	4	0
CaNt	31.075	22.49	26.30	3.33	-
survivors (x 10^5) 1	1.93	2.18	0.95	0.2	-
2	8.55	9.63	4.21	0.711	
F (x 10^{-5})	55.44	33.03	90.53	20.00	
a (x 10^{-5}) 1	16.10	10.32	27.68	16.65	
2	3.63	2.34	6.25	4.68	

Results: #314 Calculation of mutation frequency (F) and rate (a)

MNNG	0	10^{-7}M	10^{-6}M	10^{-5}M	10^{-4}M
PE	44.1	49.1	42.0	7.6	0
		0 hours mutation expression time			
total colonies	0	2	0	0	0
CaNt	-	2.35	-	-	-
survivors (x 10^5) 1	-	3.07	-	-	-
2	-	4.20	-	-	-
F (x 10^{-5})	-	0.651	-	-	-
a (x 10^{-5}) 1	-	0.765	-	-	-
2	-	0.5595	-	-	-
		42 hours mutation expression time			
total colonies	223	224	189	16	0
CaNt	55.5	55.7	48.645	7.79	-
survivors (x 10^5) 1	2.76	3.07	2.63	0.475	-
2	8.50	9.52	8.14	1.47	-
F (x 10^{-5})	80.8	72.96	7.86	33.68	
a (x 10^{-5}) 1	20.11	18.14	18.50	16.40	
2	6.49	5.85	5.98	5.30	

APPENDIX V

Selection of 8-azaguanine resistance colonies, spontaneously derived in two concentrations, 10 µg and 30 µg aza/ml with 0 and 42 hours expression time.

EXPERIMENTS 354 and 418

Method:

Cells: CHWC1/33 at 1.25 x 10^5 cells/dish, total of twenty-six 100 mm dishes

Treatment: After 4 hours incubation, dishes were randomized and arranged into six treatment blocks; two blocks of 3 dishes each and four of 5 dishes each. One set of 3 dishes was for cells counts. One set of 5 dishes received 10 ml of 10 µg aza/ml each; and another set of 5 received 10 ml of 30 µg aza/ml each. The remaining thirteen dishes received 10 ml MENAFC10 each.

After a further 42 hour incubation, the thirteen dishes containing MENAFC10 were treated by the same method as the first thirteen dishes. Selective media was renewed in the other ten dishes.

The selective media were changed every 3 or 4 days until colonies were large enough to pick.

Results: #354 Calculation of mutation rate (a)

expression time (hours)	0		42	
µg aza/ml	10	30	10	30
Cr	8	0	18	1
CaNt	4.98	-	8.44	1.76
C Nt	3.48×10^{5}	-	6.05×10^{5}	4.84×10^{5}
a	1.43×10^{-5}	-	1.40×10^{-5}	$.364 \times 10^{-5}$

#418 Calculation of mutation rate (a)

expression time (hours)	0		42	
µg aza/ml	10	30	10	30
Cr	1	2	19	3
CaNt	1.76	2.35	8.755	2.86
C Nt	2.76×10^{5}	6.9×10^{5}	1.06×10^{6}	1.91×10^{6}
a	$.638 \times 10^{-5}$	$.341 \times 10^{-5}$	8.26×10^{-6}	1.50×10^{-6}

APPENDIX VI

Cell counts and regression lines for estimation of doubling time in MENAFC10 10 µg and 30 µg aza/ml for colonies 65350, 65171, 65281, 65274 and 65691.

65350, 24th passage

cell counts ($x10^5$)

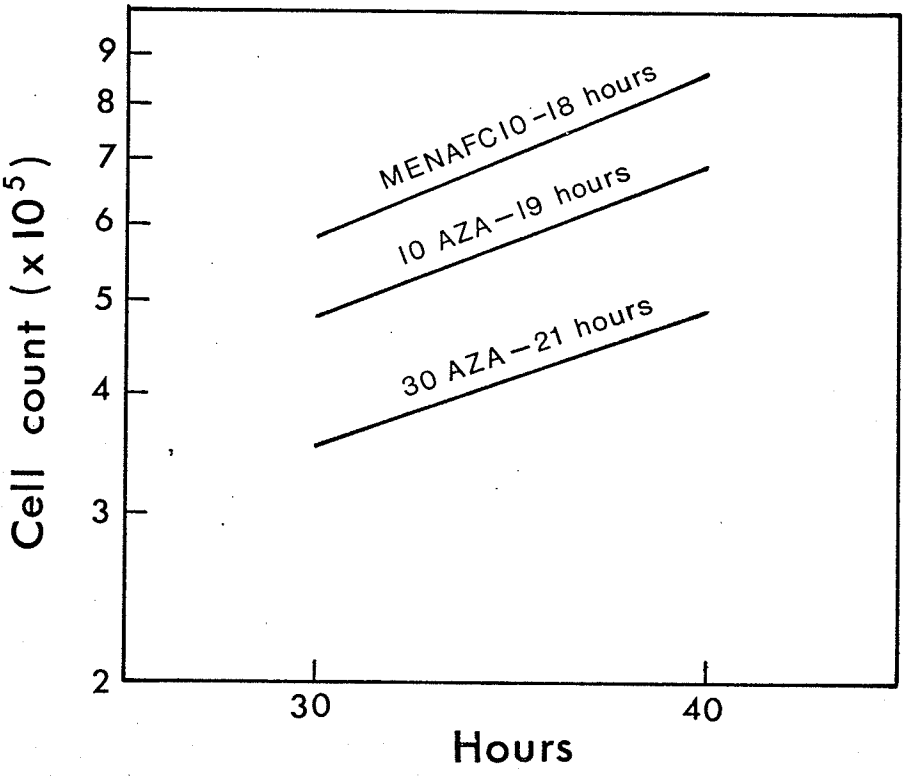
	24 hours		54 hours	
MENAFC10	5.02	4.52	10.28	16.62
	3.92	6.22	10.16	8.12
	4.12	3.32	12.04	12.64
	2.48	3.61	11.22	11.88
	3.60	4.40	17.56	15.12
10 µg aza	3.28	3.80	8.68	7.30
	3.36	3.64	11.88	12.20
	4.46	3.44	12.84	7.74
	3.52	3.00	10.46	9.86
	3.36	3.44	7.94	9.88
30 µg aza	2.66	2.08	6.86	8.24
	2.16	3.16	8.44	8.82
	1.16	3.12	3.70	6.30
	2.76	3.72	6.08	6.16
	3.02	3.10	7.72	5.60

regression lines

calculation of y when x = 30 and 40 hours

	30	40
MENAFC10	5.8×10^5	8.6×10^5
10 µg aza	4.8×10^5	6.9×10^5
30 µg aza	3.5×10^5	4.9×10^5

Regression Lines for No. 65350



65171, 20th passage

cell counts ($x10^5$)

