small quantities. This was so for at least 5 hours after injection of the labeled material.

The majority of the pyridoxine (about 70%) was excreted unchanged with a half-time of about 45 minutes. There may be 2 reasons for rapid excretion of the unchanged drug: *in vivo* substrate concentrations of pyridoxine exceed the levels in which the tissue enzyme systems responsible for its oxidation can operate, and it may be a reflection of the inability of tissue fixation or conjugation. Retention of about 28% of the label with a half-time of 17 days may represent the portion which is fixed and stored in the tissue. It is possible that previous colorimetric and microbiological assay methods are not quantitatively refined to detect this slow turnover rate.

Only one degraded metabolite of pyridoxine could be detected in small amounts in the urine excreted within 5 hours after injection. At later times, the label was so dilute it was difficult to detect the tritium beta activity (18 kv).

It would appear from the present study that pyridoxine is excreted primarily as unchanged drug in the rat, and in small amounts as 4-pyridoxic acid. Also, the very slow excretion half-time of 17 days of about 28% of the drug would tend to support the hypothesis that the "missing" pyridoxine, referred to by Snell(6) as possibly being excreted in an unidentified conjugated form, may be stored in the tissue and excreted with a rather long half-time.

Summary. Urine, blood, and fecal samples from rats injected intravenously with tritium-labeled pyridoxine hydrochloride were collected at various time intervals post injection for excretion and chromatographic study. Approximately 70% of the administered label was excreted in the urine in 5 hours. Fecal excretion was less than 0.03% in 96 hours post injection. There were 2 rates of excretion: the initial rapid rate with a half-time of about 45 minutes, followed by a slower rate with a half-time of 17 days. Chromatographic studies of urine showed that the pyridoxine was excreted primarily unchanged with a small amount of only one metabolite, most likely 4-pyridoxic acid.

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1. Scudi, J. V., Koones, H. F., Keresztesy, J. C., PROC. SOC. EXP. BIOL. AND MED., 1940, v43, 118.

2. Scudi, J. V., Unna, K., Antopol, W., *J. Biol. Chem.*, 1940, v135, 371.

3. Scudi, J. V., Buhs, R. P., Hood, D. B., *ibid.*, 1942, v142, 323.

4. Huff, J. W., Perlzweig, W. A., *ibid.*, 1944, v155, 345.

5. Rabinowitz, J. C., Snell, E. E., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 235.

6. Snell, E. E., Viatmin Metabolism, in *Proc. 4th Internat. Congress of Biochem.*, 1958, vXI, 250.

7. Verly, W. G., Hunebelle, G., *Bull. Soc. Chim. Belg.*, 1957, v66, 640.

8. Richmond, C. R., Trujillo, T. T., Martin, D. W., PROC. SOC. EXP. BIOL. AND MED., 1960, v104, 9.

9. Consden, R., Gordon, A. H., Martin, A. J. P., *Biochem. J.*, 1944, v38, 224.

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Autoradiographic Studies on Intracellular Growth of *Brucella melitensis* and Thymineless *E. coli.** (27167)

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The biochemical basis of phagocytosis has received its greatest impetus from studies concerning the events immediately following ingestion of the particle by the phagocyte. For greatest clarity in understanding the biochemical data obtained, the use of inert particles has been fruitful in avoiding the con-

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fusion which results when one tries to separate the metabolic activities of the host cell and parasite in an intracellular parasitic system.

By the use of starch particles, it has been demonstrated that the phagocytic process by polymorphonuclear leukocytes is known to make special demands on the output of cellular energy which is supplied by glycolysis(1, 2). Cyanide fails to affect ingestion or inactivation of the intracellular bacteria but iodoacetate and arsenite markedly inhibit ingestion(3,4). Increased formation of phosphatidic acids has been ascribed to production of new cell membranes as a kind of repair process following ingestion(5,6), but it has also been suggested that the increased turnover of lipid during phagocytosis may reflect a general stimulation of polymorphonuclear leukocyte metabolism accompanying the process(7).

A full understanding of the phagocytic process, however, makes other demands. For example, we are woefully ignorant of the later biochemical events which occur during the growth and multiplication and eventual destruction of organisms such as *Brucella* which typically parasitize the histiocytic type of cell for a prolonged period. Yet such information is vital to our knowledge of resistance and immunity and to our understanding of the fate of injected antigenic substances and their possible transfer from cell type to cell type in the processes which ultimately lead to antibody formation.

At present the objectives of biochemical approaches to these problems have been *via* the determination of cellular proliferation *in vitro* and on DNA, RNA, and protein synthesis of the inflammatory cells. Among these cells the peritoneal histiocyte has been of the greatest value as a tool which could be employed *in vitro* as well as *in vivo*. Such cells can be parasitized *in vivo* or *in vitro* and the parasitized cells maintained for extended periods, allowing one to observe the fate of the parasite. The results, however, have been contradictory. The findings have been reported(8).

