branches also became quite numerous. Fig. 2 depicts a network of bile canaliculi located within 2 liver cells. The intracellular branches are seen to possess a real lumen.

Discussion. In most modern textbooks of histology, no mention is made of intracellular bile capillaries. Their possible occurrence is conceded in Sharpey-Schafer's[7] and Stöhr's textbooks.[8] Their existence, however, is denied by Maximow and Bloom.[9] The study of finer morphological details with the phosphatase staining technic is considerably helped by the fact that in contrast to the dog liver, the rabbit liver, after biliary obstruction shows no appreciable increase of cytoplasmic phosphatase activity such as often blurs the outline of the dilated capillaries. Clara,[10] using special staining technics, considers the occurrence of intracellular branches in the rabbit liver as likely, but not as definitely, proved. He pointed out that an intracellular location may seemingly be present, while in reality it is due to a location of bile capillaries on the surface of the liver cells in question. While the possibility of this cannot be completely excluded, in the case of the normal rabbit liver, it is quite unlikely in that of the liver after biliary obstruction. Here, a network of dilated bile capillaries is frequently observed on the same plane within a single liver cell. Occasionally, it is found at the level of the cell nucleus.

Summary. With the aid of the histochemical phosphatase stain, intracellular bile canaliculi can be demonstrated in the normal rabbit liver. These intracellular branches become considerably more pronounced following experimental biliary obstruction.

[7] Sharpey-Schafer, E., Essentials of Histology, Descriptive and Practical for use of students. 13th edition by M. Carleton, London, New York, Toronto.

[8] Stohr, P., Lehrbuch der Histologie und der Mikroskopischen Anatomie des Menschen., 25th edition by W. V. Mollendorf, Jena 1943, p. 349.

[9] Maximow, A. A., and Bloom, W., Textbook of Histology, Philadelphia and London, 1947, p. 43.

[10] Clara, M., *Z. F. Mikr. Anat. Forsch,* 1934, **352**, 1.

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Enzymatic Hydrolysis of Benzoylarginineamide by Normal and Tuberculous Tissue of Rabbits.* (17392)

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Caseation has been described by Rich[1] in these words, "When, as a result of partial autolysis, necrotic cells lose their structure and outlines, and their remains together with intercellular materials tend to become fused into a formless, coagulated and more or less inspissated mass, the process is termed caseation." In rabbits, experimentally infected with virulent tubercle bacilli, caseation is usually observed about the third week of the disease, when according to Lurie,[2] the monocytes, which make up the bulk of the cellular material in the caseous area, have been transformed into epithelioid cells and allergy to tuberculin has developed. This suggests that the process of caseation is associated with some degree of acquired tissue immunity or resistance to infection with tubercle bacilli.

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[1] Rich, A. R., The Pathogenesis of Tuberculosis, C. C. Thomas, Publ., Springfield, Ill., 1946, p. 733 ff.

[2] Lurie, M. B., *J. Exp. Med.*, 1933, **57**, 181.

Leading investigators in this field, including Long,[3] Lurie, and Rich, have recognized that the mechanism of caseation and softening is still the key problem in tuberculosis for, as is well known, in the centers of caseous areas tubercle bacilli tend to die, whereas in areas of softening they multiply profusely. As Jaffe[4] expressed it, "The rapid liquefaction of the caseated infiltration is very dangerous, since it opens blood vessels and bronchi. Hemorrhage and aspiration result and the infection spreads rapidly to other portions of the lung." The question arises how does necrotic, caseous tissue in tuberculosis differ from similar material seen in abscess, gumma or infarct? The answer is not yet at hand. Opie and Barker[5] investigated this problem, employing hypertrophied tuberculous lymphatic glands, removed from experimentally infected dogs, as source of enzyme and 50% blood serum, previously heated to 75°C as substrate. They concluded, "When during the third week after inoculation of tubercle bacilli, the figures indicate a maximum degree of digestion, caseation is beginning. . . . After onset of caseation there is gradual and probable complete disappearance of enzymes." Rich[1] summarizes the situation as follows, "At present it remains a problem whether the failure of autolysis to occur in caseous areas is due to an inadequate content of proteolytic enzymes in the monocytes or to the presence of substances that inhibit or destroy the enzymes at the site."