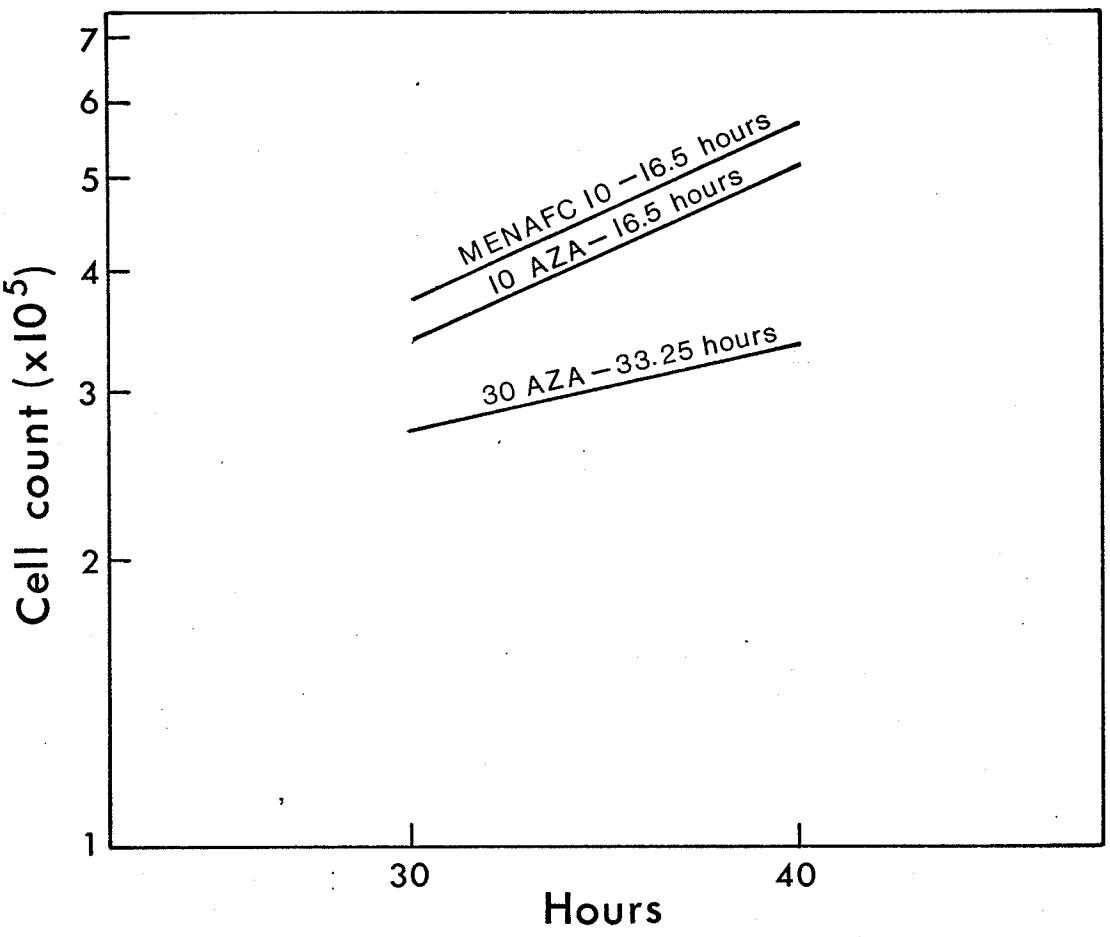
	24 hours		54 hours	
MENAFC10	2.68	2.60	7.38	7.82
	2.24	2.70	9.02	10.06
	2.20	3.10	7.40	7.02
	2.12	2.72	8.72	8.14
	2.42	2.36	9.26	10.04
10 µg aza	2.20	2.00	6.58	7.94
	2.34	2.52	5.04	9.26
	2.30	3.02	7.82	8.96
	2.26	2.72	8.44	6.76
	2.10	1.96	7.74	6.62
30 µg aza	2.26	2.40	4.57	3.94
	2.58	2.16	3.54	4.14
	2.22	2.54	4.72	4.32
	2.12	2.58	4.38	4.58
	2.18	1.82	4.40	3.98

regression lines

calculation of 7 when x = 30 and 40 hours

	30	40
MENAFC10	3.7×10^5	5.7×10^5
10 µg aza	3.4×10^5	5.1×10^5
30 µg aza	2.7×10^5	3.4×10^5

Regression Lines for No. 65171



65281, 22th passage

cell counts ($x10^5$)

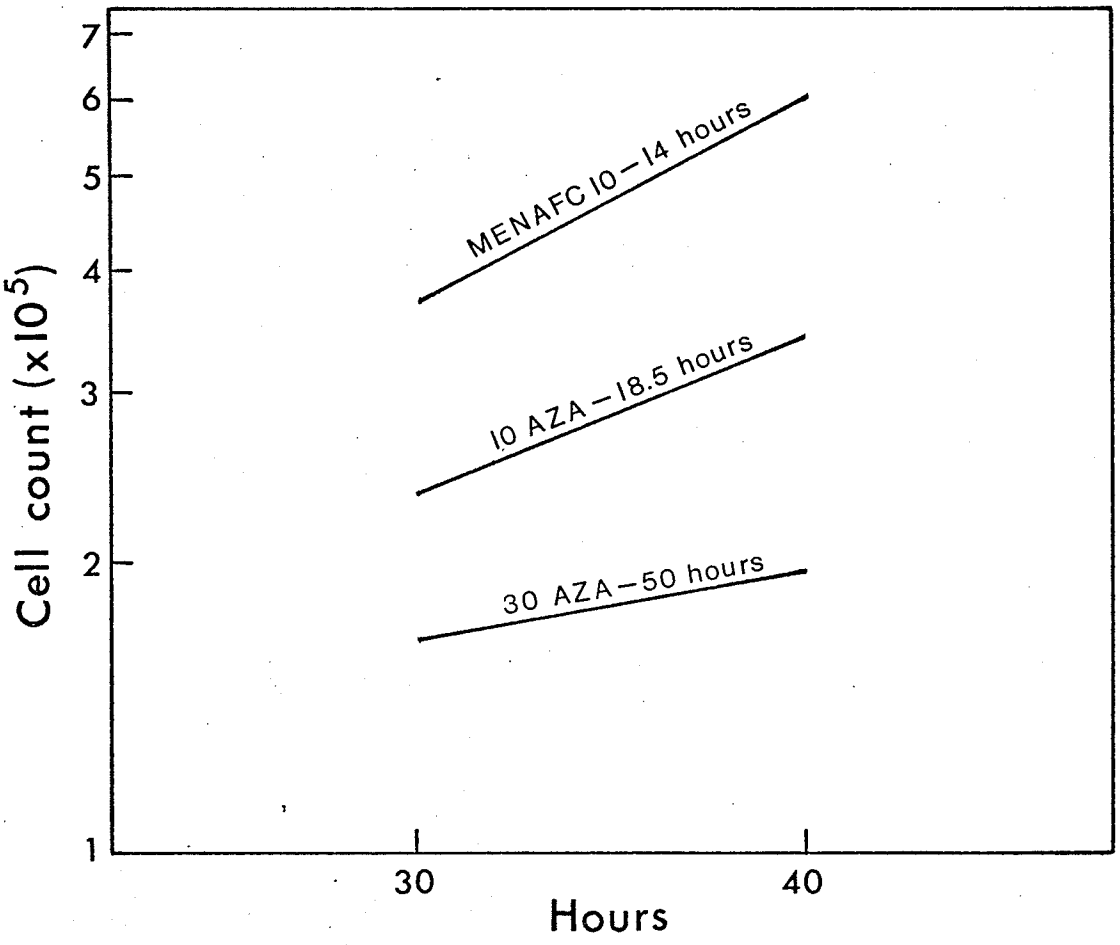
	24 hours		54 hours	
MENAFC10	2.44	2.48	6.58	10.12
	2.40	2.50	8.42	8.68
	1.94	2.52	13.46	9.66
	2.36	2.82	11.80	10.20
	2.08	0.92	7.46	8.60
10 µg aza	1.68	1.84	5.48	4.14
	1.80	1.10	5.70	5.02
	2.18	2.18	4.60	5.44
	1.36	2.16	4.48	4.02
	1.30	0.82	4.14	6.20
30 µg aza	1.46	1.76	2.50	2.80
	2.00	1.30	1.96	2.72
	1.36	1.80	1.72	2.26
	1.00	1.54	2.52	3.58
	1.40	1.38	2.12	1.64

regression lines

calculation of y when x = 30 and 40 hours

	30	40
MENAFC10	3.8×10^5	6.1×10^5
10 µg aza	2.4×10^5	3.4×10^5
30 µg aza	1.7×10^5	1.95×10^5

Regression Lines for No. 65281



65274, 6th passage

cell counts ($x10^5$)

