sequential mutations to puromycin resistance. The possibility is obviously not excluded that susceptible cells may, in addition, give rise to highly resistant ones by a single mutational event but at a rate too low to be detected by present methods.

Summary.—Cells with heritable resistance to the antibiotic puromycin or the purine analogue 8-azaguanine have been isolated from a culture of fibroblast-like mammalian cells, AMK 2-2. Resistance to puromycin, but not to azaguanine occurs in relatively small steps. Evidence has been presented to show that resistance to the former compound involves sequential mutational events.

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[2] The term mutant is used to describe a cell with a new trait stable for many generations in the absence of the specific selective agent. It is recognized that, until recombinational analysis is made, no rigorous proof of mutation is possible.

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*ESTIMATION OF MUTATION RATES WITH MAMMALIAN CELLS IN CULTURE**

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In a previous report[1] the isolation and some of the properties of cultured mammalian cells heritably resistant to metabolic analogues were described. With one of the toxic agents, puromycin, low resistance (Pm^{r-1}) and higher resistance (Pm^{r-2}) mutants were obtained. Evidence was presented to show that the increase in resistance from the parent (Pm^{s}) to the high resistance mutant involves at least two sequential mutational events.

To determine the rates of these events the classical method of Luria and Delbrück[2]

was used. The mutation rates from $Pm^s \rightarrow Pm^{r-1}$ and from $Pm^{r-1} \rightarrow Pm^{r-2}$ have been estimated to be approximately 4×10^{-6} and 1×10^{-4} mutations per cell per generation, respectively.

The purpose of this report is to detail the procedures used and to show the evidence for the validity of the mutation rate measurements.

Materials and Methods.—The exact origin of the cultured mammalian cell used in this work, AMK 2-2, is in doubt, but recent studies[3] have suggested its derivation from Earle's mouse fibroblast strain L.[4] Other materials and culture methods have been previously described.[1]

Experiments to estimate mutation rates were initiated by inoculating each of 25 to 50 test tubes with 100 cells in 2 ml of cloning medium (basal medium enriched with 15 per cent human and 5 per cent horse serum). When sufficient growth had occurred (9–15 days), the cells were detached with a rubber spatula and dispersed by vigorous aspiration. The suspended cells from 4 or 5 tubes, chosen at random, were enumerated in a Levy counting chamber and the average was taken as the number of cells per tube. Aliquots of the cultures were then added to Petri dishes (60 mm) containing puromycin and cloning medium to a final volume of 4 ml. To add the antibiotic with greater accuracy it was mixed with the cloning medium and dispensed with it.

On the 4th and again on the 8th day of incubation (37°, atmosphere = 5 per cent CO_2-air), the medium was replaced with fresh medium containing the same concentration of puromycin as initially. After an additional 4 days, the colonies were enumerated following fixation and staining.

Reliability of the Plating Method.—To determine whether plating of the AMK cells yields fluctuations larger than those ascribable to random sampling, parallel platings were made from single cultures both in the presence and absence of puromycin (Table 1). As can be seen from the table, the agreements obtained between

TABLE 1

PARALLEL PLATINGS FROM THE SAME CULTURE

Sample No.	Experiment No. 27A	28A	29A	9B	9B	25B
1	11	11	6	3	1	12
2	11	11	6	8	2	18
3	9	15	2	7	2	20
4	11	8	11	3	1	13
5	14	14	8	5	5	14
6	6	10	6	5	4	24
7	8	9	9	4	4	12
8	11	7	1	6	5	18
9	12	7		10	3	19
10	9	11		6	3	17
11	13	11		6	0	...
12	9	10		10	4	...
13	14	15	...	...	...	...
Cell culture	Pm^{r-1}	Pm^{r-1}	Pm^{r-1}	Pm^{s*}	Pm^s	Pm^{r-1}
Number of cells plated	10	10	5	1×10^5	1×10^5	4,800
Puromycin, μg per ml	...	...	...	12	12	40
Mean	10.6	10.7	6.1	6.1	2.8	16.7
Variance	5.6	7.2	11.2	5.5	2.7	15.3
χ^2	6.3	7.5	12.9	9.9	10.6	8.2
P	0.9	0.8	0.08	0.5	0.5	0.5

* The designation Pm^s will be used to describe Pm^{s-2} cultures (see reference 1).

