change in proliferation rate, however slight, was accompanied with a proportionate change in uptake. A discrepancy appears in the Jensen experiment at the 5th to 6th day when the rate of uptake was larger than during the 1st to 2nd day period. Otherwise, the changes were in proportion to the proliferation.

Two of the human cell types, HEp-2 and WISH, have pronounced epithelial-like morphology, and the magnitude of glucose uptake in pre-confluent populations of these was much less than in the other cell types. During the period of formation of multilayers, 4th to 8th day, glucose uptake increased, especially in the HEp-2 cells derived from malignant tissue, but retained, nevertheless, a degree of correlation with rate of proliferation.

Although this report specifically illustrates the relationship between glucose uptake and proliferation (and possibly morphology), it also stresses the use of perfusion systems in many types of animal cell culture studies. Nutritional and metabolic investigations, in particular, may be facilitated when they are conducted (a) in controlled *in vitro* environments, (b) within well-defined phases of population development, and (c) in dense as well as dilute populations.

Summary. Perfused animal cell cultures with controlled pH and glucose concentrations exhibited direct correlations of rates of glucose uptake with rates of proliferation and population density. Further, rapidly proliferating cells of fibroblast-like morphology consumed glucose at *ca* 4-fold the rates observed in cells of epithelial morphology increasing at comparable rates.

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1. Willmer, E. N., J. Exptl. Biol., 1941-42, v18, 237.

2. Wilson, H., Jackson, E. B., Brues, A. M., J. Gen. Physiol., 1941-2, v25, 689.

3. Westfall, B. B., Evans, V. J., Shannon, J. E., Jr., Earle, W. R., J. Natl. Cancer Inst., 1953, v14, 655.

4. Fulton, W. C., Sinclair, R., Leslie, I., Biochem. J., 1956, v63, 18p.

5. Bryant, J. C., Schilling, E. L., Earle, W. R., J. Natl. Cancer Inst., 1958, v21, 349.

6. Zwartouw, H. T., Westwood, J. C. N., Brit. J. Exptl. Pathol., 1958, v39, 529.

7. Munyon, W. H., Merchant, D. J., Exptl. Cell Research, 1959, v17, 490.

8. Bryant, J. C., Schilling, E. L., Peppers, E. V., Evans, V. J., Abstracts Tissue Culture Assoc. Meeting, 1964, Baltimore, Md., p55.

9. Kruse, P. F., Jr., Myhr, B. C., Johnson, J. E., White, P. B., J. Natl. Cancer Inst., 1963, v31, 109.

10. McCoy, T. A., Maxwell, M. D., Kruse, P. F., Jr., Cancer Research, 1959, v19, 591.

11. Kruse, P. F., Jr., Schoolar, E. J., Jr., White, P. B., Biochim. et Biophys. Acta, 1960, v41, 375.

12. Somogyi, M., J. Biol. Chem., 1952, v195, 19.

13. McCarty, K., Exptl. Cell Research, 1962, v27, 230.

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Complement Dependence of Human Serum Toxicity for Melanoma S91 Cells.* (30390)

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Toxicity of normal human serum to cultured mammalian cells(1,2) and to the ascites cells of various mouse tumors(3-5) has been extensively studied. Inability to produce suspensions of single cells of sufficiently high viability from solid tumors has hindered a study of the effects of serum on this type of cell. In view of the observation by Abramoff *et al*(6) that the S91 melanoma contains antigenically distinctive components it is pertinent to determine if normal human serum is cytotoxic to cells of this solid tumor. For these reasons it was considered of interest to

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study the complement dependence of tumor cell cytotoxicity of individual human sera which might be suspected of containing tissue-reacting antibodies(7,8). A further study was made to determine if toxicity might be related to "Wassermann" antibody, sheep red blood cell hemolytic antibody content of the serum, a clinically manifest disease, or recent medication.