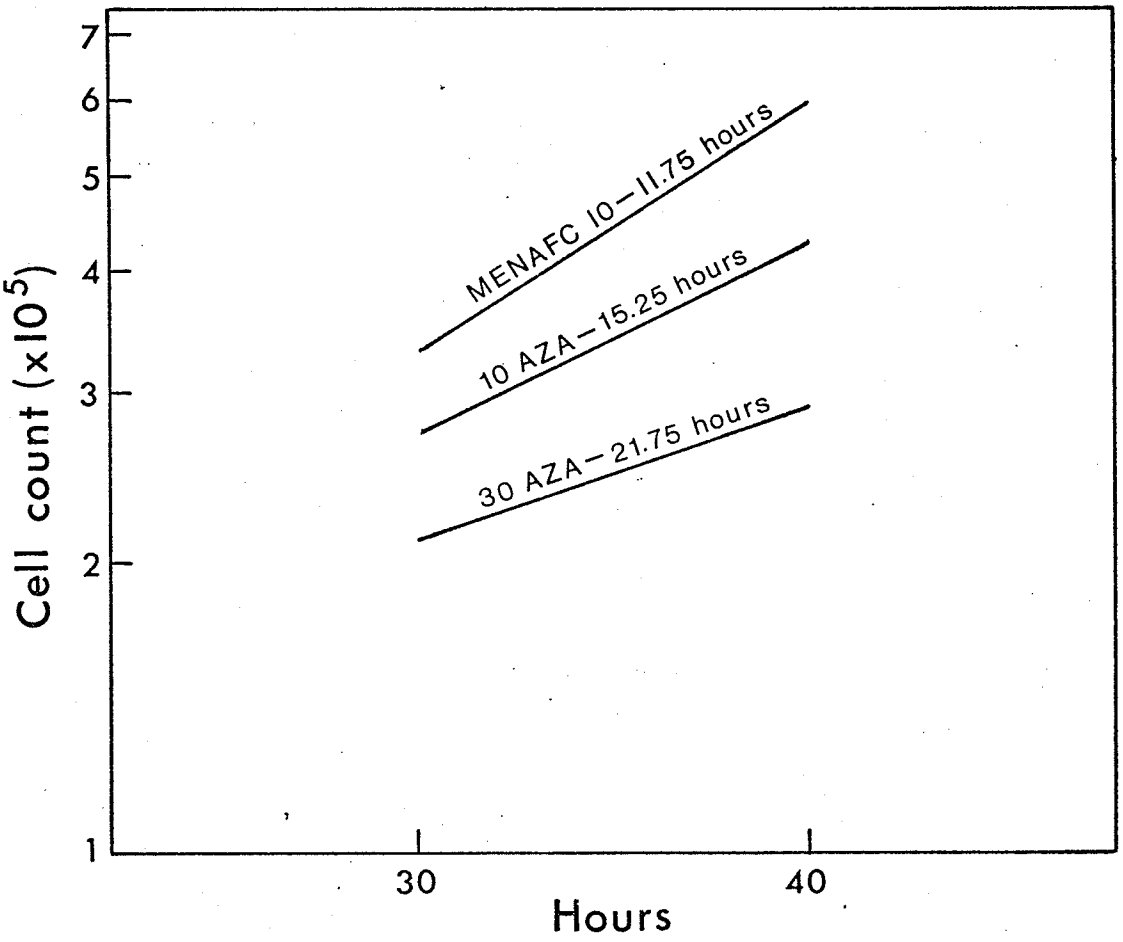
	24 hours	54 hours
MENAFC10	2.06	9.18
	1.26	9.32
	1.58	9.04
	1.86	11.10
	1.82	10.66
10 µg aza	1.78	8.54
	1.56	6.04
	1.58	7.46
	1.70	5.12
	2.16	5.88
30 µg aza	1.66	3.88
	1.80	4.82
	2.16	4.42
	1.24	
	1.46	2.89

regression lines

calculation of y when x = 30 and 40 hours

	30	40
MENAFC10	3.3×10^5	6.0×10^5
10 µg aza	2.7×10^5	4.4×10^5
30 µg aza	2.1×10^5	2.9×10^5

Regression Lines for No. 65274



65691, 20th passage

cell counts ($x10^5$)

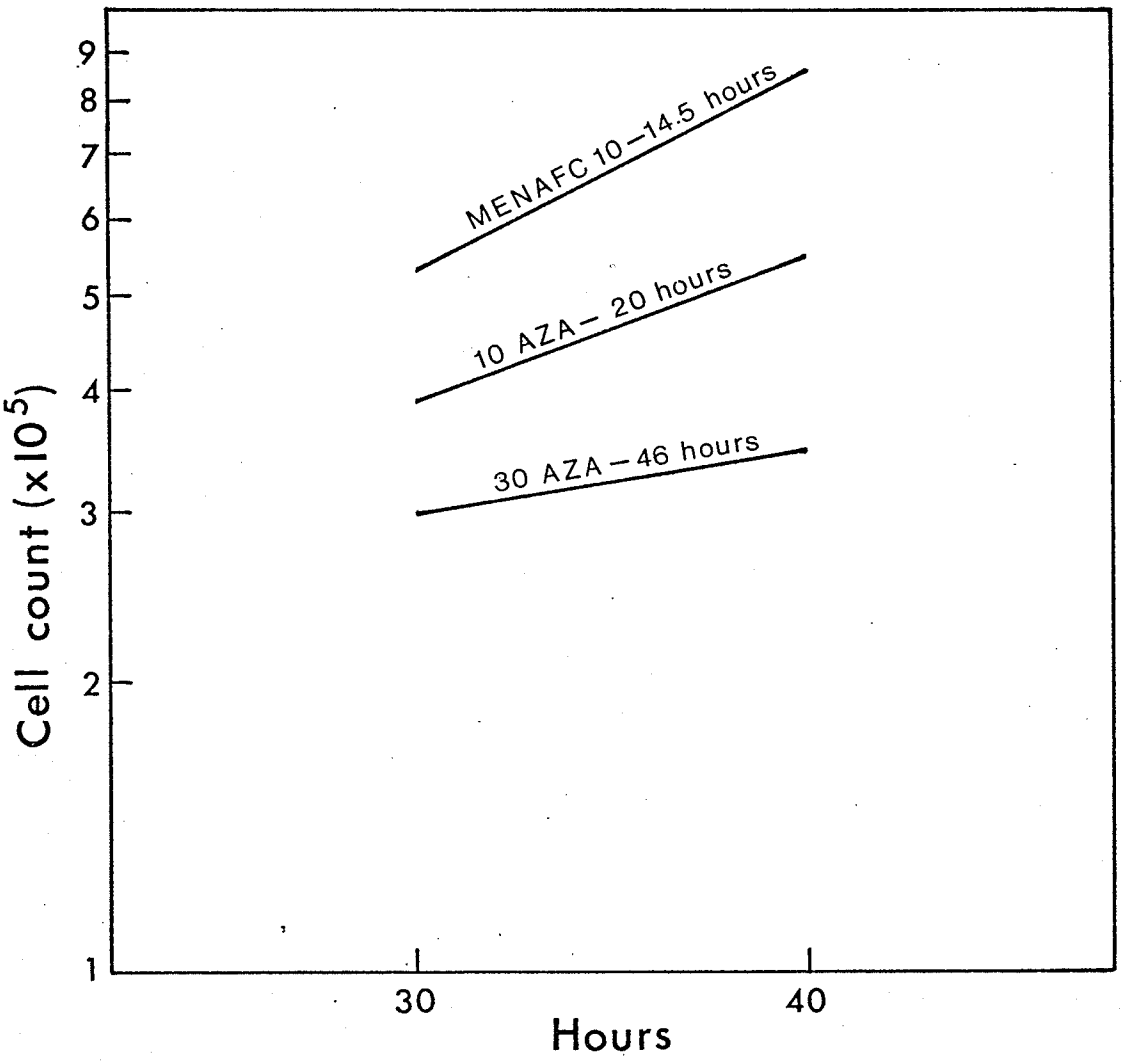
	24 hours		54 hours	
MENAFC10	3.90	3.82	12.60	10.00
	3.30	3.24	11.55	12.10
	3.52	4.08	12.18	13.25
	2.90	3.02	14.10	15.10
	2.80	3.60	15.32	15.80
10 µg aza	2.24	3.34	7.16	6.94
	2.18	4.56	7.78	6.82
	2.62	3.20	6.62	7.94
	2.24	3.26	10.70	8.48
	3.18	2.90	8.50	7.20
30 µg aza	1.84	2.96	4.22	5.16
	2.60	2.90	4.36	3.54
	2.88	2.44	3.60	3.10
	2.54	3.66	3.22	5.04
	2.48	2.70	5.52	3.76

regression lines

calculation of y when x = 30 and 40 hours

	30	40
MENAFC10	5.4×10^5	8.6×10^5
10 µg aza	3.9×10^5	5.5×10^5
30 µg aza	3×10^5	3.5×10^5