We have studied the behavior of Cathepsin II during various stages of caseation since this endocellular proteolytic enzyme (BA-amidase)[6] is probably concerned in tissue breakdown and in protein synthesis. If this or similar enzymes should play a significant role in the mechanism of caseation and/or softening, there is hope that their action may be influenced by known activators or inhibitors.

Experimental procedures. In order to produce caseous tissue, albino rabbits were infected with tubercle bacilli as follows: Animals of Group 1 were injected intratracheally, without previous immunization, with a dose of 0.001 mg of a virulent Ravenel culture of tubercle bacilli. Those of Group 2 were first immunized by means of 2 successive weekly intravenous injections of 1 mg of a lowly virulent R1 strain of *M. tuberculosis* and then 2 weeks later injected intratracheally in the same manner as the animals in Group 1. They were x-rayed at frequent intervals in order to follow the progress of the infection. Five to 8 animals of each group were killed at varying intervals of time—from 5 to 24 weeks after intratracheal infection, by exsanguination from the carotid artery. The organs (lungs, kidneys, liver, and spleen) were removed, weighed, and small sections taken for histologic study. The remainder of the tissues were frozen intact in a dry ice chamber and aliquots (15-20 g) were chipped in the frozen state and placed, together with 10 times their weight of ice water containing chipped ice, into a Waring blendor and homogenized. Under these conditions the blendor remained ice-cold during the one or 2 minutes of homogenation. Aliquots of the homogenates were used for semi-micro-Kjeldahl determinations and enzyme studies. Enzymatic hydrolysis of BAA (benzoylarginineamide) was estimated by the method of Greenstein and Leuthardt,[7] which determines the amount of ammonia N liberated. The reaction constant was calculated from the equation $K = \frac{1}{t} \log \frac{100}{100-x}$ where x is percentage hydrolysis and t is time in hours. The activity was expressed in units per mg of nitrogen. Under the conditions of these experiments the first order reaction constant for the liberation of ammonia from BAA was proportional to the amount of homogenate used and hence to the enzyme (BA-amidase) concentration.[8]

In carrying out the tests 1.0 ml of organ homogenate was added to 1.0 ml solution of

[3] Long, E. R., *Arch. Path.*, 1939, **28**, 719.

[4] Jaffe, R. H., *Arch. Path.*, 1934, **18**, 712.

[5] Opie, E. L., and Barker, B. I., *J. Exp. Med.*, 1908, **10**, 645.

[6] Bergmann, M., A Classification of Proteolytic Enzymes, in "Advances in Enzymology," edited by F. F. Nord and C. H. Werkman, Interscience Publ., Inc., New York, 1941, **1**, 63-99, and 1942, **2**, 49-68.

[7] Greenstein, J. E., and Leuthardt, F. M., *J. Nat. Cancer Inst.*, 1945-46, **6**, 203.

TABLE I.
Comparison of Benzoylarginine Amidase Activity of Organs of Rabbits Killed at Various Time Intervals After Primary or Reinfection with Virulent Tubercle Bacilli.

Group	When killed	No. of rabbits	Liver K/g N Avg.	S.E.	Sign	Spleen K/g N Avg	S.E.	Sign	Kidneys K/g N Avg	S.E.	Sign	Lungs K/g N Avg	S.E.	Sign
A	Normal controls	14	28	2.1		24	2.7		24	1.9		8.6	0.8	
					After primary infection									
B	5 wk	5	20	1.1	+	21	3.1	—	20	1.7	++	9.8	1.4	—
C	16 wk	5	21	0.9	±	31	6.9	—	25	1.4	—	11.7	4.1	—
					After reinfection									
D	5 wk	5	26	1.9	—	31	2.4	—	30	4.1	—	17.2	2.2	++
E	16-20 wk	5	17	2.1	++	27	4.2	—	38	6.0	++	21.0	2.9	++

S.E. = Standard error (±). Sign—indicates the significance of the experimental averages when compared with the controls. A minus (—) means that the Fischer "t" test gave a "P" value of 0.05 indicating probable significance. A plus (+) denotes a "P" value greater than 0.02 and less than 0.05, indicating statistical significance. Two plus (++) indicates values less than 0.02 or a high degree of significance.