Materials and methods. Tumor. The transplantable mouse melanoma S91 which originated at the Roscoe B. Jackson Memorial Laboratory and was carried in DBA/1 mice was used in this study. The tumor was maintained by mouse to mouse transfer of single cell suspensions aseptically prepared in commercial Earle's solution with the aid of a Snell cytosieve(9).

Preparation of cell suspension. Freshly excised tumors, trimmed of necrotic and connective tissues, were minced with iris scissors. Cell suspensions prepared according to the enzymatic digestion method of Madden and Burk(10) were repeatedly found to consist of 93 to 98% viable cells as measured by the dye exclusion test using 0.25% trypan blue(10,11). Tumor fragments were digested with freshly prepared 0.25% trypsin and 0.04% deoxyribonuclease (DNase) in veronal buffer. The modified, divalent cation-free, Earle's solution used by Madden and Burk was replaced by veronal buffer containing 0.1% gelatin and adjusted to pH 7.4 with N/10 HCl because of the excellence of the latter buffer for use in systems employing complement(12).

Human sera. Blood samples were collected from patients attending a social hygiene clinic at the Milwaukee Public Health Department. Individual specimens were classified as being: 1) "Reactive" or "Non-reactive" to the VDRL non-treponemal antigen test for syphilis(13); 2) having come from patients with gonorrhea; or 3) having come from presumably normal donors with no apparent venereal disease.

Absorption procedures. VDRL "Reactive" sera were absorbed with VDRL antigen. Absorbing antigens were prepared by precipitating 5 ml of commercial VDRL cardiolipin lecithin antigen with 25 ml of 0.85% saline. To remove natural SRBC hemolytic antibody, test sera were absorbed with one-half their volume of thrice washed, packed SRBC held at 0°-1°C for 30 minutes.

Portions of specimens 6 and 15 were each absorbed with melanoma S91 cell suspensions prepared by mechanical and enzymatic means.

Each absorbed specimen along with its absorption control and unabsorbed counterpart was assayed for complement (C′) activity and reactivity with the absorbing antigen (if other than tumor cells) before and after being tested.

Titration of complement. Complement activity of fresh human serum, before and after reaction in the cytotoxic test, was assayed using the standard one-fifth volume titration technique of Kolmer(13). However, the sera were not preincubated with the hemolytic system.

In vitro *cytotoxic test.* In the cytotoxic tests the suspensions of single, viable, S91 cells were adjusted with veronal buffer to contain 4×10^6 cells per 0.5 ml so as to provide approximately 2×10^6 cells/ml in a test system of 2.0 ml final volume of which 0.5 ml was human serum. The tubes were shaken and incubated for 1 hour at 37°C. Approximately 0.5 ml of the contents of a tube to be assayed for cell viability was mixed with an equal volume of 0.25% trypan blue and a random count of at least 200 cells was made.

The cytotoxic index (C.I.) was calculated as a function of the survival control; $C.I. = \frac{C-E}{C}$ where C = percent of living, nonstained cells in the control, and E = percent of living cells in the experimental tube(14).

In those experiments in which the relative amount of human C′ used in the cytotoxic reaction was to be measured, the tubes were first centrifuged for 5 minutes at $200 \times g$ after incubation and the supernatants decanted and stored at −43°C until a C′ assay could be performed. The sedimented cells were resuspended in veronal buffer before measuring a volume to be mixed with trypan blue. An additional control, consisting of a portion of the human test serum diluted 1:4

TABLE I. Cytotoxicity of Human Serum to S91 Melanoma Cells.