Regression Lines for No. 65691



1102 (clone of 65691), 38th passage

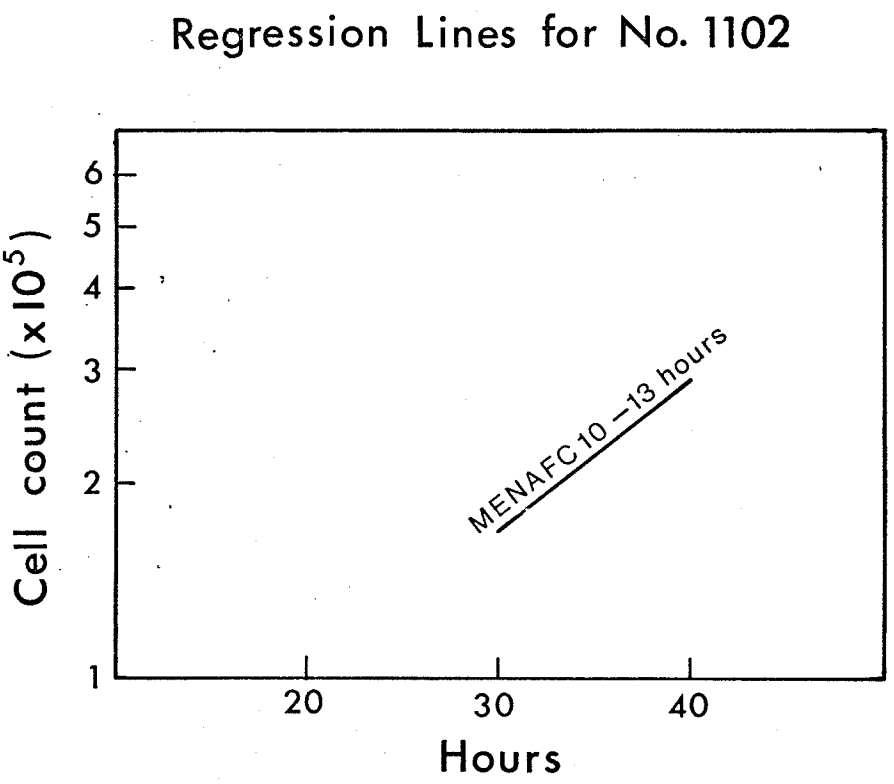
Cell counts ($x10^5$) in MENAFC10

18 hrs.	24 hrs.	42 hrs.	48 hrs.	66 hrs.	72 hrs.
.62	1.18	1.82	3.26	10.9	10.6
.58	1.10	2.12	3.40	10.0	12.1
.60	1.12	1.76	3.12	12.7	14.0

regression line

calculation of y when x = 30 and 40 hours

	30	40
MENAFC10	1.69×10^5	8.42×10^5



APPENDIX VII

Colonies selected at 10 µg aza/ml, 0 hours expression time.

Colony 354-08-01. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
44	71.5	$.45 \times 10^5$	1.07×10^5	4.57	15.6
45	96.5	$.321 \times 10^5$	2.21×10^6	6.11	15.8
46	74	$.354 \times 10^5$	7.55×10^5	4.41	16.8
47	99	$.302 \times 10^5$	1.785×10^6	5.89	16.8
48	94	$.321 \times 10^5$	1.865×10^6	5.86	16
49	72	$.336 \times 10^5$	6.05×10^5	4.17	17.3
50	70.5	$.363 \times 10^5$	7.10×10^5	4.29	16.4
51	99.5	$.355 \times 10^5$	2.115×10^6	5.9	16.9
52	66	$.329 \times 10^6$	5.82×10^5	4.14	15.9
					16.4

10 µg aza/ml

44	71.5	$.45 \times 10^5$	4.55×10^5	3.34	21.4
45	96.5	$.364 \times 10^5$	1.185×10^6	5.02	19.2
46	74	$.356 \times 10^5$	1.115×10^5	4.97	14.9
47	99	$.312 \times 10^5$	1.15×10^6	5.2	19
48	94	$.322 \times 10^5$	1.77×10^6	5.78	16.3
49	72	$.329 \times 10^5$	4.30×10^5	3.71	19.4
50	70.5	$.344 \times 10^5$	7.05×10^5	4.36	16.2
51	99.5	$.355 \times 10^5$	3.925×10^6	6.79	14.7
52	66	$.325 \times 10^5$	1.92×10^5	2.56	25.8
					16.2

Colony 354-08-02. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
27	77.5	$.27 \times 10^5$	6.15×10^5	4.51	17.2
38	98	$.246 \times 10^5$	2.9×10^6	6.88	14.2
39	70	$.348 \times 10^5$	7.0×10^5	4.33	16.2
40	72	$.36 \times 10^5$	9.0×10^5	4.64	15.5
41	71.5	$.32 \times 10^5$	5.65×10^5	4.14	17.3
42	96.5	$.396 \times 10^5$	1.705×10^6	5.43	17.8
43	68.5	$.341 \times 10^5$	4.7×10^5	3.78	18.1
44	99	$.319 \times 10^5$	2.99×10^6	6.55	15.1
45	75	$.299 \times 10^5$	6.0×10^5	4.33	17.3
					16.5

10 µg aza/ml

37	77.5	$.27 \times 10^5$	4.4×10^5	4.03	19.2
38	98	$.264 \times 10^5$	1.72×10^6	6.03	16.3
39	70	$.34 \times 10^5$	6.0×10^5	4.14	16.9
40	72	$.36 \times 10^5$	4.35×10^5	3.59	20.1
41	71.5	$.348 \times 10^5$	4.0×10^5	3.52	20.3
42	96.5	$.4 \times 10^6$	1.585×10^6	5.31	18.2
43	68.5	$.317 \times 10^5$	5.4×10^5	4.09	16.7
44	99	$.324 \times 10^5$	1.525×10^6	5.56	17.8
45	75	$.305 \times 10^5$	4.7×10^5	3.95	19
					18.3

Colony 354-08-03. Cell counts and estimation of doubling time t/g in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
40	72	$.52 \times 10^5$	1.595×10^6	4.94	14.6
41	52	$.319 \times 10^5$	4.8×10^5	3.91	13.3
42	70.5	$.288 \times 10^5$	5.8×10^5	4.33	16.3
43	68.5	$.313 \times 10^5$	7.15×10^5	4.51	15.2
44	77.5	$.286 \times 10^5$	1.39×10^6	5.6	13.8
45	97	$.278 \times 10^5$	1.25×10^6	5.49	17.7
46	93	$.275 \times 10^5$	1.59×10^6	5.85	15.9
47	68.5	$.318 \times 10^5$	8.0×10^5	4.65	14.7
48	75	$.32 \times 10^5$	1.236×10^6	5.27	14.2
49	69.5	$.306 \times 10^5$	1.032×10^6	5.08	13.7
					14.9

10 µg aza/ml

40	72	$.52 \times 10^5$	7.5×10^5	3.85	18.7
41	52	$.3 \times 10^5$	1.45×10^5	2.27	22.9
42	70.5	$.29 \times 10^5$	8.15×10^5	4.81	14.7
43	68.5	$.326 \times 10^5$	4.35×10^5	3.74	18.3
44	77.5	$.278 \times 10^5$	7.2×10^5	4.69	16.5
45	97	$.288 \times 10^5$	1.37×10^6	5.57	17.4
46	93	$.274 \times 10^5$	1.465×10^6	5.74	16.2
47	68.5	$.322 \times 10^5$	1.06×10^6	5.04	13.6
48	75	$.318 \times 10^5$	6.6×10^5	4.38	17.1
49	69.5	$.308 \times 10^5$	6.24×10^5	4.34	16
					17.1

Colony 354-08-04. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
39	73.5	$.498 \times 10^5$	5.85×10^5	3.55	20.7
40	70.5	$.351 \times 10^5$	5.1×10^5	3.86	18.3
41	69	$.306 \times 10^5$	3.70×10^5	3.6	19.2
42	77.5	$.296 \times 10^5$	8.0×10^5	4.76	16.3
43	72.5	$.32 \times 10^5$	4.35×10^5	3.76	19.3
44	94.5	$.305 \times 10^5$	1.585×10^6	5.7	16.6
45	70	$.317 \times 10^5$	4.15×10^5	3.71	18.9
					18.5

10 µg aza/ml

39	73.5	$.498 \times 10^5$	2.7×10^5	2.44	30.1
40	70.5	$.34 \times 10^5$	4.05×10^5	3.57	19.7
41	69	$.324 \times 10^5$	2.55×10^5	2.98	23.2
42	77.5	$.306 \times 10^5$	6.45×10^5	4.4	17.6
43	72.5	$.323 \times 10^5$	4.5×10^5	3.8	19.1
44	94.5	$.3 \times 10^5$	8.75×10^5	4.87	19.4
45	70	$.35 \times 10^5$	3.9×10^5	3.48	20.1
					21.3