TABLE 2

RESISTANT COLONIES (Pm^{r-1}) FROM SIMILAR CULTURES OF PARENT CELLS

Experiment No. →	1	4		5		7
Aliquot Plated, ml →	0.8	0.3	0.9	0.15	1.5	1.0
Culture No.	Number of Resistant Colonies					
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	21
4	0	0	0	0	0	0
5	0	0	0	24	171	4
6	0	0	1	0	0	0
7	0	0	0	0	0	0
8	1	0	0	0	1	0
9	3	2	4	3	2	0
10	0	11	42	0	0	0
11	0	0	0	0	0	0
12	0	2	10	0	0	0
13	0	0	0	0	0	0
14	0	...	...	0	0	0
15	0	...	...	0	0	0
16	2	...	...	0	0	...
17	0	...	...	0	0	...
18	0	...	...	0	0	...
19	0	...	...	0	2	...
20	18	...	...	0	0	...
21	0	...	...	1	10	...
22	0	...	...	0	0	...
23	0	...	...	0	0	...
24	0	...	...	0	0	...
Average per sample	1.0	1.2	4.5	1.2	7.8	1.6
Variance (corrected for sampling)	12.7	...	131	...	1,206	26.3
Average per culture	2.5	7.7	9.9	15.5	10.3	3.1
Cells per culture	1.0×10^6	6.7×10^5		4.0×10^5		3.3×10^5
Puromycin, μg per ml	12.0	12.0		11.4		8.0
Mutation rate*	0.8×10^{-6}	4.0×10^{-6}		6.2×10^{-6}		3.3×10^{-6}
Standard deviation / Average (found)	3.6	2.5		4.5		3.2
Standard deviation / Average (calc.)	1.7	1.0		1.2		1.4

* Mutations per cell per generation.

the variance and the mean indicate Poisson distributions, confirmed by application of the χ^2 test. Hence, it is unlikely that large errors of unknown origin occurred.

Mutation Rate of Parent Cells ($Pm^s \rightarrow Pm^{r-1}$).—The number of cells capable of producing colonies in the presence of 8–12 μg per ml of puromycin was measured in a series of similar cultures as described under "Materials and Methods" (Table 2). Mutation rates were calculated with equation 8 of Luria and Delbrück.[2] As can be seen from the table, reasonable agreement was obtained in the four experiments, the average value being 3.6×10^{-6} mutations per cell per generation. Also shown are the ratios of the standard deviations to the average numbers of resistant colonies. The similarities of the calculated and experimentally determined ratios are in agreement with the assumption that the mutational events occurred in the absence of puromycin. It is also of interest to note that the exact concentration of puromycin did not appear to affect the calculated mutation rates.

Mutation Rate from Low to High Resistance ($Pm^{r-1} \rightarrow Pm^{r-2}$).—Estimation of the high resistance mutants in similar cultures of the low resistance ones was carried out as with the parent cells (Table 3). The mutation rates were calculated by the "median" method of Lea and Coulson,[5] the average value being 0.8×10^{-4} mutations per cell per generation.

Control Experiments.—The fluctuations of the numbers of resistant cells as shown

in Tables 2 and 3 were much higher than can be accounted for by sampling errors (Table 1). The question arises, however, what factors in addition to mutation and growth of mutants may have contributed to the fluctuations observed. The following possibilities were considered.

(1) *Some of the culture tubes initially received one or more resistant cells:* This possibility, seriously considered for the studies with Pm^{r-1} cells, cannot be excluded. With three experiments (including experiment 11, Table 3), however, examination of the Pm^{r-1} cells (20 dishes, 100 cells per dish) used to prepare the series of similar cultures revealed no Pm^{r-2} cells.

(2) *The extent of growth in the culture tubes was highly variable:* Before use, culture tubes were screened for acid production and macroscopically for growth. Cell numbers were then determined in 4 or 5 of the remaining tubes chosen at random. Batches of cultures with differences greater than ±15 per cent, rarely encountered, were discarded.

(3) *Manipulation resulted in depression of viable cell numbers in some but not all culture tubes:* Detachment of the cells from the culture tubes and vigorous aspiration of the suspension had no detectable effect on viability. Thus, when 20 cells

TABLE 3

HIGH RESISTANCE COLONIES (Pm^{r-2}) FROM SIMILAR CULTURES OF LOW RESISTANCE CELLS (Pm^{r-1})

