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

TABLE I. Influence of Sucrose, Mg, ADP and Hexokinase on TBRS Production with TPN or TPNH.

0.4 ml microsomes (135 μg nitrogen) of normal rat liver in either 0.15 *M* KCl or 0.25 *M* sucrose added to 2.6 ml Tris* at pH 7.4 (0.025 *M*) containing 9.8 mg glucose and 25.0 mg KCl When used: TPN or TPNH at 0.6 μmoles; ADP at 1.44 mg; hexokinase (Calbiochem) at 2 μg; $MgCl_2 \cdot 6H_2O$ at 3.8 mg. Incubation in Dubnoff shaker, at 80 to 100 oscillations/minute, temperature 37°C, for 1 hour.

	Optical density at 535 mμ			
	No $MgCl_2$		Plus $MgCl_2$	
	.15 *M* KCl	.25 *M* sucrose	.15 *M* KCl	.25 *M* sucrose
Controls	.04	.06	.04	.06
+ hexokinase + ADP	.07	.08	.07	.07
+ TPN (+ either ADP or hexokinase)	.07	.09	.06	.10
+ TPN + ADP + hexokinase	.44	.58	.46	.54
+ TPNH	.17	.19	.07	.10
+ TPNH + hexokinase	.38	.25	.12	.13
+ TPNH + ADP	.59	.56	.30	.36
+ TPNH + hexokinase + ADP	.56	.66	.33	.36

* Tris (hydroxymethyl) aminomethane buffer.

heated for 10 to 15 minutes in a boiling water bath, cooled to room temperature, and the color intensity read at 535 mμ in a Beckman Model B Spectrophotometer.

Results. The following conclusions were drawn from the data presented in Table I. (a) The use of 0.25 *M* sucrose to prepare and test liver fractions does not result in impairment of TBRS production as has been suggested by many investigators(2,3,9,10 and others). (b) The enzymatic formation of TBRS in the presence of TPN requires ADP and hexokinase in contrast to its production with TPNH where addition of ADP only is required. (c) The presence of Mg^{++} appears to inhibit the production of TBRS by the TPNH system but not that by the TPN system.

In Table II are presented data from a representative experiment which demonstrates the influence of ferrous iron with time on the pyridine nucleotide systems for production of TBRS. It is apparent that iron is more stimulatory in the TPNH system than in that employing TPN. Moreover, iron frequently prevents maximum production of TBRS by the latter. The inhibitory action of Mg^{++} on the TPNH system is again demonstrated by these data.

Table III depicts an experiment using fractions prepared from a normal human liver (accidental death of a 26-year-old female). The autopsy liver sample was taken approximately 2 hours after death and held frozen until use. Macroscopically and microscopically the tissue appeared normal. The time that the tissue was frozen prior to testing was approximately the same as that subsequently used for tumor specimens. Frozen samples were thawed and tested and the results are recorded in Table III. They show that ascorbate catalyzed TBRS production is comparable to that previously obtained with fresh and frozen normal rat liver fractions (15). In contrast, the enzymatic reaction mediated by pyridine nucleotides was not observable in this human tissue with TPNH and barely detectable with TPN, employing

TABLE II. Influence of Ferrous Sulfate (Exsiccated) with Time on TBRS Production by Normal Rat Liver Microsomes.

0.4 ml microsomes in 0.25 *M* sucrose added to 2.6 ml Tris buffer containing glucose at 9.8 mg, KCl at 25.0 mg, $MgCl_2 \cdot 6H_2O$ at 3.8 mg, hexokinase at 2 μg, and ADP at 1.44 mg. When used, TPN and TPNH were added at 0.6 μmoles and ferrous sulfate at 46.5 μg. Incubation at 37°C in Dubnoff shaker.

	Optical density at 535 mμ			
	TPN	TPN + Fe	TPNH	TPNH + Fe
Zero time	.05	.05	.08	.09
15 min	.24	.38	.33	.59
30 min	.45	.55	.27	.64
1 hr	.81	.66	.40	.70
No $MgCl_2$ —1 hr	.83	.70	.75	1.15

TABLE III. Production of TBRS with Normal Human Liver* Fractions.

Tris system: 0.4 ml of normal human liver fractions in 0.25 *M* sucrose added to 2.6 ml Tris buffer (pH 7.4, 0.025 *M*) containing 1.44 mg ADP and 2 μg hexokinase (Calbiochem).
Complete system: To the above was added glucose at 9.8 mg, KCl at 25.0 mg, $MgCl_2 \cdot 6H_2O$ at 3.8 mg. When used: TPN or TPNH at 0.6 μmoles, ascrobic at 120 μg, and ferrous sulfate (exsiccated) at 100 μg. Incubation in Dubnoff shaker, at 80 to 100 oscillations/minute, temperature 37°C, for 1 hr.