0.1 molar citrate buffer (pH 5.1) containing 0.02 mmols of BAA and 0.01 mmols of cysteine hydrochloride. Digestion was carried out in large test tubes in a 39° bath. After 2 and 4 hours, the tubes were removed, 1 ml of saturated potassium carbonate and 3 ml of water were added and the whole aerated as described by Greenstein and Leuthardt. The ammonia liberated was absorbed in a 2% boric acid solution containing indicator (methyl red-bromcresol green) and titrated with N/14 HCl. A correction blank for zero time was set up in tubes which were treated immediately with saturated K_2CO_3.

Pathologic findings. The rabbits were killed by exsanguination, at various time intervals after infection, as indicated in Tables I-III. At autopsy the kidneys and lungs showed varying degrees of caseation. Microscopically, these areas were surrounded by inflammatory cells or granulation tissue. There was no caseation in the livers or spleens. In animals which were killed 5 and 16 weeks after primary infection, the lungs were markedly enlarged and weighed from 25 to 135 g as contrasted with an average normal of 10 g. Two protocols are given to illustrate the pathologic findings.

I. *Pathologic findings after primary infection.* Rabbit 8, killed 16 weeks after infection with a virulent Ravenel culture, showed partial consolidation of the lower lobe of the left lung. There were numerous scattered caseous tubercles from 2 to 5 mm in diameter on the surfaces and also within the parenchyma of the lung. The cortices of the kidneys were studded with numerous, firm gray, elevated tubercles, each 2 to 4 mm in diameter. The spleen and liver showed no gross changes.

Microscopically, there was extensive caseation in both lungs and kidneys. The latter also showed extensive necrosis. There was evidence of hemorrhage and some tuberculous involvement in the spleen.

II. *Pathologic findings after reinfection.* Rabbit 66 was reinfected June 1, 1948 after having received 2 intravenous immunizing doses of an R1 culture of *M. tuberculosis* and killed 3 months after intratracheal infection. X-rays, made 6/9/48 and 8/18/48, showed in the fourth interspace of the right lung several areas of density, peripheral to the heart shadow, suggesting consolidation. The remainder of the right lung presented no abnormalities. In the left lung, the bronchial shadows at the hilum of the lung were accentuated. In the fourth interspace there were several spots suggestive of foci of consolidation.

Gross anatomic findings. The lungs were well aerated. Several small tubercles were seen on the pleural surfaces. The surface of each kidney showed a single large, prominent tubercle. The spleen was enlarged and showed several tubercles on the surface. The liver was not involved.

Histological findings. In sections of the lungs small miliary tubercles were seen whose centers stained well. At the periphery of one tubercle there was an area which was densely

TABLE II.
Illustrative Analytical Data on Normal and Infected Rabbits.