The evidence against host cell multiplication heretofore has been based on absence of uptake of tritiated thymidine by the rabbit peritoneal histiocyte nucleus(9). However, this work was predicated upon a choice of time periods which prevented both the observation of cell proliferation *in vitro* and labelling of the nucleus by tritiated thymidine. Later observations on mouse histiocytes, medium-sized lymphoid cells, and cells with basophilic-staining cytoplasm demonstrated clearly the *in vitro* incorporation of tritiated thymidine(10). Of equal significance was the finding that there was an increase in the proportion of mouse cells incorporating the label beginning on the third day and reaching a peak at the seventh day after peritoneal stimulation by diphtheria toxoid(10).

A previous report described the proliferation and uptake of tritium-labelled thymidine by the rabbit peritoneal histiocyte both *in vivo* and *in vitro*(11). This paper presents data extending those in(11) and concerns the intracytoplasmic growth of *Brucella melitensis* and of a thymineless strain of *E. coli*. It is shown that an intracellular transfer of the tritium-label takes place from the histiocyte nucleus to the *E. coli* but not to the *Brucella*, and that the histiocyte draws label to its own nucleus from the labelled microorganism.

Methods. Introduction of inflammatory exudates and harvest of cells was carried out as in(1).

Tritiated thymidine was diluted in 50 ml of modified Tyrode‡ at 37°C, and 0.5 μc/g weight was injected. The specific activity of the undiluted tracer was 0.36-2.7 curie/millimol. The cells were centrifuged and resuspended in medium containing 40% serum in Tyrode. Smears fixed at 37°C were immersed in absolute methanol for 15 minutes. Leighton tubes containing (0.25×10^6 cells per tube in 2 ml medium) were prepared, coverslips were fixed after 3 hours. In this way one is provided with two sources of material. If the smears were too thick and the

‡ NaCl, 7.7 g; KCl, 0.2 g; $MgSO_4$, 0.1 g; dextrose, 4 g; NaH_2PO_4, 0.05 g; water 750 ml. To this is added: $NaHCO_3$, 0.5 g, water, 250 ml which has been separately sterilized by filtration. The 2 solutions were aseptically combined.

TABLE I. Percentage of Labelled Nuclei in Histiocytes Infected with *E. coli* and *B. melitensis*.

Age of cell culture	Exp. 166			Exp. 167			Exp. 160		
	Control	Rev I	−15T	Control	Rev I	−15T	Control	Rev I	K12
3	0, 0	0, 1	0, 0	0, 0	0, 0	0, 0	1	1	1
24	2, 4	1, 0	1, 5	0, 1	0, 0	3, 4	0	0	0
48	49, 50	49, 50	3, 4	39, 35	33, 24	3, 3	5	3	0
72	66, 77	78, 81	9, 6	56, 77	68, 74	14, 9	16	20	2
96	78, 81	95, 86	30	—	—	—	—	—	—

Ratio of histiocytes to bacteria in these 3 experiments:

Exp. 166: Rev I, 1:100 −15T, 1:30
" 167: Rev I, 1:70 −15T, 1:15
" 160: Rev I, 1:27 K12, 1:20

radiation was self-absorbed. there still remained the coverslip preparation.

Results. The ability of the attenuated strain of *Br. melitensis,* Rev I, to effect a transfer of histiocytic nuclear label was studied first, since the Rev I strain multiplies freely in the histiocytes *in vitro.*

Histiocytes which had been previously labelled *in vivo* were harvested, washed, injected with Rev I, and maintained in Leighton tubes in a "cold-medium" for 4-5 days. Daily samples were taken. During this period, multiplication of both *Brucella* and phagocytes occurred. Since the phagocytic DNA is localized exclusively in the nucleus, cytoplasmic label would indicate transfer of thymidine from the host cell to the *Brucella.* No transfer was detected in 4 separate experiments, although the autoradiographs were exposed up to 6 months.

Similar findings have been reported for Ornithosis virus grown in HeLa cells(12) and it was suggested that the pathway of thymidylic-acid synthesis in this virus may not involve thymidine. Since this could also be the case with brucellae, the thymineless mutant of *E. coli* suggested itself, in the event that it was capable of intracellular growth.

The −15T strain and a K12 strain were used; both strains were streptomycin-sensitive. The −15T mutant was grown on the following liquid medium: KH_2PO_4 13.6 g; 4.25 ml 6 N KOH; 1 drop of H_2SO_4 (25%); $(NH_4)_2SO_4$ 8 g; $MgSO_4 \cdot 7H_2O$ 0.8 g; $FeSO_4 \cdot 7H_2O$ 2 mg; glucose 2 g; thymidine 10 mg; water 1 liter.

The histiocytes were infected with *E. coli* at ratios from 1:15 to 1:30, and were incubated in medium containing 0.5 μc/ml thymidine. At 3 hours one could see some cytoplasmic label; by 24 hours 90% of the histiocytes were heavily labelled. (The extracellular bacteria were label-free.) The thymineless mutant multiplied surprisingly well in the histiocyte. The cells contained hundreds of labelled bacteria. All extracellular multiplication was arrested by the presence of streptomycin in the medium. It was also clear the *E. coli*-infected histiocytes were permeable to thymidine even in the absence of synthesis of their own DNA.

