without complementing reagents. It appears that thrombokinase can activate prothrombin, unaided by other factors(5). But thrombin is produced much faster if the system is complemented by platelets or cephalin, provided that ionic calcium is also added. Production of thrombin is further accelerated by the serum reagent. Together, these reagents enormously magnify the effect of kinase. When minute amounts of kinase are assayed in this system, serious uncertainties are involved(2). Therefore, it is of interest that assays in this complex system paralleled those with oxalated prothrombin; although the former system was 2500 times as sensitive.

In some respects, thrombokinase resembles trypsin, which also can activate prothrombin in presence of oxalate. TAMe is a good substrate for trypsin(6); and others have anticipated the possibility that TAMe might be a substrate for natural activator of prothrombin (7). The present results suggest that TAMe may well be a substrate for thrombokinase; but more detailed study of this point is desirable.

Summary. Thrombokinase prepared from bovine plasma was further purified by continuous flow paper electrophoresis. Thrombokinase was assayed by its capacity to activate prothrombin in the presence of oxalate, and also by production of thrombin in a system containing prothrombin, cephalin, calcium and bovine "barium carbonate serum." Curves of these 2 assays were similar and formed a peak ahead of the thrombin peak. TAMe esterase activity appeared in 2 peaks which corresponded respectively to thrombin and thrombokinase peaks.

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Genetics of Human Cell Lines I. 8-Azaguanine Resistance, a Selective "Single-Step" Marker.* (25053)

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From the methodological point of view, the mammalian cell grown *in vitro* can be regarded as a unicellular microorganism. Thus, methods developed for quantitative work with microbes can be adapted to the genetic study of human cells, provided suitable selective markers are available. Plating and colony-counting technic, so useful for assay of viable microbial cells, was introduced into tissue culture methodology by Puck *et al.*(1). Our purpose was to find a suitable mutational system for mammalian cells in which, under selective conditions, mutant cells survive and form well developed colonies while parental population is completely inhibited or eliminated. Mutation from 8-azaguanine (AG) sensitivity to resistance satisfied the foregoing criteria(2). Moreover, the property of 8-azaguanine resistance appears to be an excellent genetic marker, since it is not associated with modifications in morphological or cultural characteristics of cells either in presence or absence of the selective agent.

Materials and methods. Strain *Detroit-98* (D98), derived from human sternal marrow by Berman and Stulberg(3), was kindly supplied by Dr. H. Moser of Cold Spring Harbor Labs. A single-cell-derived clone D98S was used throughout these studies. *Media.* The basic medium was essentially that described by Eagle(4), containing 10% com-

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plete horse serum. Sterilization was effected by filtration through Selas No. 2 filter. *Cultivation and plating procedures.* The methods were based on those developed by Puck *et al.*(1) and adapted for strain D98 by Moser and Tomizawa(5). Cells were grown on glass surface of 60-mm Petri dishes at 37°C in 5-10% CO_2 atmosphere. The inoculum was prepared from a 3- to 6-day-old culture of vigorously growing cells, washed serum-free with balanced salt solution before detachment and dispersion in the same solution containing 0.25% pancreatin (Nutritional Biochemical Corp., Cleveland) (5-minute incubation at 37°C followed by gentle manual shaking). The action of pancreatin was terminated by addition of serum-containing medium, after which the cell suspension was filtered aseptically through fine cheesecloth to remove any cell clumps. The cell density of this essentially monodispersed suspension was assayed in a hemacytometer. For colony counts, cells appropriately diluted in 5 ml of serum-containing medium were plated and incubated 6 to 12 days, with a complete medium change every 2 to 3 days. Growth was assayed either by counting and measuring colonies or by protein determination. *Protein determination* is based in part on protein staining method of Durrum(6) and its modifications for electrophoretic(7) and cytochemical(8) purposes. Adaptation of these methods for quantitative protein determination in mammalian cell cultures, suggested by Dr. A. W. Kozinski of this laboratory, permits determination of total protein without disturbing colonies on the glass. Thus a colony count may be performed on the same plate after completion of protein determination. The assay is based on spectrophotometric determination of bromphenol blue selectively bound by the protein component of $HgCl_2$-fixed cells and subsequently eluted with alkaline acetone. The procedure consists of: (1) thorough washing of glass-attached cells with balanced salt solution to remove all serum proteins; (2) 15-minute staining at 37°C with aqueous solution containing 0.1% bromphenol blue and 5% $HgCl_2$; (3) three consecutive washes with 0.5% acetic acid each lasting 6 minutes; (4) quantitative elution with 3-ml portions of aqueous acetone (20 ml acetone 0.2 ml 10 N NaOH, 5 ml water, freshly prepared); (5) adjusting the collected extracts to 10-ml volume and measuring optical density at 595 mμ wave length against a blank prepared similarly from cell-free plate preincubated with serum-containing medium. The actual amount of cell protein/plate is ascertained from predetermined calibration curves. The same procedure was also used for cell suspensions, with centrifugation as an added step. *Colony count.* After fixation in Bouin's fluid or after protein determination, colonies were stained with Giemsa solution. The dry dishes were then inserted into a projector (Bausch & Lomb, "Tri-Simplex" micro-projector), and well-focused images of colonies were scored by an electronic counter (built by Philip D. Mintz of this laboratory).

