THE ENZYMES OF THE TUBERCLE BACILLUS

H. J. CORPER AND H. C. SWEANY

From the Laboratories of the Municipal Tuberculosis Sanitarium of the City of Chicago

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The tubercle bacillus and the related acid fast bacilli (lepra, smegma, etc.,) have for a great many years been looked upon as distinct and different organisms, biologically and chemically, from the ordinary rapidly growing bacteria, and it is rather singular that until recently the tubercle bacillus should have been considered not to possess the ordinary well-known enzymes. This lack of knowledge as to the presence of enzymes in the tubercle bacillus is, of course, explicable on the basis that not until within recent years have sufficiently delicate quantitative methods been available for the demonstration of these interesting catalytic substances in such a sluggish organism as the tubercle bacillus and in amounts so small or of so slight an activity as those characteristic of this organism. It is worthy of note also that these problems could not have been solved before the days of the use of antiseptics in studying enzymatic action. During the course of investigations carried out to find a suitable antigen for use in complement fixation tests for tuberculosis, it was deemed advisable to understand more fully the part played by the enzymes in the tubercle bacillus and for this purpose the experiments to be reported upon were carried out.

LITERATURE

In order to study the action of the enzymes of various bacteria upon different substances and thus gain a better insight into the nature of these enzymes, Eijkman (1901) used a simple plate method called the "auxanographic" method of Beijerinck or the diffusion method of Wijsman. This method, which consists in mixing various substrates (casein, blood, starch, tallow,

cellulose, etc.,) with nutrient agar and observing the effect of the growths of various organisms upon the substrates, was of necessity crude and gave only gross results. The criterion of enzymatic action was merely the formation of a zone of clearing or change in the media surrounding the colonies. Thus Eijkman found that the following gelatin liquefying or digesting organisms also cause the solution of casein: *B. anthracis*, *B. pyocyaneus*, *Staphylococcus pyogenes aureus*, *B. Metschnikowi*, *B. cholerae*, *B. fluorescens*, *B. prodigiosus*, *B. indicus*, *B. ruber*, *B. subtilis*, *B. megatherium*, and *B. mesentericus*; while the following organisms, not peptonizing gelatin, also failed to dissolve casein: *B. typhosus*, *B. coli-communis*, *B. mallei*, *B. pestis*, *B. diphtheriae* and *B. lactis cyanogenes*. He concludes, therefore, that the same enzyme probably digests both substances.

As the hemolytic action of a number of bacteria (*B. diphtheriae* *B. anthracis*, *B. fluorescens*, *B. mesentericus*, *B. prodigiosus* and *B. indicus*) did not vary hand in hand with the gelatin liquefying property, he concludes that the hemolytic and tryptic enzymes are not identical, but suggests that different tryptic enzymes may be present in different bacteria and these might in some cases attack blood or gelatin more vigorously. Varying grades of diastatic action by pathogenic organisms were observed with starch. *B. anthracis* and *B. cholerae* intensely digested it, while *B. diphtheriae* and *B. dysenteriae* (Kruse) had only a faint action and *B. mallei*, *B. pyocyaneus* and *Staphylococcus pyogenes aureus* were negative in this respect. Fat splitting power was also found to be present in some organisms and absent in others. None of those forms tested were able to split beeswax. In all tests with inulin, (Eijkman, 1913) keratin and cellulose negative results were obtained. Elastic tissue prepared from calf lung was digested by *B. pyocyaneus* (culture and bouillon filtrate),—which action was destroyed at 80°C.,—*B. anthracoides* and a bacillus isolated from a case of lung gangrene. It was noteworthy that all the elastic tissue dissolving organisms liquefied gelatin, but the reverse did not hold true.

Fontes (1911) was unable to demonstrate enzymes, zymases or oxidases in cultures or tuberculins of bovine and human tubercle

bacilli. Proteolytic enzymes were also not found in tuberculous pus free from other organisms. Opie and Barker (1908) had earlier observed that tuberculous tissues in the early stages contained a proteolytic enzyme acting in neutral and weakly acid solution. With the advance of caseation the one acting in alkaline solution disappears while the other (lympho-protease) is retained. After complete caseation the latter also disappears. No enzymes were demonstrable in tuberculous exudates in human beings (Opie and Barker, 1909).

Gosio (1905) using the tellurite and selenite reduction test devised by him was able to demonstrate reductases in all the bacteria tested including the various tubercle bacilli: avian, bovine and human. He examined 173 microorganisms and divided them into three classes, dependent upon the reaction obtained: (1) a decided reaction, (2) a less intense, but fully evident reaction (in this class he included a bovine, a human and an avian tubercle bacillus and a so-called pseudo-tubercle-bacillus (Rabinowitsch)), (3) a very slight reaction.