Colony 354-08-05. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
34	96.5	$.613 \times 10^5$	2.09×10^6	5.09	19
35	68	$.31 \times 10^5$	2.1×10^5	2.76	24.6
36	77.5	$.77 \times 10^5$	1.74×10^6	4.5	17.2
37	119.5	$.348 \times 10^5$	7.65×10^6	7.78	15.4
38	70.5	$.306 \times 10^5$	1.02×10^6	5.06	13.9
39	145.5	$.306 \times 10^5$	1.66×10^6	5.76	25.3
40	95.5	$.33 \times 10^5$	1.26×10^6	5.25	18.2
41	67.5	$.3024 \times 10^5$	6.4×10^5	4.4	15.3
42	100	$.307 \times 10^5$	1.965×10^6	6	16.7
					18.4

10 µg aza/ml

34	96.5	$.62 \times 10^5$	1.324×10^6	4.42	21.8
35	68	$.34 \times 10^5$	3.85×10^5	3.5	19.4
36	77.5	$.77 \times 10^5$	1.05×10^6	3.76	20.6
37	119.5	$.312 \times 10^5$	1.6×10^6	5.68	21
38	70.5	$.32 \times 10^5$	3.5×10^5	3.45	20.4
39	145.5	$.35 \times 10^5$	1.525×10^6	5.45	26.7
40	95.5	$.305 \times 10^5$	2.4×10^6	6.3	15.2
41	67.5	$.288 \times 10^5$	3.05×10^5	3.4	19.9
42	100	$.305 \times 10^5$	1.09×10^6	5.16	19.4
					20.3

Colony 354-08-08. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
17	72.5	$.54 \times 10^5$	1.665×10^6	4.95	14.6
18	97.5	$.33 \times 10^5$	1.895×10^6	5.84	16.7
19	74.5	$.379 \times 10^5$	6.45×10^5	4.09	18.2
20	98.5	$.387 \times 10^5$	2.26×10^6	5.87	16.8
21	77.5	$.345 \times 10^5$	1.5×10^6	5.44	14.2
22	98	$.3 \times 10^5$	1.85×10^6	5.95	16.5
23	68.5	$.37 \times 10^5$	7.05×10^5	4.25	16.1
24	78.5	$.352 \times 10^5$	1.15×10^6	5.03	15.6
25	89.5	$.345 \times 10^5$	1.42×10^6	5.36	16.7
					16.2

10 µg aza/ml

17	72.5	$.54 \times 10^5$	2.7×10^5	2.32	31.3
18	97.5	$.324 \times 10^5$	1.52×10^6	5.55	17.6
19	74.5	$.304 \times 10^5$	4.85×10^5	4	18.6
20	98.5	$.388 \times 10^5$	1.78×10^6	5.52	17.8
21	77.5	$.356 \times 10^5$	1.08×10^6	4.92	15.8
22	98	$.3 \times 10^5$	8.5×10^5	4.82	20.3
23	68.5	$.34 \times 10^5$	3.6×10^5	3.4	20.1
24	78.5	$.36 \times 10^5$	6.6×10^5	4.2	18.7
25	89.5	$.33 \times 10^5$	1.302×10^6	5.3	16.9
					19.7

Colony 518-04-01. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
6	76	$.53 \times 10^5$	6.25×10^5	3.56	21.3
7	93	$.375 \times 10^5$	1.167×10^6	4.96	18.8
8	121.5	$.389 \times 10^5$	1.167×10^6	4.91	24.7
9	98.5	$.389 \times 10^5$	2.07×10^6	5.73	17.2
10	91.5	$.331 \times 10^5$	2.125×10^6	6	15.3
11	72	$.34 \times 10^5$	9.25×10^5	4.77	15.1
12	77	$.37 \times 10^5$	7.85×10^5	4.41	17.5
13	93.5	$.314 \times 10^5$	1.64×10^6	5.71	16.4
14	71	$.328 \times 10^5$	1.41×10^6	5.43	13.1
					17.7

10 µg aza/ml

6	76	$.53 \times 10^5$	5.4×10^5	3.35	22.7
7	93	$.378 \times 10^5$	1.11×10^6	4.88	19.1
8	121.5	$.377 \times 10^5$	1.41×10^6	5.22	23.3
9	98.5	$.367 \times 10^5$	1.08×10^6	4.88	20.2
10	91.5	$.324 \times 10^5$	8.4×10^5	4.7	19.5
11	72	$.336 \times 10^5$	2.6×10^5	2.95	24.4
12	77	$.364 \times 10^5$	6.25×10^5	4.11	18.7
13	93.5	$.3 \times 10^5$	8.25×10^5	4.78	19.6
14	71	$.33 \times 10^5$	2.35×10^5	2.83	25.1
					21.4

Colony 518-12-01. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
6	76	$.575 \times 10^5$	1.115×10^6	4.28	17.8
7	93	$.335 \times 10^5$	1.11×10^6	5.05	18.4
8	122	$.303 \times 10^5$	1.375×10^6	5.5	22.2
9	99	$.303 \times 10^5$	1.25×10^6	5.37	18.4
10	91	$.275 \times 10^5$	1.5×10^6	5.77	15.8
11	73	$.3 \times 10^5$	5.35×10^5	4.16	17.5
12	73	$.321 \times 10^5$	7.2×10^5	4.49	16.3
13	96	$.36 \times 10^5$	1.410×10^6	5.29	18.1
14	93.5	$.31 \times 10^5$	1.81×10^6	5.87	15.9
					17.8

10 µg aza/ml

6	76	$.575 \times 10^5$	3.05×10^5	2.41	31.5
7	93	$.305 \times 10^5$	7.45×10^5	4.61	20.2
8	122	$.298 \times 10^5$	7.55×10^5	4.66	26.2
9	99	$.302 \times 10^5$	1.185×10^6	5.29	18.7
10	91	$.284 \times 10^5$	6.15×10^5	4.44	20.5
11	73	$.308 \times 10^5$	4.7×10^5	3.93	18.6
12	73	$.310 \times 10^5$	2.7×10^5	3.12	23.4
13	96	$.36 \times 10^5$	1×10^6	4.8	30.8
14	93.5	$.3 \times 10^5$	7.9×10^5	4.72	19.8
					23.3

Colony 518-14-01. Cell counts and estimation of doubling time t/g in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
6	75.5	$.477 \times 10^5$	2.04×10^6	5.42	13.9
7	92	$.286 \times 10^5$	7.25×10^5	4.66	19.7
8	120	$.29 \times 10^5$	1.09×10^6	5.23	22.9
9	100	$.327 \times 10^5$	1.305×10^6	5.32	18.8
10	92.5	$.261 \times 10^5$	2.25×10^6	6.43	14.4
11	149	$.27 \times 10^5$	5.4×10^6	7.64	19.5
12	93	$.24 \times 10^5$	1.65×10^6	6.10	15.2
13	93	$.33 \times 10^5$	1.585×10^6	5.59	16.6
					17.6

10 µg aza/ml

6	75.5	$.477 \times 10^5$	4.75×10^5	3.32	22.7
7	92	$.285 \times 10^5$	3.65×10^5	3.67	25.1
8	120	$.292 \times 10^5$	9.05×10^5	4.95	24.2
9	100	$.362 \times 10^5$	1.115×10^6	4994	20.2
10	92.5	$.245 \times 10^5$	5×10^5	4.35	21.3
11	149	$.27 \times 10^5$	1.35×10^6	5.64	26.4
12	93	$.24 \times 10^5$	7.65×10^5	4.99	18.6
13	93	$.306 \times 10^5$	9.15×10^5	4.90	18.9
					22.2

Colony 518-17-01. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
6	76	.56 $x10^5$	6.25 $x10^5$	3.48	21.8
7	71	.375$x10^5$	5.1 $x10^5$	3.77	18.8
8	71.5	.377$x10^5$	5.05 $x10^5$	3.74	19.1
9	74.5	.364$x10^5$	5.4 $x10^5$	3.89	19.2
10	74	.324$x10^5$	1.1 $x10^6$	5.09	14.5
11	91	.3 $x10^5$	1.05 $x10^6$	5.13	17.7
12	74	.314$x10^5$	8.8 $x10^5$	4.81	15.4
13	100	.352$x10^5$	2.04 $x10^6$	5.86	17.1
14	65	.34 $x10^5$	9.95 $x10^5$	4.87	13.3
					17.4

10 µg aza/ml

6	76	.56 $x10^5$	2.75 $x10^5$	2.3	33
7	71	.385$x10^5$	3.8 $x10^5$	3.3	21.5
8	71.5	.38 $x10^5$	2.2 $x10^5$	2.53	28.3
9	74.5	.364$x10^5$	4.5 $x10^5$	3.63	20.5
10	74	.3 $x10^5$	3.75 $x10^5$	3.64	20.3
11	91	.3 $x10^5$	5.3 $x10^5$	4.14	22
12	74	.318$x10^5$	2.6 $x10^5$	3.03	24.4
13	100	.364$x10^5$	1.56 $x10^6$	5.42	18.5
14	65	.39 $x10^5$	2.82 $x10^5$	2.85	22.8
					23.5

APPENDIX VIII

Colonies selected at 10 µg aza/ml, 42 hours mutation expression time.