Results. When above procedures are followed, strain D98S exhibits a consistent plating efficiency of greater than 0.6, *i.e.*, over 60% of microscopically assayed cells give rise to well defined colonies (Fig. 1), which can easily be counted, providing their number does not exceed 5000/plate. Above this figure, colonies coalesce, and total protein synthesis after 14 days' incubation becomes less than proportional to the number of cells in the inoculum (Fig. 2).

A series of plates was prepared, each seeded with approximately 5000 cells, to which AG was added in graded concentrations. Fig. 3 represents the survival curve 1 of the wild-

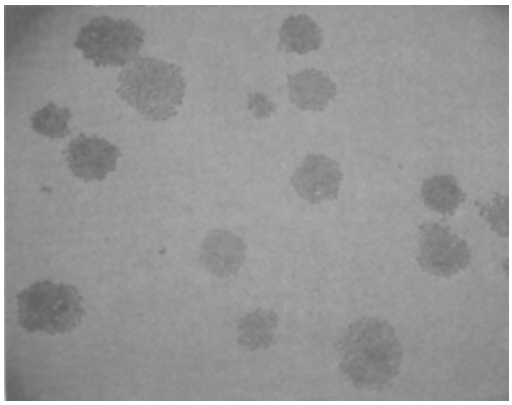

FIG. 1. 6-day-old colonies of strain D98S grown on glass, Bouin fixed, Giemsa stained.

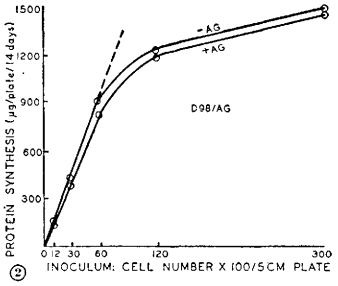

FIG. 2

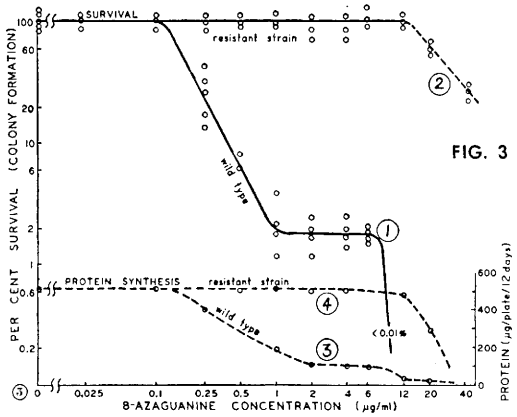

FIG. 3

FIG. 2. Relationship between inoculum size (colony-forming cells) and total growth determined as amount of protein synthesized (14 days) by AG-resistant strain D98/AG grown in presence (+AG) or absence (–AG) of 8 μg AG/ml medium.

FIG. 3. 12-day growth determined as amount of protein synthesized and survival (colony formation) of AG-sensitive (wild type—1, 3) and AG-resistant (2, 4) isolates derived from strain D98S grown at increasing concentrations of AG. Inoculum was 5000 cells/plate.

type strain D98S. It is apparent that in presence up to 0.1 μg AG/ml survival and colony-forming ability of each cell is virtually unaffected. There is a sharp drop in plating efficiency between 0.1 and 1 μg AG/ml, followed by a plateau extending to approximately 10 μg AG/ml, at which level 1 to 2% of cells survive and form colonies. These colonies were isolated and found to be stable, AG-resistant clones (D98/AG) as reflected by survival curve 2. Their growth, as measured by protein synthesis, was not affected by AG in concentrations up to 12 μg/ml (curve 4), while protein synthesis of the wild-type strain was increasingly suppressed above 0.1 μg/ml.

Thus the wild-type population contains approximately 1 to 2% mutant cells, exhibiting to 100-fold increase in AG resistance without impairment of plating efficiency or growth rate either in the presence or in the absence of AG (Fig. 2 and 3). Fig. 4 shows appearance of colonies on 2 plates seeded with equal inoculum of AG-sensitive strain D98S, incubated in absence (A) and in presence (B) of AG (6 μg/ml).

