system may follow the usual dose of nitrogen mustard, particularly in patients with widespread disease and in those patients who have had previous treatment with other similar agents or x-ray."

In an attempt to clarify the problem of this effect of previous exposure to x-ray and subsequent response to nitrogen mustard, a similar experimental study was devised utilizing the rabbit as the experimental animal. There was a marked difference in response of the two groups. In the control group which received no previous x-ray, but did receive 1.5 mg/kg nitrogen mustard, there were no deaths and no depression of hemopoietic activity. In the x-irradiated group, with all animals showing normal hemopoietic activity prior to receiving nitrogen mustard, 3 of 5 animals died following injection. In 2 of these, the cause of death was not demonstrated at autopsy. Blood counts were not obtained prior to death. In the third, bone marrow and peripheral blood count revealed marked depression of hemopoietic activity and death secondary to massive hemopericardium. That this complication was, in part, due to thrombocytopenia is suggested by the fact that no evidence of bleeding due to cardiac puncture in the control group was noted.

Summary. Rabbits which had been exposed to whole body x-irradiation showed a significant mortality rate following injection of 1.5 mg/kg body weight nitrogen mustard in contrast to the control group where no deaths occurred. This difference was noted even though bone marrows of the x-irradiated group were functionally normal when nitrogen mustard was administered.

1. Conrad, M. E., Crosby, W. H., *Blood*, in press, 1960.

2. Mrazek, R. G., Jr., Wachowski, T. J., *J.A.M.A.*, 1955, v159, 160.

3. Council on Pharmacy and Chemistry, *ibid.*, 1956, v161, 150.

Received Sept. 27, 1960. P.S.E.B.M., 1960, v105.

Effects of Brucellaphage on Extracellular and Intracellular *Brucella abortus*.* (26136)

ROSSLYN W. I. KESSEL, MOSHE ARONSON,† AND WERNER BRAUN
Institute of Microbiology, Rutgers, The State University, New Brunswick, N. J.

During studies on the activity of a brucellaphage(1) capable of lysing smooth *Brucella abortus* it was observed that UV-inactivated phage preparations retained their ability to inhibit multiplication of *Br. abortus* organisms. Similar findings have been reported for *Escherichia coli*(2). Efforts now have been made to ascertain whether these inhibitory effects were caused by the protein moiety of the phage, and also whether such effects could be exerted intracellularly as well as extracellularly. An opportunity to examine this latter aspect was afforded by concurrent studies on intracellular multiplication of *Br. abortus* within monocytes maintained *in vitro*(3-5).

Methods. Smooth organisms of *Br. abortus*, strain SA-S(4), were maintained and grown using tryptose agar, buffered beef extract broth (BBE), or, as described below, within monocytes. Brucellaphage(1) was propagated by mixing a BBE suspension of 24-hour-old *Br. abortus* organisms with phage in the ratio of 1:5, allowing adsorption for 30 mintues at 37°C, and plating the mixture upon tryptose agar. Twenty-four hours later the surface of the agar was washed with saline, a few ml of chloroform was added to the washings to kill all remaining viable brucellae, and the mixture was allowed to stand overnight at 4°C. Phage plus bacterial debris were then sedimented by centrifugation,

* Supported by a grant from Nat. Science Foundation and a contract with U. S. Army Chem. Corps, Fort Detrick, Frederick, Md.

† On leave of absence from Israeli Inst. for Biol. Research, Ness-Ziona, Israel.

and the phage was eluted from the sediment into phosphate buffer at pH 7.0. Successive elutions and centrifugation (1 to 5), yielded phage preparations having a titer of about 1 x 10^{10} plaque-forming units (pfu) per ml. Phage was titrated by plaque count; serial dilutions being placed onto respread, young (8-hour-old) surface cultures of susceptible *Br. abortus*. UV-inactivation (99%) of phage was achieved with a "Universal Illuminator" (40 cm, 2 minutes), heat-inactivation (99.99%) by heating to 70°C for 10 minutes.

Cultures of guinea-pig monocytes were prepared according to the methods of Pomales-Lebrón and Stinebring(3), except that monocytes were allowed to ingest brucellae while both were in suspension at 37°C for 1 hour. Following such periods of ingestion, the monocytes were washed free from extracellular brucellae and resuspended in serum-Hanks' medium containing streptomycin (10 γ per ml). One-ml portions of this brucella-containing monocyte suspension were then mixed with either phage or buffer (the latter served as control) and introduced into Porter flasks. Thus, in any given experiment, phage-exposed and unexposed monocytes were identical in regard to prior history and brucella content. After 2 hours incubation at 37°C, when the monocytes had become attached to the floor of the flask, the supernatant medium was replaced by streptomycin-containing serum-Hanks' medium free of phage. After desired periods of time, number of intracellular brucellae was estimated by viable counts of bacteria released from broken monocytes(5), and by direct microscopic observations on stained monocytes adhering to flying coverslips that were contained within the culture flasks. In addition, number of monocytes per flask was estimated, after staining(3), by microscopic counts on 10 separate, measured areas of the coverslip.

