respect to heat and pH stability and thromboplastin generating qualities(9). This result is consistent with a regulatory hypothesis, but closer analysis(7), showed that it is also consistent with a "combining subunit" hypothesis of AHF structure (2 structural loci) because of the physiological nature of our assays.

It occurred to us that a comparison of the extent of "new synthesis" of AHF in heterozygotes and homozygotes for v.W.d. might provide a crucial test of the regulatory hypothesis. If the only defect in v.W.d. is diminished AHF production because of reduced "effector" substance, both abnormal genotypes should respond strongly and essentially equally when given a large transfusion of hemophilia A plasma deficient in AHF but rich in the hypothetical "effector." The data presented here clearly show that persons who can be presumed to be *homozygous* for v.W.d. synthesize only 1/8 as much AHF as presumed *heterozygotes* under these conditions. This appears to mean that it is not possible to explain the defect in v.W.d. or the control of AHF biosynthesis with the "regulatory" model proposed earlier(3) and suggests that this idea can now be discarded.

Summary. Two persons believed to be homozygous for v.W.d. and their heterozygous parents were transfused with hemophilia A plasma. The parents showed extensive "new synthesis" of AHF while the children showed only 1/8 as much "new synthesis" as an unrelated group of v.W.d. heterozygotes. This result is taken to mean that the v.W.d. locus is not occupied by a regulatory gene. The result is consistent, however, with the idea that both the autosomal and the X-locus which affect AHF biosynthesis are occupied by structural genes coding sub-units of the molecule. There is still no direct evidence that the latter hypothesis is correct, however.

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Inhibition of Influenza Virus Cytotoxicity by Ammonium Ion in Commercial Medium #199. (29939)

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During studies on the cytotoxicity of influenza virus in tissue culture, variations were encountered in the susceptibility of a rabbit heart cell line to a standard, massive inoculum of influenza B (Lee) virus. The variations in viral susceptibility of the cells were traced to the presence of inhibitory concentrations of ammonium ion in 2 commercial lots of medium #199(1) from different manufacturers. The inhibitory effect of ammonium ion on influenza virus has been previously shown by several investigators(2,3,4,5).

Materials and methods. A. *Cell line.* 1. A line of cells isolated from normal rabbit myo-

cardium by Dr. Robert Pumper in this laboratory was used for the virus studies. It has been designated as University of Illinois Chicago Rabbit 1 (UICR1) and is routinely grown on a medium consisting of 10% calf serum and 90% medium #199. Monolayer cultures for virus testing were prepared by seeding 2×10^5 cells per ml in Leighton tubes. After 3 or 4 days of incubation at 37°C the growth medium was replaced by maintenance medium consisting of medium #199 only or Hanks' balanced salt solution (BSS) (6), supplemented with 1% calf serum. Sodium bicarbonate was added at a final concentration of 0.065% and penicillin and streptomycin at 100 units and 0.1 mg/ml, respectively. B. *Virus.* 1. Influenza B virus, Lee strain, was propagated in the chorioallantoic cavity of embryonated chicken eggs, and prior to testing had undergone an undetermined number of allantoic passages, but had not been passed in tissue culture. Stock seed virus was harvested from the allantoic sac, pooled, titered for egg infectivity and stored frozen in 2 ml quantities at —54°C. For tissue culture experiments one-tenth ml of undiluted seed virus having a 50% egg infectious dose (EID_{50}) of $10^{7.2}$ was added to the cell cultures. The cytopathic effect (CPE) was graded from 0 to 4; 0 indicating no CPE or complete cell protection, 1+ as 25%, 2+ as 50%, 3+ as 75%, and 4+ as 100% of the cells destroyed. The concentration of virus employed was sufficient to give 3 to 4+ CPE in a minimum of 17 hours in control cultures. Hemadsorption tests (7) were done on all cultures using 0.5% chicken red blood cells to confirm infection. C. *Determination of ammonia.* 1. A modification of the Conway microdiffusion method, followed by colorimetric determinations of nesslerized samples (9) was used for ammonium analysis. Microdiffusion was performed for 4 hours in the cold (4°C) to prevent hydrolysis of the glutamine.

Results. The variations in viral susceptibility of the cells were traced to ammonium ion in medium #199 by employing 2 different lots of media from each of 3 major commercial sources. Four lots found to be non-inhibitory contained from 3 to 11 μg ammonia nitrogen (NH_3-N) per ml of medium. Cells maintained in these media showed 3 to 4+ CPE within 24 hours after addition of the virus inoculum. Two lots from different manufacturers contained 57 and 63 μg NH_3-N per ml of medium and completely inhibited virus CPE for a period of 48 hours, the normal test period. The complete inhibition of virus CPE by these media, however, lasted for 5 days or longer, at which time normal cell disintegration occurred. Similar protection was observed with ammonium sulfate dissolved in non-inhibitory medium #199 at a concentration of 50 μg N per ml but not at a concentration of 10 μg N per ml of medium. Cells maintained on Hanks' BSS plus 1% calf serum were destroyed in a period of 24 hours. These findings are summarized in Table I. Hanks' BSS contained less than 1 μg NH_3-N per ml of medium.