Wells and Corper (1912) studying the lipase of *B. tuberculosis* and other bacteria noted that toluene would kill the tubercle bacillus but did not destroy the lipases, and that lipolytic enzymes are present with different activites in the organisms tested (*B. dysenteriae, Staphylococcus pyogenes aureus, B. pyocyaneus, B. coli* and *B. tuberculosis*). Of these the tubercle bacillus was least actively lipolytic. They also noted that the "auxanographic" method was not suitable for use in studying the enzymes of the tubercle bacillus.

Kendall, Day and Walker (1914c) verified and elaborated these findings noting that various strains of the human tubercle bacillus, the bovine and avian bacilli as well as the leprosy, smegma and grass bacilli, form lipase during their growth on glycerin broth. This lipase is present in the medium free from bacteria. The bodies (Kendall, Day and Walker, 1914d) of acid fast bacteria grown in nutrient broth, with glucose, mannite and glycerin as additional sources of carbon, freed from adherent media, also contained a lipase not as active or as great in amount as that in the culture media. The authors were

unable to determine whether the lipase was freed as the result of autolysis or whether it was excreted as an exo-lipase.

The above authors (Kendall, Day and Walker, 1914d) also observed that the metabolism of the smegma and grass bacillus resembles that of the rapidly growing human tubercle bacillus in two important particulars; neither glucose, mannite nor glycerin exhibits any sparing action for the protein constituents as measured by the ammonia content of the broth (the "lepra bacillus" does not present this metabolic phenomenon); and a rapidly growing strain of human tubercle bacilli grown in a medium of very simple composition consisting essentially of diammonium hydrogen-phosphat, as a combined source of nitrogen and phosphorus, and glucose, mannite and glycerin respectively, as a source of carbon, so changed this medium that 10 per cent of the ammonium nitrogen had disappeared at the end of two weeks apparently being built up into bacillary bodies. At the end of four weeks between 40 and 50 per cent of the lost ammonia nitrogen reappeared in the media. This occurred coincidently with the cessation of vegetative activity, strongly suggesting, as stated by the authors, that it is associated with a certain amount of autolysis of the bacilli, (Kendall, Day and Walker, 1914a).

That the tubercle bacilli themselves contain enzyme inhibiting substances was shown by Jobling and Peterson (1914) who prepared unsaturated fatty acid soaps from tubercle bacilli which were more actively inhibitory than soaps prepared from linseed, olive and cod liver oils.

In the following investigations it will be observed that an attempt was made to check the findings obtained in every case by as many methods as possible. Frequent failures marked the progress of the work however, because certain of the methods used were not delicate enough for the purpose and others could not be used on account of interfering (absorption and turbidity) phenomenon. This was especially true of the nephelometric methods tried.

AUTOLYTIC ENZYMES

Method

The amount of non-coagulable nitrogen was determined in all the following experiments, except where otherwise indicated, by the colorimetric micro-method as devised by Folin and Farmer (1912) using the distillation modification of Bock and Benedict, (1915). The coagulable nitrogen was removed by the addition of ten volumes of 2.5 per cent trichloracetic acid according to Greenwald (1915). The results obtained were plotted in curves, using the initial nitrogen found as the zero point, that is the amount of non-coagulable nitrogen obtained in a definite volume (1 cc. of solution) at the beginning of the experiment was subtracted from the amount of nitrogen in an equivalent amount (1 cc.) of solution subsequently.

The method used for determining quantitatively the amount of amino acid α nitrogen was that devised by Harding and MacLean (1915; 1916). Asparagin was used as a standard and since the results are merely comparative the objections to the use of asparagin raised by the above authors did not apply to the procedure.

EXPERIMENTAL PART

Series I. Liberation of nitrogenous substances by tubercle bacilli suspended in salt solution

In order to determine whether there was a liberation of non-coagulable nitrogenous substances from tubercle bacilli under certain conditions, heavy suspensions were made of virulent human tubercle bacilli in sterile physiological salt solution and divided among three graduated centrifuge tubes in as nearly equal amounts as possible (each tube containing 10 cc. of heavy suspension). At the conclusion of the experiment the amount of residual nitrogen remaining in the bacilli was always determined and found to agree fairly well within the limits of error of the method, thus serving as a check upon the use of approxi-

mately equivalent amounts of bacilli in the three tubes. One of these tubes was heated as a control and kept in the incubator, one was placed in the incubator at 37°C., without previous heating and the third was kept at room temperature during the course of the experiment in order to determine the most favorable condition suitable for the liberation of the nitrogenous substances from the bacilli.[1]

It was found that tubercle bacilli suspended in physiological salt solution in this way liberate non-coagulable nitrogenous substances at incubator temperature (37°C.), but that this liberation requires a number of days. At room temperature (i.e., about 15° to 20°C.) this does not occur to any appreciable extent within the same time.