Colony 354-02-01. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
14	76.5	.89 $x10^5$	1.88 $x10^6$	4.4	17.4
15	95.5	.27 $x10^5$	1.2 $x10^6$	5.47	17.5
16	71	.48 $x10^5$	1.09 $x10^6$	4.51	15.7
17	97.5	.217$x10^5$	1.08 $x10^6$	5.64	17.3
18	72.5	.21 $x10^5$	6.8 $x10^5$	5.02	14.4
19	96	.27 $x10^5$	1.23 $x10^6$	5.51	17.4
20	67.5	.246$x10^5$	5 $x10^5$	4.35	15.5
21	97	.25 $x10^5$	1.23 $x10^6$	5.62	17.3
22	74.5	.246$x10^5$	8.20 $x10^5$	5.06	14.7
					16.4

10 µg aza/ml

14	76.5	.825$x10^5$	1.35 $x10^6$	4.03	19.0
15	95.5	.27 $x10^5$	1.15 $x10^6$	5.41	17.7
16	71	.45 $x10^5$	5.85 $x10^5$	3.7	19.2
17	97.5	.211$x10^5$	5.25 $x10^5$	4.64	21.0
18	72.5	.21 $x10^5$	2.5 $x10^5$	3.57	20.3
19	96	.25 $x10^5$	1.34 $x10^6$	5.74	16.7
20	67.5	.268$x10^5$	3.5 $x10^5$	3.71	18.2
21	97	.21 $x10^5$	1.34 $x10^6$	6.00	16.2
22	74.5	.267$x10^5$	3.30 $x10^5$	3.63	20.5
					18.8

Colony 354-02-02. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
31	72	.5 $x10^5$	7.8 $x10^5$	3.96	18.2
32		.343$x10^5$	ND	-	
33	72	ND	2.45 $x10^5$	-	
34	96.5	.49 $x10^5$	1.19 $x10^6$	4.6	21
35	94	.356$x10^5$	6.65 $x10^5$	4.22	22.3
36	95	.306$x10^5$	3.75 $x10^5$	3.61	26.3
37	68	.375$x10^5$	5 $x10^5$	3.74	18.2
38	76.5	.375$x10^5$	9.18 $x10^5$	4.61	16.6
39	96	.306$x10^5$	1.17 $x10^6$	5.24	18.3
40	71	.351$x10^5$	6.15 $x10^5$	4.13	17.2
					19.8

10 µg aza/ml

31	72	.5 $x10^5$	3.4 $x10^5$	2.77	26
32		.34 $x10^5$	ND		
33	72	ND	4.2 $x10^5$		
34	96.5	.42 $x10^5$	7.75 $x10^5$	4.21	22.9
35	94	.331$x10^5$	7.3 $x10^5$	4.46	21.1
36	95	.321$x10^5$	3.90 $x10^5$	3.6	26.4
37	68	.390$x10^5$	2.5 $x10^5$	2.68	25.4
38	76.5	.75 $x10^5$	6.195$x10^5$	3.05	25.1
39	96	.301$x10^5$	4.2 $x10^5$	3.8	25.3
40	71	.336$x10^5$	4.65 $x10^5$	3.79	18.7
					23.7

Colony 354-02-03. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
17	65	$.525 \times 10^5$	2.25×10^5	5.42	30.4
18	144	$.45 \times 10^5$	1.645×10^6	5.19	27.7
19	168	$.329 \times 10^5$	1.01×10^6	4.94	34
20	174	$.303 \times 10^5$	3.66×10^6	6.92	25.1
21	162	$.305 \times 10^5$	5.2×10^5	4.09	39.6
22	70	$.416 \times 10^5$	3.0×10^5	2.85	24.6
					30.2

(medium change between every passage, 3 or 4 days)

10 µg aza/ml

17	165	$.525 \times 10^5$	3.5×10^5	2.74	60.2
18	144	$.35 \times 10^5$	2.85×10^5	3.03	47.5
19	168	$.314 \times 10^5$	1.035×10^6	5.04	31.3
20	174	$.311 \times 10^5$	3.4×10^6	6.77	27.7
21	162	$.34 \times 10^5$	6.35×10^5	4.22	38.4
22	70	$.445 \times 10^5$	3.20×10^5	2.85	24.6
					38.3

Colony 354-04-01. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
26	97	.53 $x10^5$	1.244$x10^6$	4.55	21.3
27	94	.311$x10^5$	5 $x10^5$	4.01	23.4
28	73	.4 $x10^5$	6.25 $x10^5$	3.64	20.1
29	95.5	.388$x10^5$	9 $x10^5$	4.54	21.0
30	73	.384$x10^5$	6.5 $x10^5$	4.08	17.9
31	98	.39 $x10^5$	1.12 $x10^6$	4.84	20.2
32	66	.29 $x10^5$	1.75 $x10^5$	2.59	25.5
33	78	.35 $x10^5$	7.8 $x10^5$	4.48	17.4
34	68.5	.312$x10^5$	4.65 $x10^5$	3.898	17.6
					20.4

10 µg aza/ml

26	97	.54 $x10^5$	6.52 $x10^5$	3.59	27.0
27	94	.326$x10^5$	2.5 $x10^5$	2.94	32.0
28	73	.5 $x10^5$	4.0 $x10^5$	3.00	24.3
29	95.5	.4 $x10^5$	6.1 $x10^5$	3.93	24.3
30	73	.4 $x10^5$	1.7 $x10^5$	2.09	34.9
31	98	.34 $x10^5$	7.6 $x10^5$	4.48	21.9
32	66	.304$x10^5$	2.4 $x10^5$	2.98	22.1
33	78	.34 $x10^5$	4.3 $x10^5$	3.66	21.3
34	68.5	.344$x10^5$	3.8 $x10^5$	3.47	19.7
					25.3

Colony 354-12-01. Cell counts and estimation of doubling time (t/g) in mass cultures for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
38	76.5	$.24 \times 10^5$	7.4×10^5	4.95	15.5
39	97.5	$.296 \times 10^5$	1.2×10^6	5.34	18.3
40	69.5	$.239 \times 10^5$	5.45×10^5	4.51	15.4
*41	146.5	$.327 \times 10^5$	1.04×10^6	4.99	29.4
42	96	$.318 \times 10^5$	1.11×10^6	5.13	18.7
43	92	$.333 \times 10^5$	1.26×10^6	5.24	17.6
44	73.5	$.328 \times 10^5$	5.7×10^5	4.12	17.8
45	74.5	$.308 \times 10^5$	5×10^5	4.02	18.5
46	95.5	$.33 \times 10^5$	1.465×10^6	5.47	17.5
47	68	$.352 \times 10^5$	5.9×10^5	4.06	16.7
					17.3

10 µg aza/ml

38	76.5	$.24 \times 10^5$	2.5×10^5	3.38	22.6
39	97.5	$.25 \times 10^5$	7.85×10^5	4.97	19.6
40	69.5	$.236 \times 10^5$	1.5×10^5	2.67	26
*41	146.5	$.3 \times 10^5$	9.3×10^5	4.95	29.6
42	96	$.372 \times 10^5$	1.05×10^6	4.82	19.9
43	62	$.327 \times 10^5$	7.2×10^5	4.46	20.6
44	73.5	$.331 \times 10^5$	4.35×10^5	3.72	19.8
45	74.5	$.305 \times 10^5$	5.15×10^5	4.08	18.3
46	95.5	$.305 \times 10^5$	1.15×10^6	5.24	18.2
47	68	$.345 \times 10^5$	3.15×10^5	3.19	21.3
					20.7

*passage 41--overgrew; counts are not valid.