Resistance to AG appears to be a highly stable property. Subculture for over 150 generations, either in absence (curve 3) or in presence (curve 2) of AG (6 μg/ml) did not alter level of resistance (Fig. 5). By subculturing resistant lines at high concentrations of AG, strains with slightly higher resistance could be isolated (Fig. 5, curve 4).

Determination of mutation rates was based on Luria-Delbrück variance test(9), modified for mammalian cells grown on glass. Each colony grown in absence of AG and firmly attached to glass may be considered as analogous to one "test tube culture"(9). On addition of the drug (AG), only colonies containing at least one resistant cell would give rise to secondary resistant colonies, while others would slough off the glass and be lost during medium changes. Mutation rate based on preliminary experiments, employing the P_0 method(9) corresponds to 5×10^{-4}/cell/generation.

Properties of AG-resistant cells. AG-sensitive cells were found to exhibit the same inhibitory threshold for AG and 8-azaguanosine (10^{-7} M). Neither guanine (1 to 50 μg/ml) nor adenine nor thymine antagonize the inhibitory action of 1 to 50 μg/AG/ml. AG-resistant lines are approximately 100 times more resistant to AG, but only 2-3 times more resistant to 8-azaguanosine.† They were also found to be more resistant to 5-fluorouracil and to ultraviolet (UV) light (Fig. 6 and Fig. 7, curve 3). It was therefore of interest to

† Strain D98/AG contains approximately 0.01% mutants (D98/AGR) resistant in a single (genetic) step to over a 100-fold higher concentration of 8-azaguanosine (AGR), with concomitant further increase in AG resistance (over 10-fold). Direct mutations from AG sensitivity (D98S) to AGR resistance (D98/AGR) were not detected in populations as large as 10^7 cells. Thus AG and AGR resistances behave as two sequential mutational steps.

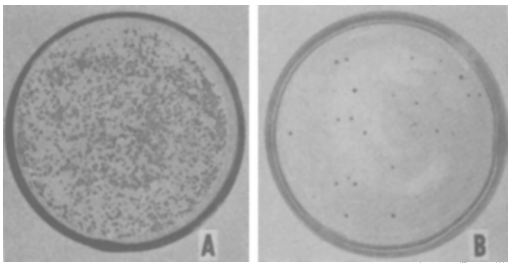

FIG. 4. Colony formation on 2 plates seeded with identical inocula (4000 cells) of AG-sensitive cells (strain D98S) in absence (A) or presence (B) of 6 μg AG/ml medium.

determine whether UV would increase mutation frequency from AG sensitivity to resistance or would select for the more UV-resistant, AG-resistant cells(10). The wild-type population was irradiated with increasing doses of UV and plated in absence (curve 1) or in the presence (curves 2 and 2a) of 6 μg AG/ml. It is apparent that AG-resistant cells not exposed to AG prior to irradiation, exhibit a "single-hit" survival curve (curves 2 and 2a), while UV survivals of parental strain D98S (curve 1) and of the established resistant line D98/AG (curve 3) are characterized by "multi-hit" curves. Curve 4 represents UV survival of the AG-resistant line at slightly inhibitory concentrations of AG (12 μg/ml). The shape of survival curve appears unchanged. It may therefore be concluded that the newly emerging AG-resistant cells, pre-existing in the wild-type population, contain only one AG-resistant "complement" (chromosome?), while the number of these "complements" increases during establishment of the resistant sublines. Further genetic and cytological studies may explain this phenomenon and indicate whether processes analogous to nondisjunction or mitotic crossing-over could be involved.

Summary. A cloned wild-type population (strain D98S) derived from human bone marrow cells, which is inhibited by 8-azaguanine (AG) in concentrations in excess of 0.1 μg/ml, contains approximately 1 to 2% cells resistant to AG in 100-fold higher concentrations. A single-step mutational process (in broad meaning of the word) appears to be involved. The AG-resistant lines are stable upon prolonged subculture either in presence or absence of drug. Mutation rate from sensitivity to resistance is of the order of 5 x 10^{-4}/cell/generation. AG resistance is a use-