Serum sample No.	Reactivity with VDRL slide test	% Viable cells: Survival control*	% Viable cells: Final serum dilution of fresh human serum 1:4	% Viable cells: Final serum dilution of fresh human serum 1:20	Cytotoxic index (C.I.): Final serum dilution of fresh human serum† 1:4	Cytotoxic index (C.I.): Final serum dilution of fresh human serum† 1:20	Cytotoxic index (C.I.): Final serum dilution of heat inactivated human serum† 1:4
1	Reactive	96.0	23.1	59.0	.76	.39	.02
2	"	94.8	10.0	63.4	.89	.33	.03
3	"	90.6	18.0	60.0	.80	.34	.01
4	"	95.7	41.7	54.9	.56	.43	.09
5	"	87.1	52.7	73.8	.40	.15	.01
6	Weakly reactive	81.3	6.0	—	.93	—	.01
7	Nonreactive	92.6	52.4	70.7	.43	.33	.02
8	"	96.7	2.9	45.9	.97	.53	.11
9	"	96.5	4.4	56.3	.95	.42	.07
10	"	93.5	7.5	61.8	.92	.34	.06
11	"	92.5	44.7	51.5	.52	.44	.08
12	"	92.5	3.3	55.3	.96	.40	.06
13	"	91.7	46.0	—‡	.50	—	.19
14	"	79.7	3.0	—	.96	—	.01
15	"	83.9	8.3	—	.90	—	.01

* Survival control contains 0.5 ml of S91 cells and 1.5 ml veronal buffer.
† Reaction mixture contains 0.5 ml undiluted human serum or serum diluted 1:5, 0.5 ml S91 cells (4×10^6 cells/0.5 ml) and 1.0 ml veronal buffer.
‡ Not done.

with veronal buffer, was included during the incubation to provide a measure of C′ deterioration in the absence of tumor cells. The residual C′ activity of a test specimen was subtracted from the number of $C'H_{100}$ units used up in the reaction.

Results. It can readily be seen from Table I that, with the exception of samples No. 4, 5, 7, 11, and 13, human serum, at a final reaction mixture dilution of 1:4, was highly toxic to mouse melanoma S91 cells. The 15 samples studied at this dilution showed a mean cytotoxic index of 0.77. Eleven of the 15 specimens, when diluted to 1:20, gave a mean C.I. of 0.37 showing that cytotoxicity diminishes with dilution. The latter observation, along with consistently high survival percentages in buffer alone, tends to indicate that cell death was due to some active cytotoxic component in the serum.

To determine whether serum was selectively cytotoxic to enzyme-treated cells, one sample of fresh undiluted serum was absorbed with S91 cells mechanically prepared with a Snell cytosieve and another sample of the same serum with enzymatically prepared cells. As shown in Table II there is no indication in these experiments that enzymatically prepared cells absorb the cytotoxic factors more effectively than do mechanically prepared cells. Therefore, toxicity of fresh human serum to S91 cells is not an artifact due to enzymatic alteration of the cells.

The consistent heat lability of serum cytotoxicity to S91 cells is readily apparent from data shown in Table I. This apparent re-

TABLE II. Absorption of the Serum Factor Cytotoxic to Mouse Melanoma S91 Cells by Mechanically and Enzymatically Prepared S91 Cells.

Serum sample No.	Unabsorbed serum: Cytotoxic index	Unabsorbed serum: $C'H_{100}$/ml serum	Serum absorbed with cytosieve prepared cells*: No. absorbing cells/ml serum	Serum absorbed with cytosieve prepared cells*: Cytotoxic index	Serum absorbed with cytosieve prepared cells*: $C'H_{100}$/ml serum	Serum absorbed with enzymatically prepared cells*: No. absorbing cells/ml serum	Serum absorbed with enzymatically prepared cells*: Cytotoxic index	Serum absorbed with enzymatically prepared cells*: $C'H_{100}$/ml serum
6	.93	198	1.33×10^6	.71	198	1.33×10^6	.66	198
15	.90	150	15×10^6	.42	120	15×10^6	.44	100

* Absorption with continuous rotation at 4°C for 18 hr.

TABLE III. Fixation of Human Complement in the Cytotoxicity Tests.