Colony 354-13-01. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
15	125.5	1.3×10^5	1.962×10^6	3.92	32
16	141.5	$.88 \times 10^5$	1.83×10^6	4.38	32.3
17	121	1.095×10^5	2.02×10^6	4.21	28.7
18	141.5	$.403 \times 10^5$	1.85×10^6	5.52	25.6
19	124.5	$.68 \times 10^5$	1.06×10^6	3.96	31.4
20	142	$.64 \times 10^5$	2.2×10^6	5.10	27.8
21	93	$.66 \times 10^5$	5.65×10^5	3.1	30
22	96	$.565 \times 10^5$	1.91×10^6	5.08	18.9
23	148	$.382 \times 10^5$	4.6×10^6	6.61	22.4
					29.7

10 µg aza/ml

15	125.5	1.3×10^5	1.67×10^6	3.68	34.1
16	141.5	$.83 \times 10^5$	1.06×10^6	3.67	38.6
17	121	1.06×10^5	2.35×10^6	4.47	27.1
18	141.5	$.47 \times 10^5$	1.71×10^6	5.19	27.3
19	124.5	$.68 \times 10^5$	1.45×10^6	4.41	28.2
20	142	$.64 \times 10^5$	1.245×10^6	4.28	33.2
21	93	$.623 \times 10^5$	6.05×10^5	3.28	28.4
22	96	$.545 \times 10^5$	9×10^5	4.05	23.7
23	148	$.36 \times 10^5$	2.04×10^6	5.82	25.4
					31

Colony 354-13-02. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
26	165.5	$.54 \times 10^5$	9.4×10^5	4.12	40.2
27	144.5	$.38 \times 10^5$	8.85×10^5	4.54	31.8
28	147	$.354 \times 10^5$	1.465×10^6	5.37	27.4
29	170.5	$.322 \times 10^5$	2.25×10^6	6.13	27.8
30	163	$.35 \times 10^5$	1.18×10^6	5.08	32.1
31	74	$.354 \times 10^5$	1.5×10^5	2.08	35.6
					32.5

10 µg aza/ml

26	165.5	$.54 \times 10^5$	2.5×10^5	2.21	74.9
27	144.5	$.25 \times 10^5$	8.75×10^5	5.13	28.2
28	147	$.35 \times 10^5$	1.145×10^6	5.03	29.2
29	170.5	$.344 \times 10^5$	3.06×10^6	6.47	26.4
30	163	$.34 \times 10^5$	6×10^5	4.14	39.4
31	74	$.36 \times 10^5$	3×10^5	3.05	24.3
					29.5

(medium change between every passage)

Colony 354-17-01. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
33	72	$.49 \times 10^5$	1.17×10^6	4.58	15.7
34	96.5	$.304 \times 10^5$	1.86×10^6	5.94	16.2
35	74.5	$.372 \times 10^5$	1.525×10^6	5.36	13.9
36	98	$.305 \times 10^5$	2.04×10^6	6.06	16.2
37	93.5	$.326 \times 10^5$	3×10^6	6.52	14.3
38	97.5	$.3 \times 10^5$	1.335×10^6	5.48	17.8
39	69.5	$.347 \times 10^5$	7.35×10^5	4.41	15.8
40	75	$.3675 \times 10^5$	1.278×10^6	5.12	14.6
41	92	$.3195 \times 10^5$	2.466×10^6	6.27	14.7
					15.5

10 µg aza/ml

33	72	$.49 \times 10^5$	5.25×10^5	3.42	21.1
34	96.5	$.315 \times 10^5$	1.755×10^6	5.8	16.6
35	74.5	$.351 \times 10^5$	6.7×10^5	4.25	17.5
36	98	$.335 \times 10^5$	1.675×10^6	5.64	17.4
37	93.5	$.335 \times 10^5$	2.15×10^6	6	15.6
38	97.5	$.301 \times 10^5$	1.665×10^6	5.79	16.8
39	69.5	$.333 \times 10^5$	5.2×10^5	3.96	17.6
40	75	$.3432 \times 10^5$	6.825×10^5	4.31	17.4
41	92	$.312 \times 10^5$	1.938×10^6	5.96	15.4
					17.3

Colony 354-17-04. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
24	96	$.38 \times 10^5$	1.25×10^6	5.04	19.0
25	76.5	$.88 \times 10^5$	8.8×10^5	3.32	23.0
26	119	$.176 \times 10^5$	1.36×10^6	6.27	19.0
27	143.5	$.16 \times 10^5$	1.52×10^6	6.57	21.8
28	93	$.18 \times 10^5$	3.6×10^5	4.32	21.5
29	77	$.288 \times 10^5$	3.55×10^5	3.62	21.3
30	94	$.355 \times 10^5$	5.9×10^5	4.05	23.2
31	93	$.345 \times 10^5$	1.195×10^6	5.11	18.2
32	78	$.359 \times 10^5$	5.5×10^5	3.94	19.8
					20.8

10 µg aza/ml

24	96	$.36 \times 10^5$	4.4×10^5	3.61	26.6
25	76.5	$.88 \times 10^5$	8.85×10^5	3.33	23.0
26	119	$.17 \times 10^5$	5.55×10^5	5.03	23.7
27	143.5	$.17 \times 10^5$	8.65×10^5	5.67	25.3
28	93	$.17 \times 10^5$	1.4×10^5	3.04	30.6
29	77	$.28 \times 10^5$	5.5×10^5	4.3	17.9
30	94	$.33 \times 10^5$	5.5×10^5	4.06	23.2
31	93	$.33 \times 10^5$	8.55×10^5	4.70	19.8
32	78	$.342 \times 10^5$	3.0×10^5	3.13	24.9
					23.9

Colony 418-02-01. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
21	72	$.5 \times 10^6$	8.58×10^5	4.1	17.6
22	69	$.71 \times 10^5$	1.67×10^6	4.56	15.1
23	98.5	$.303 \times 10^5$	9.56×10^6	4.98	19.8
24	99.5	$.956 \times 10^5$	3.69×10^5	5.27	18.9
25	119	$.738 \times 10^5$	6.3×10^6	6.42	18.5
26	70.5	$.5 \times 10^5$	2.10^6	5.33	13.2
27	98.5	$.6 \times 10^5$	3.9×10^6	6.02	16.4
28	67	$.39 \times 10^5$	6.85×10^5	4.13	16.2
29	75.5	$.41 \times 10^5$	1.175×10^6	4.84	15.6
30	95.5	$.3295 \times 10^5$	3.235×10^6	6.62	14.4
					16.6

10 µg aza/ml

21	72	$.5 \times 10^5$	9.12×10^5	4.19	17.2
22	69	$.76 \times 10^5$	5.16×10^5	2.76	25
23	98.5	$.34 \times 10^5$	9.4×10^5	4.79	20.6
24	99.5	$.94 \times 10^5$	2.34×10^6	4.64	21.4
25	119	$.702 \times 10^5$	2.6×10^6	5.21	22.8
26	70.5	$.52 \times 10^5$	6.25×10^5	3.59	19.6
27	98.5	$.6 \times 10^5$	1.46×10^6	4.60	21.4
28	67	$.38 \times 10^5$	4.75×10^5	3.64	18.4
29	75.5	$.38 \times 10^5$	9×10^5	4.57	16.5
30	95.5	$.36 \times 10^5$	2×10^6	5.80	16.5
					19.9

Colony 418-06-01. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
17	98	$.8 \times 10^5$	1.395×10^6	4.12	23.8
18	98	$.558 \times 10^5$	1.61×10^6	4.85	20.2
19	141.5	$.55 \times 10^5$	3.31×10^6	5.91	23.9
20	72	$.5 \times 10^5$	6.2×10^6	3.63	19.8
21	96.5	$.62 \times 10^5$	1.285×10^6	4.37	22.1
22	68	$.5425 \times 10^5$	7.3×10^5	3.75	18.1
23	98.5	$.438 \times 10^5$	1.175×10^6	4.75	20.7
24	99.5	$.4 \times 10^5$	1.14×10^6	4.83	20.6
25	91	$.342 \times 10^5$	1.73×10^6	5.66	16.1
					20