Rabbit No.	Body wt	Organ	Organ wt	% N	K × 10³/ml. hom.	K/g N
			Normal animals			
9	3896	Li†	78.3	2.87	52.2	19.3
		K	13.0	2.53	14.7	14.8
		S	1.39	2.37	16.0	25.8
		Lu	11.0	2.02	12.8	8.1
13	4140	Li	96.0	2.72	57.7	20.0
		K	18.9	2.39	22.2	15.7
		S	1.59	2.11	12.0	17.6
		Lu	11.74	2.12	16.1	8.4
18	2645	Li	98.0	2.23	58.6	18.0
		K	12.8	2.53	22.4	15.8
		S	1.04	3.41	3.1	8.1
		Lu	8.7	2.51	14.4	6.2
26	2870	Li	97.3	2.55	60.5	16.9
		K	12.8	2.46	31.2	22.0
		S	1.02	1.76	5.6	13.6
		Lu	9.36	2.22	11.2	6.5
			Infected animals*			
1	3286	Li	95.6	2.81	64	24.4
		K	24.3	2.34	85	28.5
		S	2.7	1.48	31	39.0
		Lu	131.0	1.80	16.3	12.5
2	2990	Li	64.0	2.61	30.8	18.5
		K	16.9	2.60	49.8	22.8
		S	1.4	2.60	18.2	12.4
		Lu	110.0	2.26	16.9	10.1
5	2590	Li	86.8	2.35	—	—
		K	25.6	2.17	45.9	21.1
		S	2.32	2.09	57.0	26.0
		Lu	18.2	2.02	11.0	4.5
6	2665	Li	73.0	2.76	45.1	21.6
		K	15.3	2.25	45.9	27.0
		S	2.0	2.14	20.3	15.7
		Lu	75.3	2.12	3.6	2.6
8	3296	Li	80.9	3.06	46.6	19.7
		K	24.6	2.17	91.1	25.5
		S	1.56	2.58	30.8	19.0
		Lu	26.3	1.71	43.0	25.8

* Rabbits killed 16 wk after primary infection with virulent tubercle bacilli.
† Li = Liver; K = Kidney; S = Spleen; Lu = Lung; hom. = Homogenate.

packed with lymphocytes and young fibroblasts. The surrounding lung tissue was fairly normal. There were a few RBC but no fluid. No PMN were seen and not all of the endothelial cells were desquamated. The liver showed no tubercles. There was some congestion and cloudy swelling, also periductal proliferation with monocytes and young fibroblasts. The hepatic lobules were fairly well demarcated as a result of the interstitial reaction. In sections of the kidneys there were many areas of caseation necrosis surrounded by large giant cells. There were also closely-packed collections of cells (monocytes, lymphocytes, and young fibroblasts), the centers of which showed softening. Congestion, cloudy swelling and beginning desquamation of the tubular epithelium were evident in addition to many giant cells. The spleen showed large areas of necrosis with disintegration of the nuclei and absence of cellular outlines. The Malpighian follicles were prominent.

TABLE III.
Total Nitrogen, Protein N, and N.P.N. of Tuberculous and Normal Lungs.

Rabbit No.	Total N, mg	Total protein, N	N.P.N., mg	N.P.N. as % of total N
		Tuberculous rabbits		
1	2360	1950	410	17.3
2	2500	2190	310	12.4
6	1480	1270	44	14.1
8	446	381	65	14.5
		Normal rabbits		
9	222	202	20	9.0
11	265	251	14	5.3
13	248	222	26	8.9
18	221	206	15	6.8

16 weeks following primary infection the animals showed diffuse caseous consolidation involving both lungs almost completely. These data show that most of the increase in N is due to protein and not to N.P.N.

Results and discussion. The biochemical data which are summarized in Tables I-III indicate that homogenates of lungs and kidneys of tuberculous rabbits which are diffusely infiltrated with caseous material are enzymatically very active. While there are individual variations, there is, nevertheless, a statistically significant difference in the rates at which these homogenates hydrolyze BAA which depends upon whether we are dealing with tissues from animals with primary infection or with reinfection. In the former, the rate of hydrolysis of lung tissue was not significantly altered as compared with the normal. Likewise, the kidneys showed either normal rates or moderately decreased activities. After reinfection, however, both lung and kidney homogenates revealed marked increases in hydrolytic activity. These data are strikingly similar to the immunologic results of Lurie,[2] who showed that the lungs and kidneys of normal rabbits possess little innate power to destroy virulent tubercle bacilli after primary infection. But as the result of immunization and reinfection there is more complete destruction of these microorganisms. This increase in bactericidal action is associated with an acceleration of the localized inflammatory process and a heightened physiologic activity of the tissue phagocytes. The liver and spleen, having a high innate capacity to destroy virulent tubercle bacilli, do not call forth any appreciable inflammatory response. The observation that liver homogenates have decreased enzymatic activity after primary as well as reinfection may perhaps be explained by the fact that a loss of liver Cathepsin II may accompany the loss of labile liver protein during the state of malnutrition late in the tuberculous infection.[8] It is also of interest to note that the loss of enzyme activity from the liver takes place earlier following primary infection than following reinfection.