Results. Effects of phage on extracellular brucellae. When equal volumes of suspensions of phage (5 x 10^9 pfu per ml) and smooth *Br. abortus* (1 x 10^9 per ml BBE) were mixed, incubated on a shaker at 37°C for 30 minutes, diluted, and plated upon tryptose agar, approximately 35% of the cells were killed (judged by their inability to form detectable colonies). Table I, which shows data typical of a large series, also contains data demonstrating that a comparable inhibition of bacterial growth was obtained when UV-inactivated, but not when heat-inactivated, phage preparations were employed. The possibility of ascribing the inactivating effects exerted by UV-irradiated phage to the remaining 1% plaque-forming phage is made unlikely by the observation that untreated phage preparations diluted to a comparable pfu titer failed to affect the multiplication of susceptible bacteria. Our inability to obtain more than 40 to 45% "killing" by either untreated or UV-treated phage preparations is probably due to a lack of optimal conditions for phage adsorption onto bacteria in the present experiments.

TABLE I. Survival of *Brucella abortus*, SA-S, Following Exposure to Untreated or Treated Brucellaphage.

Brucellae exposed to:	Brucellae recovered*	% inhibition
Buffer	103 ± 5.7	—
Untreated phage	66 ± 2.8	36
UV-inactivated phage	58 ± 3.1	44
Heat-inactivated "	98 ± 4.6	None

* After plating 0.02-ml aliquots of a 10^{-5} dilution of the phage-bacterium mixture. Averages from 4 separate determinations.

Effects of phage on intracellular brucellae. When the effects of untreated, UV-inactivated, and heat-inactivated phage preparations were tested with brucellae that had previously been ingested by monocytes, the results were essentially identical with those obtained in tests with extracellular brucellae; *i.e.*, untreated or UV-treated phage, even when added following the period of ingestion of brucellae by monocytes, interfered with intracellular multiplication of the bacteria. Thus, inspection of stained preparations, obtained from replicate cultures at various times following exposure to phage, revealed a slight but consistent retardation of intracellular multiplication of brucellae in cultures exposed to untreated or UV-treated phage. Typically, the number of smooth brucellae within individual monocytes increases rapidly after a lag of approximately 12 hr(4); however, cultures exposed to untreated or

TABLE II. Distribution of *Brucella abortus*, SA-S, within Individual Brucella-Containing Monocytes Cultivated *In Vitro* in Presence or Absence of Phage Preparations. Data obtained by microscopic examination of stained material.

Time of exam.*	Treatment of cultures	% of monocytes containing 1-6	7-10	>10 brucellae
2	Control	77 ± 6.1†	19 ± 3.0	4 ± 1.2
	Active phage	83 ± 7.0	13 ± 3.1	4 ± 1.4
	UV-treated phage	85 ± 7.9	10 ± 2.2	5 ± 1.2
	Heat-treated "	89 ± 6.2	7 ± 1.2	4 ± 1.1
24	Control	46 ± 4.2	36 ± 5.9	18 ± 3.5
	Active phage	63 ± 4.4	24 ± 6.8	13 ± 2.9
	UV-treated phage	65 ± 4.5	28 ± 6.1	7 ± 2.0
	Heat-treated "	48 ± 3.9	36 ± 5.7	16 ± 3.1

* In hr after introduction into culture flasks.
† Each figure represents avg value obtained by pooling data from 3 separate experiments. Within each experiment at least 2 coverslips were read for each determination.

UV-treated phage regularly contained smaller numbers of brucellae per monocyte after 24 hours than cultures exposed to no phage or to heat-treated phage (Table II). (For any one of the experiments here summarized all of the phage-free and phage-exposed cultures originated from the same large sample of brucella-containing monocytes.) This effect of phage was also discernible by viable counts of brucellae harvested from phage-free and phage-exposed monocyte cultures, particularly when large numbers of brucellae were employed in the inoculum (Table III). The effect, though still discernible, was less pronounced in cultures with smaller inocula (Table IV).

The foregoing data are based on the fate of monocytes containing one or more brucellae. Also, in many, but not all, experiments with cultures exposed to untreated or UV-treated phage, an increased "0 class" was observed. That is, throughout the testing period the percentage of monocytes harboring one or more brucellae tended to be less (as low as 50%) in phage-exposed cultures than in phage-free cultures or in cultures exposed to heated phage (where the typical value was 80%). Since this difference was detectable as early as 2 hours after addition of phage, and since all the monocytes were equally infected initially, this difference between phage-exposed and phage-free cultures suggests that in the former an actual intracellular destruction of bacteria may occur.