10 µg aza/ml

17	98	$.88 \times 10^5$	1.45×10^5	.72	13.6
18	98	$.58 \times 10^5$	3.9×10^5	2.75	35.6
19	141.5	$.55 \times 10^5$	1.04×10^6	4.24	33.4
20	72	$.52 \times 10^5$	$.75 \times 10^5$	.53	135.8
21	96.5	$.6 \times 10^5$	5.6×10^5	3.22	30
22	68	$.56 \times 10^5$	3.25×10^5	2.54	26.8
23	98.5	$.423 \times 10^5$	4.2×10^5	3.31	29.8
24	99.5	$.42 \times 10^5$	4.0×10^5	3.25	30.6
25	91	$.4 \times 10^5$	3.0×10^5	2.91	31.3
					31.1

Colony 418-16-01. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
19	71	$.81 \times 10^5$	1.14×10^6	3.81	18.6
20	116	$.456 \times 10^5$	7.2×10^6	7.30	15.9
21	125	$.432 \times 10^5$	4.75×10^6	6.78	18.4
22	67	$.475 \times 10^5$	5.7×10^5	3.58	18.7
					17.9

10 µg aza/ml

passage 16, 17, 18, 19 each from MENAFC10 but no growth in 10 µg aza/ml.

Colony 418-16-02. Cell count and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
22	101	.6 $x10^5$	$4.128x10^6$	6.1	16.6
23	67	.89 $x10^5$	1.57 $x10^6$	4.14	16.2
24	74	.31 $x10^5$	1.42 $x10^6$	5.52	13.4
25	99	.64 $x10^5$	2.69 $x10^6$	5.39	18.4
26	119	$.537x10^5$	9.75 $x10^6$	7.5	15.9
27	71	.59 $x10^5$	1.05 $x10^6$	4.15	17.1
28	98.5	.42 $x10^5$	2.27 $x10^6$	5.76	17.1
29	66	.54 $x10^5$	1.11 $x10^6$	4.36	15.1
30	77.5	$.355x10^5$	$1.185x10^6$	5.06	15.3
					16.1

10 µg aza/ml

22	101	.6 $x10^5$	$1.752x10^6$	4.87	20.7
23	67	.88 $x10^5$	6.45 $x10^5$	2.87	23.3
24	74	.32 $x10^5$	6.10 $x10^5$	4.25	17.4
25	99	.61 $x10^5$	2.74 $x10^6$	5.49	18.0
26	119	.54 $x10^5$	2.75 $x10^6$	5.67	21.0
27	71	.55 $x10^5$	1.16 $x10^6$	4.4	16.1
28	98.5	.46 $x10^5$	9.85 $x10^5$	4.42	22.3
29	66	$.591x10^5$	1.64 $x10^6$	4.79	14.5
30	77.5	$.394x10^5$	1.59 $x10^6$	5.33	14.5
					18.6

Colony 418-16-03. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
16	72(?)	1.2×10^5	2.2×10^6	4.2	17.1
17	94	$.62 \times 10^5$	2.47×10^6	5.32	17.7
18	73	$.494 \times 10^5$	9.8×10^5	4.31	16.9
19	96	$.48 \times 10^5$	1.64×10^6	5.09	18.9
20	72.5	$.49 \times 10^5$	1.07×10^6	4.45	16.3
21	144.5	$.32 \times 10^5$	9.35×10^6	8.2	17.6
22	116.5	$.374 \times 10^5$	1.54×10^5	5.36	21.7
23	72	$.308 \times 10^5$	3.2×10^5	3.38	21.3
24	95	$.32 \times 10^5$	1.53×10^6	5.58	17.0
					18.3

10 µg aza/ml

16	72(?)	1.28×10^5	1.5×10^6	3.55	20.3
17	94	$.62 \times 10^5$	2.09×10^6	5.08	18.5
18	73	$.418 \times 10^5$	4.3×10^5	3.36	21.7
19	96	$.98 \times 10^5$	1.835×10^6	4.26	22.5
20	72.5	$.48 \times 10^5$	8.3×10^5	4.11	21.9
21	144.5	$.33 \times 10^5$	3.19×10^6	6.59	21.9
22	116.5	$.319 \times 10^5$	1.02×10^6	5.0	23.3
23	72	$.306 \times 10^5$	4.25×10^5	3.8	18.9
24	95	$.315 \times 10^5$	9.5×10^5	4.91	19.3
					20.4

APPENDIX IX

Colonies selected at 30 µg aza/ml, 42 hours mutation expression time.

Colony 418-28-01. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
20	74.5	$.87 \times 10^5$	1.518×10^6	4.13	18.0
21	68.5	$.76 \times 10^5$	1.54×10^6	4.34	15.8
22	99	$.28 \times 10^5$	1.14×10^6	5.35	18.5
23	99.5	$.684 \times 10^5$	3.12×10^6	5.51	18.1
24	120.5	$.312 \times 10^5$	3.75×10^6	6.91	17.4
25	141	$.292 \times 10^5$	2.83×10^6	6.6	21.4
26	72	$.28 \times 10^5$	5.3×10^5	4.24	17.0
27	97	$.258 \times 10^5$	1.725×10^6	6.06	16.0
28	69	$.345 \times 10^5$	9.75×10^5	4.82	14.3
					17.4

10 µg aza/ml

20	74.5	$.87 \times 10^5$	9.0×10^5	3.37	22.1
21	68.5	$.75 \times 10^5$	7.73×10^5	3.37	20.3
22	99	$.27 \times 10^5$	7×10^5	4.7	21.1
23	99.5	$.7 \times 10^5$	1.67×10^6	4.58	21.7
24	120.5	$.334 \times 10^5$	1.41×10^6	5.4	22.3
25	141	$.282 \times 10^5$	2.45×10^6	6.44	21.9
26	72	$.28 \times 10^5$	2.5×10^5	3.16	22.8
27	97	$.25 \times 10^5$	1.095×10^6	5.45	17.8
28	69	$.329 \times 10^5$	4.45×10^5	3.76	18.4
					20.9

Colony 533-02-01. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
6	120	$.339 \times 10^5$	1.596×10^6	5.32	22.6
7	96	$.399 \times 10^5$	7.2×10^5	4.17	23.0
8	98.5	$.36 \times 10^5$	7.3×10^5	4.34	22.7
9	44.5	$.511 \times 10^5$	2.5×10^5	2.29	19.4
					21.9

10 µg aza/ml

6	120	$.375 \times 10^5$	2.19×10^6	5.87	20.4
7	96	$.381 \times 10^5$	6.65×10^5	4.13	23.2
8	98.5	$.399 \times 10^5$	7.95×10^5	4.31	22.9
9	44.5	$.366 \times 10^5$	1.6×10^5	2.13	20.9
					21.9

Colony 533-13-01.

MENAFC10

6	120	$.304 \times 10^5$	3.4×10^6	6.81	17.6
7	96	$.34 \times 10^5$	2.0×10^6	5.88	16.3
8	98	$.36 \times 10^5$	3.41×10^6	6.57	14.9
9	44.5	$.341 \times 10^5$	2.75×10^5	3.01	14.8
					15.9

10 µg aza/ml

6	120	$.304 \times 10^5$	1.5×10^6	5.62	21.4
7	96	$.3 \times 10^5$	1.22×10^6	5.35	19.9
8	98	$.366 \times 10^5$	2.095×10^6	5.84	16.8
9	44.5	3.34×10^5	1.94×10^6	2.54	17.5
					18.9

Colony 533-15-01. Cell counts and estimation of doubling time (t/g) in mass culture for:

MENAFC10

passage	t(hrs)	No	Nt	g	t/g
7	48	$.525 \times 10^5$	2.5×10^5	2.75	21.3
8	72	$.5 \times 10^5$	3.25×10^5	2.70	26
9	73	$.39 \times 10^5$	2.3×10^5	2.56	28.1
10	96	$.46 \times 10^5$	7.2×10^5	3.97	24.2
					25

10 µg aza/ml

7	48	$.51 \times 10^5$	2.2×10^5	2.11	22.7
8	72	$.44 \times 10^5$	3.4×10^5	2.95	26
9	72	$.408 \times 10^5$	1.85×10^5	2.18	33.0
10	96	$.444 \times 10^5$	6.48×10^5	3.87	24.8
					26.6