Serum sample No.	Fresh serum, C′ activity/ml	C′ deterioration control,* C′ activity/ml	Residual C′ activity/ml, serum reacted with S91 cells†	Cytotoxic index	Amount of C′ used in cytotoxic reaction/0.5 ml serum
11	264 $C'H_{100}$	200 $C'H_{100}$	80 $C'H_{100}$	.52	60 $C'H_{100}$
12	332 $C'H_{100}$	200 $C'H_{100}$	100 $C'H_{100}$	.96	50 $C'H_{100}$

* 0.5 ml serum plus 1.5 ml veronal buffer incubated 1 hr at 37°C.
† 0.5 ml serum, 0.5 ml S91 cells (4×10^6/0.5 ml), and 1.0 ml veronal buffer incubated 1 hr at 37°C.

TABLE IV. Effect of Absorption with VDRL Antigen on Cytotoxic Index of VDRL Reactive and VDRL Nonreactive Sera.

	Before absorption			After absorption		
Serum No.	VDRL slide test reactivity	C′ activity per ml	Cytotoxic index	VDRL slide test reactivity	C′ activity per ml	Cytotoxic index
1	R*	N.D.†	.76	N.R.	N.D.	.04
2	R	N.D.	.89	N.R.	N.D.	.21
4	R	150 $C'H_{100}$	.56	N.R.	40 $C'H_{100}$	.12
5	R (64 dils)‡	120 $C'H_{100}$	.40	R (5 dils)	40 $C'H_{100}$	.01
7	N.R.§	N.D.	.43	N.R.	N.D.	.31
8	N.R.	150 $C'H_{100}$	.97	N.R.	150 $C'H_{100}$	.95
9	N.R.	198 $C'H_{100}$	.95	N.R.	150 $C'H_{100}$	.93
10	N.R.	198 $C'H_{100}$	.92	N.R.	150 $C'H_{100}$	.91

* R = Reactive. † N.D. = Not done. ‡ Dils = Highest saline dilution of serum at which a reactive reading was observed in the VDRL slide test. § N.R. = Nonreactive.

quirement for complement was verified by assaying activity following incubation in the cytotoxic system. The relative amount of complement fixed was measured by subtracting residual values from the number of $C'H_{100}$ remaining in the complement deterioration control. This difference was equated to the number of $C'H_{100}$ units fixed per ml of test serum. Since the cytotoxic test employed 0.5 ml of serum the difference was halved to indicate the number of $C'H_{100}$ units used in killing 4×10^6 S91 cells by 2.0 ml of a 1:4 dilution of serum (Table III).

More conclusive evidence regarding the requirement for complement in this cytotoxic reaction is shown in Table IV. Sera from patients being followed for clinically manifest syphilis were observed not only to react in the VDRL slide test for syphilis but also were found to be toxic to S91 cells. Absorption of these sera with washed VDRL antigen particles resulted in reduction of both VDRL slide test reactivity and tumor cell cytotoxicity. On the other hand, sera from individuals free of diagnosed syphilis and nonreactive to the VDRL test, after absorption with VDRL antigen, showed little or no change in their C.I. The absorption procedure was carried out by incubating serum-VDRL antigen mixtures at 37°C for one hour followed by incubation for 15-18 hours at 7°C. As might be expected the VDRL reactive serum absorbed with VDRL antigen was found to be complement-deficient. Furthermore, the complement activity and cytotoxicity of VDRL nonreactive sera, after absorption with VDRL antigen, were not appreciably changed.

The cytotoxic factor in human serum does not appear to be related to reactivity of the sera in the VDRL slide test for syphilis. All of the sera studied, except specimens No. 8, 11, 12, and 15 were collected from patients with either clinical gonorrhea or syphilis. Specimens No. 11 and 12, which were toxic to S91 cells, were taken from healthy male adults with no apparent disease and no history of recent or past antibiotic treatment or chemotherapy. Therefore, correlation of serum cytotoxicity with a disease process or the presence of antibiotics in the serum seems unlikely. Furthermore, it can be seen from Table V that there is no correlation between the hemolytic antibody titer and the cytotoxicity of any of the serum samples studied

TABLE V. Effect of SRBC Absorption on Cytotoxic Index and Hemolytic Antibody Titer of Unheated Normal Human Serum.