TABLE III. Viable Counts of Intracellular *Brucella abortus*, SA-S, Harvested from Phage-Free or Phage-Exposed Monocyte Cultures with High Initial Brucella Counts.*

Time of exam.†	Treatment of cultures	Viable brucellae ($\times 10^5$)/10^6 monocytes‡
2	Phage-free	3.26 ± .32
	" -exposed	1.60 ± .15
24	" -free	12.66 ± 1.39
	" -exposed	1.56 ± .25
48	" -free	39.63 ± 5.61
	" -exposed	7.04 ± .82

* Cultures initiated with approximately 125 × 10^6 brucellae/10^6 monocytes.
† In hr after introduction into culture flasks.
‡ Avg of duplicate determinations upon each of 2 cultures.

TABLE IV. Viable Counts of Intracellular *Brucella abortus*, SA-S, Harvested from Phage-Free or Phage-Exposed Monocyte Cultures with Low Initial Brucella Counts.*

Time of exam.†	Treatment of culture	Viable brucellae ($\times 10^4$)/10^6 monocytes‡
2	Phage-free	6.3 ± .37
	" -exposed	5.2 ± .34
	UV phage-exposed	5.4 ± .41
24	Phage-free	92.3 ± 8.62
	" -exposed	48.5 ± 6.33
	UV phage-exposed	51.1 ± 4.90

* Cultures initiated with approximately 25 × 10^6 brucellae/10^6 monocytes.
† In hr after introduction into culture flasks.
‡ Avg of duplicate determinations upon each of 2 cultures.

Discussion. The finding that untreated, or UV-treated, brucellaphage is capable of interfering with intracellular and extracellular multiplication of *Br. abortus*, whereas heat-treated phage lacks this capability, suggests that the protein moiety of the phage is responsible. It remains uncertain in the case

of the interference with the intracellular multiplication of brucellae: 1) whether the entire, untreated or UV-treated, phage particle is phagocytosed and subsequently enters into a direct bacterium-phage interaction, 2) whether only the active protein moiety diffuses to the susceptible bacterial site, or 3) whether the phage-protein, in conjunction with monocyte constituents, can cause changes in intracellular conditions that secondarily affect bacterial multiplication. The last hypothesis seems unlikely in view of the similarity between results obtained in extra- and intracellular systems. Also, preliminary tests, in which monocytes containing many brucellae were exposed to untreated phage, showed, 24 hours later, slight but consistent increases in phage titers (3.37×10^4 per 10^6 monocytes) over those obtained from brucella-free cultures (1.06×10^4 per 10^6 monocytes) or from cultures initially containing intracellular heat-killed brucella (1.67×10^4 per 10^6 monocytes). These observations would suggest that phage is actually phagocytosed and may interact with intracellular bacteria in the same manner as with extracellular bacteria. Finally, the observation that in phage-exposed cultures there is a decreased proportion of monocytes that contain stainable brucellae suggests the possible occurrence of additive effects between phage (or phage-protein) and cellular factors that can lead to the intracellular destruction of brucellae.

Summary. Studies on the interactions between brucellaphage and *Br. abortus,* either under extracellular conditions or when the bacteria resided intracellularly within guinea pig mononuclear phagocytes maintained *in vitro,* showed: 1) inhibitory effects of untreated or UV-treated phage on the multiplication of brucellae, and 2) a lack of such effects in the case of heat-treated phage. These findings suggest an association between brucellacidal, or possibly brucellastatic, effects and phage protein. It was demonstrated that the effects on intracellular bacteria can occur even when exposure to phage is delayed until 2 hours after phagocytosis of the bacteria.

1. Stinebring, W. R., Braun, W., *J. Bact.,* 1959, v78, 736.

2. Luria, S. E., Delbrück, M., *Arch. Biochem.,* 1942, v1, 207.

3. Pomales-Lebrón, A., Stinebring, W. R., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 78.

4. Braun, W., Pomales-Lebrón, A., Stinebring, W. R., *ibid.,* 1958, v97, 393.

5. Stinebring, W. R., Kessel, R. W. I., *ibid.,* 1959, v101, 412.

Received Sept. 28, 1960. P.S.E.B.M., 1960, v105.

Creatine Synthesis After Creatinine Loading and After Nephrectomy.* (26137)

RALPH GOLDMAN AND JOHN X. MOSS

Department of Medicine, University of California Medical Center, Los Angeles and Veterans Administration Hospital, Sepulveda, Calif.

Bloch and Schoenheimer(1) established that in mammals glycine and amidine (from arginine) react to form guanidinoacetic acid (GAA) and Du Vigneaud(2) demonstrated that the latter compound is methylated to form creatine. The two enzymes necessary for this synthesis are arginine-glycine transamidinase and guanidinoacetate methylferase. It had been thought for many years that the first of these enzymes was found only in the mammalian kidney(3). However, Walker (4) has demonstrated that the pancreas contains significant amounts of transamidinase, and could be a site of GAA synthesis. We have reported(5) that we were able to recover creatine-C^{14} after injecting nephrectomized rats with glycine-2-C^{14}, indicating that there is, in fact, a non-renal route of creatine synthesis. Horner(6) has reported essentially the same observation. However, in our study

* This work was supported by grant from Nat. Heart Inst.