During the studies with *E. coli,* it appeared that the multiplication of *E. coli*-infected histiocytes was inhibited. Therefore, the percentage of labelled nuclei of controls and of histiocytes infected with *B. melitensis* strain Rev I, as well as with *E. coli,* respectively, were compared, using histiocytes harvested from the same rabbits. Table I gives the readings on duplicate coverslips for each time period and parasite.

The results in Table I corroborate those found in Mackaness chambers(8) using Rev I-infected histiocytes, *i.e.,* there was no interference with *in vitro* multiplication of the histiocytes. On the other hand, there was a dramatic retardation of multiplication of *E. coli*-infected histiocytes, the appearance of which suggested granular degeneration even from the first day. The cause of the degeneration is not known. The *E. coli* ceases to multiply intracellularly in 48 hours (Table II, column 3).

When histiocytes were allowed to ingest thoroughly washed, labelled *E. coli* and maintained in Leighton tubes in a "cold" medium,

TABLE II. Appearance of Nuclear Label in Cells Which Had Ingested Labelled *E. coli*.

(1)	(2)	(3)	(4)
	Nuclear label	Cytoplasmic label	Labelled *E. coli*
Hr in culture	Cold *E. coli*, hot medium		Hot *E. coli*, cold medium
	%	%	%
3	0, 0	0, 3	0, 2
24	1, 5	93	0, 2
48	3, 4	89	5, 2
72	9, 6	55	5, 5
96	30	12	14, 12

the histiocytes utilized bacterial thymidine for synthesis of their own DNA, as shown in Table II, column 2. Histiocytes which had ingested dead, labelled *E. coli* also made use of the bacterial thymidine as indicated by nuclear labelling.

Discussion. From a very preliminary study of the peritoneal histiocyte-*Brucella* system, no transfer of tritium-labelled thymidine from host cell nucleus to intracellular microorganism could be detected. The experiments with the thymine-requiring *E. coli* indicate that this organism could serve as a tool since it grows within histiocytes and utilizes thymidine while growing there. The growth of *E. coli* was accompanied by inhibition of proliferation of the parasitized histiocyte as determined by reduced cell numbers and nuclear labelling. A similar observation has been reported for cells infected with variola and cowpox viruses(13).

The interpretation of the divergent results with the attenuated strain of *B. melitensis* and *E. coli* requires much more study in both "immune" and "normal" phagocytes. The results thus far suggest that *in vitro* the peritoneal histiocyte of normal rabbits is less able to retard intracellular growth of *E. coli* than of *B. melitensis*, strain Rev I. A partial significance of these results lies in the clue which is given to the pathogenicity of certain strains of *E. coli* for man. The finding that the rabbit peritoneal histiocyte seems especially susceptible to intracellular growth by a thymineless *E. coli* when maintained in a medium containing thymidine suggests that pathogenicity of *E. coli* in certain situations may indeed be a nutritional factor once the organism invades beyond the polymorphonuclear leukocytes.

Summary. Rabbit peritoneal histiocytes labelled with tritiated thymidine were infected with *Brucella*. No transfer of the labelled nuclear material from the host to the parasite was detected, although both types of cells multiplied during the course of the experiment. When unlabelled histiocytes ingested labelled *E. coli*, living or dead, label passed to the histiocyte nucleus. Uptake of tritium-labelled thymidine by peritoneal histiocytes was markedly reduced after ingestion of *E. coli* but not by ingestion of the attenuated strain of *B. melitensis*.

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1. Sbarra, A. J., Karnovsky, M. L., *Biol. Chem.*, 1959, v234, 1355.
2. Gelzer, J., Suter, E., *Experientia*, 1959, v15, 417.
3. Cohn, Z. A., Morse, S. I., *J. Exp. Med.*, 1960, v111, 667.
4. Becker, H., Munder, G., Fisher, H., *Z. Physiol. Chem.*, 1958, v313, 266.
5. Sbarra, A. J., Karnovsky, M. L., *J. Biol. Chem.*, 1960, v235, 2224.
6. Karnovsky, M. L., Hoelzl Wallach, D. F., *J. Biol. Chem.*, 1961, v236, 1895.
7. Elsbach, P., *J. Exp. Med.*, 1959, v110, 969.
8. Elberg, S., *Bact. Revs.*, 1960, v24, 67.
9. Watts, J. W., Harris, H., *Biochem. J.*, 1959, v72, 147.
10. Speirs, R. S., Osada, Y., Jansen, V., PROC. SOC. EXP. BIOL. AND MED., 1961, v106, 251.
11. Aronson, M., Elberg, S., *Proc. Nat. Acad. Sci.*, in press.
12. Pelc, S. R., Crocker, T., *Biochem. J.*, 1961, v78, 20p.
13. Kato, S., Kameyama, S., Kamahora, J., *Bikens J.*, 1960, v3, 183.

Received November 30, 1961. P.S.E.B.M., 1962, v109.