Serum sample No.	Unabsorbed			Absorbed with SRBC		
	C.I.	Hemolytic Ab titer*	$C'H_{100}$/ml	C.I.	Hemolytic Ab titer	$C'H_{100}$/ml
5	.40	256	N.D.†	N.D.	N.D.	N.D.
11	.52	640	N.D.	.63	160	N.D.
12	.96	640	N.D.	.72	160	N.D.
13	.50	128	200	.33	16	200
14	.96	256	150	.95	8	150

* Reciprocal of highest dilution of serum yielding a trace of hemolysis after incubation with an equal volume of 1% SRBC at 37°C for 1 hr.
† Not done.

since absorption of these sera with washed SRBC resulted in 2-, 3-, and 5-fold reductions in their hemolytic antibody titers and a corresponding reduction in their cytotoxicity was not observed. Therefore, lack of correlation between the cytotoxicity and natural SRBC antibody titers of the sera studied makes it seem unlikely that these two properties reside in the same factor.

Discussion. In this study the cytotoxicity of human sera to mouse melanoma S91 cells has been analyzed using Madden and Burk's enzymatic method(10) of preparing viable single cells from solid tumors. These tumor cells were susceptible to toxicity of all of the sera tested. This cytotoxic factor has been shown to be complement-dependent. This observation is in agreement with the finding of other investigators who have studied the effects of human serum on a variety of mammalian cell lines propagated *in vitro,* as well as on cells freshly harvested from ascitic tumors(1-5,15,16). The observation that heating of serum at 56°C for 30 minutes destroys its cytotoxicity is presumptive evidence that complement is required for cytotoxicity. Demonstration that an independent antigen-antibody system (VDRL antigen-VDRL reactive serum), which fixes complement and thereby inhibits the cytotoxicity of the serum, further indicates complement as being essential. This conclusion is further substantiated by data showing the cytotoxic index of a serum to be unaffected when that serum is reacted with the VDRL antigen in the absence of the VDRL or "Wassermann" antibody, a condition in which complement is not fixed. In addition, measurement of the complement activity of unheated human serum, before and after reaction with 4×10^6 S91 cells, showed that 50 to 60 $C'H_{100}$ units had been fixed in the cytotoxic test when the values were corrected against the activity remaining in a C′ deterioration control. The mean C.I. of 8 fresh specimens from this study, which was observed to be 0.79, was diminished to 0.05 following heat-inactivation. The addition of 44 $C'H_{100}$ guinea pig serum, which alone gave a mean C.I. of 0.09, restored only 29% of the original 0.79 to these heated sera. This apparent requirement for human complement is undergoing further study.

The cytotoxic system, as described in this study, was found to be independent of the "Wassermann" and sheep red blood cell hemolytic antibody content of the sera studied. Furthermore, it was found that sera of disease-free and medication-free individuals were as cytotoxic as the sera of those who had a clinically detectable disease and/or recent medication. Until the natural cytotoxic factor of human serum may be identified with a nonspecific sensitizing substance, as intimated by Mayer(17), one is tempted to speculate that it is a natural antibody of the type long known to be active against heterologous erythrocytes and bacteria(18).

The cytotoxicity test used in these studies is a modification of that reported by Gorer and O'Gorman(19). Variations of this procedure have been employed by Ginsburg *et al* (3), Bolande and McClain(4), Landy *et al* (5) and Terasaki *et al*(14). Completely lysed cells probably are not detected by this method since loss of cytoplasmic content would leave nothing behind to bind the dye. However, this assay does permit a valid comparison of the percent of injured cells in the ex-

perimental tube as opposed to the survival control(20). The *in vitro* cytotoxic test not only provides a convenient assay of cell injury but, as pointed out by Winn(21), it enables the investigator to detect destruction of fractions of cell populations.