	Optical density at 535 mμ					
	Mitochondria + microsomes (230 μg nitrogen)		Mitochondria (569 μg nitrogen)		Microsomes (283 μg nitrogen)	
	Tris	Complete	Tris	Complete	Tris	Complete
Control	.09	.09	.19	.09	.1	.09
+ TPN	.04	.09	.19	.22	.06	.11
+ TPNH	.02	.03	.15	.09	.02	.02
+ ascorbic	.64	.54	.95	.94	.81	.64
Control + Fe	.44	.18	.60	.25	.47	.21
+ TPN + Fe	.11	.25	.50	.43	.16	.34
+ TPNH + Fe	.2	.12	.50	.25	.28	.24
+ ascorbic + Fe	.0	.0	.50	.25	.08	.11

No reaction was present at zero time in any of the variables tested.
* From macroscopic and microscopic examination, the tissue appeared normal. It was from a patient without obvious disease signs. The time that the tissue was frozen prior to testing was approximately the same as that used for the tumor specimens.

mitochondria in the complete system. Surprisingly, iron stimulates TBRS production significantly in Tris buffer but only slightly in the complete system. Addition of both pyridine nucleotides to the iron stimulates Tris-system depressed TBRS production, whereas in the complete system only TPN stimulated the iron catalyzed reaction as found with rat tissue (Table II). The ascorbate mediated TBRS production is inhibited by iron.

Two tumors present in human livers were likewise examined. The first was a primary adenocarcinoma of the colon that had metastasized to the liver. Four sections were tested; one from a cancerous nodule, 2 with apparently bordering normal tissue, and one section of liver far removed from the obvious cancer area. Microscopic examination, however, showed that the macroscopically normal appearing liver was also in a necrotic condition and could not be classified as normal. The second tumor was a cholangioma and again 4 sections were taken as described for the adenocarcinoma. Microscopic examination showed that little representative normal liver tissue was present. The apparently macroscopically normal liver showed inflammatory lesions and necrotic changes with many abnormal cells. No production of TBRS was found with either of the 2 tumors under the conditions outlined in Table III for a normal human liver.

These results are in agreement with those of other investigators who found that tumors and rapidly proliferating tissues, in contrast to normal tissues of many types in addition to livers, do not produce upon incubation with ascorbic acid substance(s) that react with thiobarbituric acid(12,13,14,16,17).

Summary and conclusions. It has been shown that normal human liver will produce TBRS by the chemically mediated reaction catalyzed with ascorbate or iron but that the enzymatic production of TBRS is either nonexistent or only weakly active under the conditions tested. In contrast the 2 human tumors studied were unable to produce TBRS chemically or enzymatically under the conditions used. Hochstein and Ernster(2) and Hochstein *et al*(3) reported that the ADP stimulation of their TPNH system was due to the presence of iron in the ADP employed. It does not appear likely that an iron contamination will account for the production of TBRS by the TPN system of Thiele and Huff(1) since ADP does not increase TBRS in this TPN system unless hexokinase is present. Furthermore, hexokinase was shown not to influence the TPNH system (Table I).

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An Electrophoretic Study of the Neurotoxin(s) of Nine Strains of *Clostridium tetani.* (29941)

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The observations of investigators working with *Cl. tetani* have frequently suggested the existence of biological differences in the toxins produced by different strains of this organism(1-6).

After Pillemer *et al*(7) crystallized the neurotoxin of the Harvard strain, little attention was given to the possibility that more than one neurotoxin might be produced until Largier(8) reexamined the concept of qualitative differences among the toxins produced by different strains of *Cl. tetani.* In a comparison of 2 strains, he found no differences in physical or immunological properties.

The present study was designed to examine a larger number of strains from a variety of sources for possible qualitative differences. Starch zone electrophoresis was used to examine the electrophoretic mobility of the neurotoxins of 9 strains of *Cl. tetani.*

Materials and methods. The 9 strains of *Cl. tetani* used were: Massachusetts C_2 (Mass. State Biological Laboratories); Harvard (Dept. of Public Health, State of Michigan); F/3 (Connaught Laboratories, Toronto, Canada); Tulloch II #127 (NIH); Wellcome G.S. #761 (South African Inst. of Medical Research); 301; 43/50-51 (Statens Seruminstitut, Copenhagen, Denmark); CN 1339 (T61B) (Wellcome Research Laboratory, Beckenham, England); Pease TP (Lederle Laboratories).

The Massachusetts strain was grown in the medium described by Latham, Bent and Levine(9). For all other strains the medium developed by Mueller and Miller was used(10). To obtain a filtrate containing only those macromolecules produced by *Cl. tetani* the cellophane bag technique described by Polson and Sterne(11) was employed. A bag of Visking dialysis tubing containing 100 ml of saline was attached to the glass apparatus described by Koch and Kaplan

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