While the data clearly indicate that homogenates of certain organs which contain large amounts of tuberculous caseous material are not enzymatically inert but may show rates of hydrolysis of BAA which are far greater than the corresponding normal values, we have no precise information as to the origin of this added cathepsin. There are 3 possibilities: (a) the inflammatory exudate (or "granulation tissue") which surrounds the necrotic or caseous material is rich in BA-amidase, (b) the caseous material itself, being derived from the breakdown of inflammatory cells is very active enzymatically or (c) the normal, adjacent tissue has acquired an increased rate of hydrolysis. That the first of these two possibilities is the most likely to be true is suggested from the following data:

Ten rabbits which were killed 5 or 16 weeks after primary intratracheal infection with virulent tubercle bacilli had lungs weighing 25 to 125 g, as compared with an average normal value of 10 g. Microscopically, there was caseous and cellular infiltration and chemically there was proof of an accumulation of

[8] Schultz, J., *J. Biol. Chem.*, 1949, **178**, 451.

protein nitrogen (Table III). In spite of this increase in organ weight and the possibility of dilution of enzyme, the rates of hydrolysis of BAA were not significantly different from the normal. Analyses made of bits of caseous material removed and freed from adjacent lung tissue showed them to be enzymatically very active.

The present report confirms the work previously published by Weiss and Halliday[9] that livers and spleens of normal rabbits usually show greater activity of Cathepsin II (BA-amidase) than the lungs. The rate of hydrolysis of lung homogenates is about one-third that of liver. This is of interest since the lungs[2] cannot destroy virulent tubercle bacilli as effectively as do other organs. Considering the size of the liver, it is probably one of the greatest sources of cathepsin in the body.

Summary and conclusions. 1. Caseous material was produced in the lungs and kidneys of rabbits by intratracheal infection with a virulent, Ravenel culture of tubercle bacilli. The animals were killed at intervals of from 5 to 20 weeks after infection. The lungs and kidneys, together with the livers and spleens, were removed immediately after exsanguination and death of the animals, preserved at a very low temperature in a dry ice refrigerator, and examined for enzymatic activity.

2. Homogenates of tuberculous lung tissue containing very large amounts of caseous material can hydrolyze benzoylarginineamide (BAA) at pH 5.1 at a more rapid rate than those of homologous normal tissues. The lungs and kidneys of animals which were first immunized with a non-virulent culture before reinfection with a virulent strain of *M. tuberculosis* were found to have greater enzymatic activity than those with a primary infection.

3. These results are analogous to those of Lurie who showed an increased capacity of rabbit tissue to destroy virulent tubercle bacilli under similar circumstances. They are also of interest since we have previously demonstrated[9] a parallelism between bactericidal action and the rate of hydrolysis of BAA in innate organ immunity.

4. It is not clear whether the observed increased rate of enzymatic activity has its origin in the caseous ("necrotic") material itself or in the surrounding inflammatory exudate ("granulomatous tissue"). The following hypothesis is suggested: The lungs and kidneys of rabbits possess little innate resistance to infection with virulent tubercle bacilli. Hence the latter multiply freely and bring about an inflammatory exudate which is the source of the added enzyme. The liver and spleen, on the other hand, are organs of high natural immunity. They do not permit rapid multiplication of tubercle bacilli (Lurie[2]). The intense inflammatory response with enzyme-laden cells is therefore not called forth.

5. The decreased rate in BA-amidase activity observed in liver homogenates after primary and reinfection, may perhaps be due to the loss of labile liver protein which occurs during the malnutrition accompanying the late stages of a virulent tuberculous infection.

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[9] Weiss, C., and Halliday, N., *J. Immunol.*, 1944, **49**, 251.

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