TABLE I. Inhibition of Influenza Virus CPE by Ammonium Ion in Commercial Medium #199.

Medium	NH_3-N (μg/ml)	CPE (hr)* 24	48
Co. # I-#199, lot 1	57	0	0
" 2	7	4+	–
Co. # II-#199, lot 1	11	3+	4+
" 2	63	0	0
Co. #III-#199, lot 1	10	4+	–
" 2	3	4+	–
50 μg N/ml as $(NH_4)_2SO_4$ in Co. #III-#199, lot 2	53	0	0
10 μg N/ml as $(NH_4)_2SO_4$ in Co. #III-#199, lot 2	13	4+	–
Hanks' BSS + 1% calf serum	2	4+	–

* $10^{7.2}$ EID_{50} influenza B (Lee) virus.

The inhibitory role of the ammonium ion in the 2 lots of medium #199 was further demonstrated by adjusting the medium to pH 12 with 1 N NaOH and lyophilizing the preparations for 24 hours to remove the ammonia. Lyophilized preparations were reconstituted with sterile distilled water and neutralized with 1 N HCl. The treated samples contained 1 μg or less NH_3-N per ml of medium and were no longer inhibitory.

Discussion. The source of the high levels of ammonium ion in the inhibitory medium #199 is not known. Decomposition of glutamine may occur on storage (8) and contribute in part to the ammonium content of the medium. However, even if complete decomposition of the glutamine normally incorporated

in medium #199 (100 mg/liter) should occur, this would not explain the high levels present. In addition, medium #199 which had been stored at refrigerator temperature for 2 years had a low ammonium ion concentration (11 μg NH_3-N/ml). There is an obvious need for careful regulation and standardization in the preparation of commercial medium #199.

Summary. Variations in susceptibility of a cell line to the cytotoxic effects of influenza virus were traced to the presence of inhibitory concentrations of ammonium ion in 2 commercial lots of medium #199. Ammonium ion in concentrations of 57 and 63 μg NH_3-N per ml of medium completely inhibited the cytopathic effects of a concentrated inoculum of influenza B (Lee) virus. This study emphasizes the important need for careful regulation and standardization in the preparation of commercial cell culture media.

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Thiobarbituric Reacting Substance(s) Produced in Fractions of Normal Liver and Tumors. (29940)

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Recent investigations(1,2,3) have shown that the aerobic production in tissues of substance(s) reacting with thiobarbituric acid (TBA) results from a pyridine nucleotide-dependent enzymatic reaction and an ascorbate catalyzed non-enzymatic process. The thiobarbituric reacting substance(s) (TBRS) has been correlated with lipid peroxide formation and involvement of the latter has been demonstrated in phospholipid oxidation, inhibition of energy and electron transfer enzyme systems and in mitochondrial swelling(4-11). The presence of TBRS in normal tissue but not in tumor or rapid proliferating tissue makes it of interest in the cancer field(12-15).

There are presented here the results of experiments which clarify and expand those factors related to production of TBRS by liver. The methods used were the same as those previously described(1,15).

Methods. Preparations of all liver fractions were done at 2° to 5°C. A 10 to 20% liver suspension (w/v) was homogenized in either 0.24 *M* sucrose or 0.15 *M* KCl. Nucleic and cellular debris was removed by centrifugation at 600 × *g* for 10 minutes. A mitochondrial and microsomal fraction was obtained by centrifugation at 10,000 × *g* for 10 minutes and at 30,000 × *g* for 1 hour, respectively. These fractions were suspended in either 0.25 *M* sucrose or 0.15 *M* KCl, filtered through gauze, and adjusted with the appropriate suspending fluid to the microgram nitrogen content noted in the experiments. Twenty ml beakers containing 3.0 ml of the substances noted in the tables were incubated at 37°C in a water bath shaker oscillating at 80 to 100 times per minute. At the times indicated in the tables the reaction was stopped with 0.3 ml of 4.8 *M* trichloroacetic acid. The contents of the beakers centrifuged and 1.0 ml of the supernatants (or aliquots of the supernatants in a final of 1.0 ml water diluent) was added to 4 ml of a 0.5% aqueous solution of 2-thiobarbituric acid. After mixing the tubes were