Experiment No.→	1	2		4		11
Aliquot plated, ml→	1.5	0.3	1.0	0.25	0.75	0.65
Culture No.	Number of Resistant Colonies					
1	4	7	9	5	16	22
2	1	18	37	18	33	17
3	53	10	20	2	8	13
4	1	2	3	17	73	1
5	14	9	29	2	10	6
6	0	72	207	5	12	267
7	1	3	0	6	39	12
8	0	3	3	1	9	8
9	3	4	38	0	0	23
10	6	1	3	24	108	15
11	4	21	89	24	72	22
12	45	8	8	2	17	2
13	18	4	5	28	99	45
14	4	6	7	8	28	2
15	15	4	24	5	5	100
16	5	7	20	11	62	7
17	2	4	11	0	3	21
18	0	6	20	5	20	9
19	48	3	2	12	42	...
20	12	3	8	2	18	...
21	0	0	7	2	5	...
22	16	...	...	34	124	...
23	1	...	...	1	9	...
24	0	...	...	4	11	...
25	11	...	...	...	...	...
Average per sample	10.6	9.3	26.2	9.1	34.3	32.9
Variance (corrected for sampling)	229	...	2088	...	1272	3899
Average per culture	14.1	62.0	52.4	72.8	91.6	101
Cells per culture	20,000	48,000		88,000		163,000
Puromycin, μg/ml	40	40		40		38
Mutation rate*	0.9×10^{-4}	0.9×10^{-4}		1.0×10^{-4}		0.5×10^{-4}
Standard deviation / Average (found)	1.4	1.7		1.0		1.9
Standard deviation / Average (calc.)	1.3	1.0		0.9		0.9

* Mutations per cell per generation.

TABLE 4

EFFECT OF COMBINING SAMPLES FROM PAIRS OF CULTURES*

Culture No.	Number of Resistant Colonies: Individual Cultures	Combined Cultures: Found	Combined Cultures: Calculated
1	4	2	3
2	2		
3	0	16	21
4	41		
5	15	4	8
6	0		
7	2	11	8
8	14		
9	1	0	1
10	0		
11	2	1	2
12	2		
13	4	8	12
14	20		
15	3	11	11
16	18		
17	1	4	1
18	0		
19	0	0	2
20	3		
21	59	37	32
22	2		
23	0	18	11
24	21		

* The cultures (2 ml), initiated with 100 Pm^{r-1} cells, were allowed to grow to 30,000 cells per tube. Plated were 1 ml aliquots of the suspended cells from each tube (individual cultures) and 1.0 ml aliquots of equal mixtures (combined cultures) prepared as indicated. The puromycin concentration was 40 μg per ml.

from cultures 7, 9, 11, and 13 were plated after use in experiment 4 (Table 3), 22, 19, 22, and 17 colonies were formed, respectively.

(4) *Differences in growth rate:* Doubling times of parent and mutant clonal isolates in the absence of puromycin were found to be indistinguishable, about 24 hours. The growth rate of resistant cells in mixtures containing relatively large numbers of more susceptible cells has, however, not been studied.

(5) *Unrecognized variables in plating efficiency with puromycin:* The number of colonies formed in the presence of puromycin appears to be a valid measure of the number of resistant cells present in the inoculum. In evidence is the acceptable proportionality obtained when different numbers of cells are plated from the same test cultures (experiments 4 and 5, Table 2; experiments 2 and 4, Table 3).

Further support was obtained by estimating resistant cells in mixtures of suspensions from pairs of culture tubes (Table 4). As can be seen from the table, the numbers of colonies yielded by the combined cultures were similar to the calculated values.

Discussion and Summary.—The availability of mutant mammalian cells in culture, manipulatable by procedures similar to those used with other microorganisms, has made possible the estimation of two mutation rates by the method of Luria and Delbrück.[2] The heritable changes studied, the acquisition of resistance to the antibiotic, puromycin, and the conversion of these mutants to ones with greater resistance, were calculated to occur at rates of approximately 4×10^{-6} and 1×10^{-4} mutations per cell per generation, respectively. It will be of the greatest interest

to determine whether these mutational processes can be used to study mutagenesis and antimutagenesis.[6]

The Luria-Delbrück analysis provides additional information, of course, not directly concerned with the rate of mutation. From the results obtained it may be concluded that the heritable increases in puromycin resistance are not dependent upon the presence of the antibiotic. Thus, cellular changes not involving mutation *per se*, like those encountered with bacteria[7,8] and protozoa,[9] may be ruled out to explain the origin of the puromycin-resistant cells. It should be noted that the work of Law[10] with A-methoperin resistant leukemic cells in mice led him to the same conclusions.

The question arises whether the changes studied result from genic or chromosomal alterations. With cultured mammalian cells, which commonly undergo chromosomal alterations,[11] the possibility of chromosomal rather than intragenic change is attractive. Unfortunately, methods which could supply at least presumptive evidence[12] on this point cannot yet be applied to cultured mammalian cells.

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*AN INFECTIOUS RIBONUCLEIC ACID DERIVED FROM INFLUENZA-INFECTED CELLS**

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Several workers have recently reported the preparation of infectious ribonucleic acid from virus-infected cells of fluids of tissue cultures infected with animal viruses (poliovirus, mengo encephalitis, encephalomyocarditis, foot-and-mouth disease), using the phenol method described by Gierer and Schramm[1] for TMV. We have