Summary. Evidence is presented indicating that viable cells, freshly prepared from a solid mouse melanoma, are killed when reacted with normal human serum. Serum cytotoxicity was shown to be complement dependent by 1) heat lability, 2) residual complement testing, and 3) through diminution by reacting the serum with a secondary antigen for which it has a demonstrable antibody. Apparent absorption of toxicity from VDRL-reactive human sera by VDRL antigen was shown to be due to concomitant depletion of complement. The toxicity factor could not be correlated with "Wassermann" antibody or the sheep red blood cell antibody content of the sera. Furthermore, the cytotoxic activity could not be related to recent antibiotic therapy or clinically manifest venereal disease of the serum donors.

1. Fedoroff, S., Doerr, J., J. Nat. Cancer Inst., 1962, v29, 331.
2. Bolande, R. P., Lab. Invest., 1960, v9, 475.
3. Ginsburg, I., Dishon, T., Bloch, M., Gross, J., Proc. Soc. Exp. Biol. and Med., 1961, v107, 235.
4. Bolande, R. P., McClain, J. P., ibid., 1960, v103, 345.
5. Landy, M., Michael, J. G., Trapani, R-J, Achinstein, B., Woods, M. W., Shear, M. J., Cancer Res., 1960, v20, 1279.
6. Abramoff, P., Chinchinian, H., Saunders, J. W., Jr., J. Nat. Cancer Inst., 1959, v22, 919.
7. Kahn, R. L., Serology in Syphilis Control, Williams & Wilkins, Baltimore, 1942.
8. Muschel, L. H., Simonton, L. A., Wells, P. A., Fife, E. H., Jr., J. Clin. Invest., 1961, v40, 517.
9. Snell, G. D., J. Nat. Cancer Inst., 1953, v13, 1511.
10. Madden, R. E., Burk, D., ibid., 1961, v27, 841.
11. Schrek, R., Am. J. Cancer, 1936, v28, 389.
12. Wallace, A. L., Osler, A. G., Mayer, M. M., J. Immunol., 1950, v65, 661.
13. Serologic Tests for Syphilis, A Manual, U. S. P.H.S. Publ. 411, 1959.
14. Terasaki, P. I., Esail, M. L., Cannon, J. A., Longmire, W. P., Fr., J. Immunol., 1961, v87, 383.
15. Willheim, R., Ivy, A. C., Janacek, H. J., Exp. Med. and Surg., 1957, v15, 300.
16. Bolande, R. P., Todd, E. W., A. M. A. Arch. Path., 1958, v66, 720.
17. Mayer, M. M., Cancer Res., 1961, v21, 1262.
18. Skarnes, R. C., Watson, D. W., Bact. Rev., 1957, v21, 273.
19. Gorer, P. A., O'Gorman, P., Transplant. Bull., 1956, v3, 142.
20. Reif, A. E., Norris, H. J., Cancer Res., 1960, v20, 1235.
21. Winn, H. J., Ann. N. Y. Acad. Sci., 1962, v101, 23.

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Effect of Nicotinic Acid on Serum Triglyceride, Cholesterol and Chylomicrons in Rats. (30391)

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The serum cholesterol lowering effect of nicotinic acid has been studied in various animal species since it was first reported to be of interest in the treatment of hypercholesterolemia in man(1). Recent clinical studies have shown that, in addition to reduced serum cholesterol, nicotinic acid treatment resulted in lowered blood triglyceride(2). In this paper are reported experiments in which reductions in serum cholesterol and triglyceride were obtained in the rat, and data are presented on the effects of nicotinic acid in reducing the number of serum chylomicrons present after fat ingestion.

Methods. A) *Serum triglyceride and cholesterol in fasted rats.* Individually housed male Charles River rats (150-180 g) were fasted 24 hours. Water was given *ad libitum.* Nicotinic acid suspended in distilled water was administered intragastrically to treat-