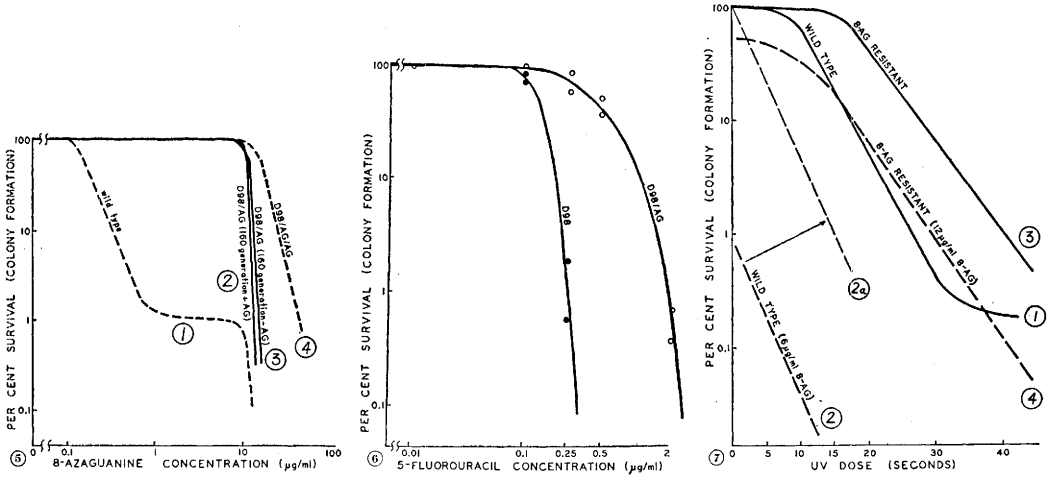

FIG. 5. Survival of AG-sensitive (1) and of AG-resistant mutant lines at increasing concentrations of AG. Curves 2 and 3 represent survival of AG-resistant strains after subculture for 160 generations in presence (2) or absence (3) of 5 μg AG/medium. Survival of more resistant line D98/AG/AG, obtained by serial subculture of D98/AG at 20 to 40 μg AG/ml, is depicted by curve 4.

FIG. 6. Survival of AG-sensitive (D98) and AG-resistant (D98/AG) strains at increasing concentrations of 5-fluorouracil.

FIG. 7. Ultraviolet survival of AG-sensitive (1, 2) and AG-resistant (3, 4) cell lines grown in absence (1, 3) or presense (2, 4) of AG. Curve 2a is parallel to curve 2. UV intensity 120 μW/cm^2.

ful genetic marker, since at selective AG concentrations (a wide plateau at 1-10 μg/ml) colony formation by resistant cells is not impaired and sensitive cells are destroyed. A method for protein determination is described, based on measurement of eluted bromphenol blue from $HgCl_2$-fixed, glass-attached cells.

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Mechanism of Inhibition of Gastric Secretion in the Rat Following Bile Duct Ligation.* (25054)

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Madden *et al.*(1) noted that simultaneous bile duct ligation in the Shay rat preparation prevented formation of rumenal ulcers. Ambrus(2) found that gastric secretory output from gastric fistula rats was slightly decreased when the bile duct was ligated. To date, this interesting phenomenon has not been adequately explained. On the basis of some evidence(3) that bile, when present in the intestine, is a gastric secretagogue, it was thought that removal of bile from the intestine could lead to decrease in gastric secretory activity. Actually, it has been postulated on the basis of a possible secretagogue effect of bile that continuous entrance of bile into the intestine in the rat, by virtue of absence of gall bladder, could explain the large interdigestive gastric secretion so characteristic of the rat(4). Our data show that gastric secretion in the rat is inhibited by bile duct ligation. This inhibition is due, not to removal of bile from the intestine, but to inhibitory effects of bile salts on gastric secretion.

Materials and methods. Gastric secretion was measured by pylorus ligation method(5) in 151 male Holtzman rats weighing from 160 to 220 g. Prior to experiments, the rats were fasted for 48 hours in individual cages with wide mesh wire bottoms to prevent coprophagia. Water was given *ad lib.* during fasting period. Following the 48 hour fast, the rats were operated under light ether anesthesia and under clean conditions and the pylorus gently ligated with a 4-0 silk ligature. Simultaneous ancillary procedures were done and will be described in relation to specific test groups. Exactly 4 hours later (6 hours in one group) the animals were killed by exsanguination, the stomachs removed and gastric content studied individually for volume and free acidity. The rats were divided into 5 groups.

Results. Group 1. Simultaneously with pyloric ligation, the proximal portion of bile duct was divided between 2 ligatures. In random controls, the duct was merely exposed. There was inhibition in volume and free HCl concentration of gastric content in bile duct ligated rats (Table I). There was relatively greater inhibition in volume than in free HCl concentration.

Group 2. An external biliary fistula was created simultaneously with pyloric ligation. This was accomplished by catheterizing proximal portion of bile duct with a fine polyethyl-

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† Markle Scholar in Medical Sciences.