Series II. Determination of an autolysis of human tubercle bacilli

In order to determine whether the non-coagulable nitrogenous substances liberated at incubator temperature are merely dissolved out from the bacillary bodies of these substances or whether they are formed as a result of enzymatic action as suggested by Kendall, Day and Walker, tubercle bacilli in equal amounts in two tubes were suspended in sterile physiological salt solution and one was incubated at 37°C., as a control, while to the other was added 3 cc. toluene before incubation. The toluene, as has been shown by Wells and Corper (1912), kills the bacilli but leaves the enzymes intact.[2]

It appeared that there was a definite liberation of non-coagulable nitrogen in both tubes, rather more rapid and reaching a maximum earlier under toluene than in sterile physiological salt solution. A suggested explanation of the difference may be found in the fact that the bacilli are killed rapidly by the toluene and therefore more rapidly disintegrate as a result of enzymatic action, an autolysis.

[1] Charts depicting these results and those of Series II have been published in the Jour. of Infectious Diseases, Vol. 19, 1916, p. 315–21.

[2] See note under series I.

Series III. Determination of an autolysis of bovine tubercle bacilli

The experiments of series I and II were performed with human tubercle bacilli; this series is in a certain sense a repetition and check on the above except for the fact that bovine tubercle bacilli were used. These results are plotted in chart I.

They bear out the findings with the human tubercle bacilli in that the autolysis occurs more rapidly under toluene (antiseptic) than in merely aseptic conditions.

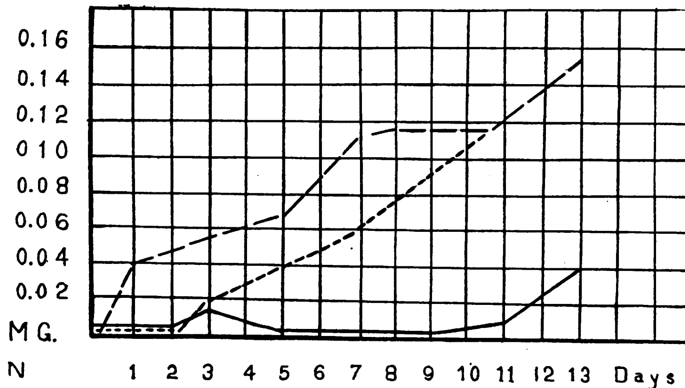

CHART I. AUTOLYSIS OF TUBERCLE BACILLI NON-COAGULABLE NITROGEN CURVES

Continuous line is heated control at incubator temperature. Dotted line is aseptic autolysis at incubator temperature. Dash line is antiseptic (toluene) autolysis at incubator temperature.

Series IV. Liberation of amino acids during autolysis of tubercle bacilli

As a check upon the above findings and in order to see whether the increase in non-coagulable nitrogen runs parallel with an increase in liberated amino acid α nitrogen, the above experiments were repeated and the autolysate was tested, at various intervals during incubation, for α amino acid content by the

Harding and MacLean colorimetric method. The results are plotted in chart II.

It is to be noted again that even though equivalent amounts of human tubercle bacilli were used in each test the curve for the antiseptic (toluene) autolysis is higher than the aseptic curve and that autolysis as indicated by the liberation of amino acids is more rapid at incubator (37°C.) than at room tempera-

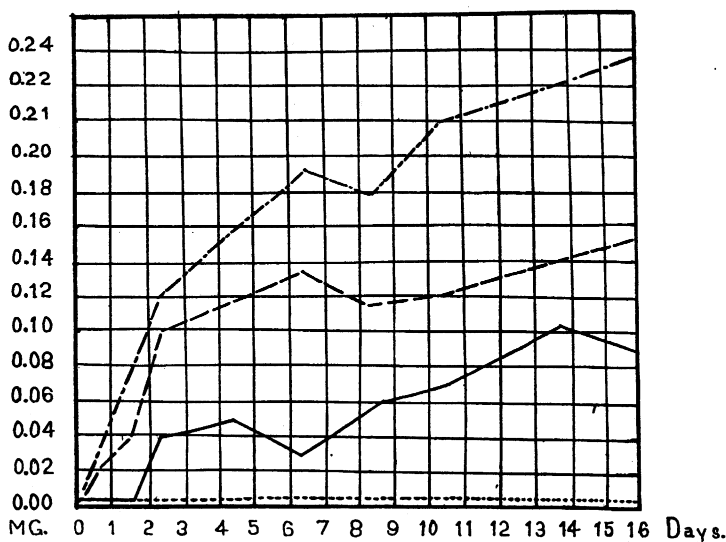

CHART II. AUTOLYSIS OF TUBERCLE BACILLI. AMINO ACID CURVES

Solid line is aseptic autolysis at room temperature. Dotted line is heated control. Long dash line is aseptic autolysis at incubator temperature. Dash dot line is antiseptic (toluene) autolysis at incubator temperature.

ture (these experiments were carried out in a warmer room than those reported in series I which probably accounts for the greater height attained by the room temperature curve).

Series V. Liberation of antigenic substances during autolysis

In order to determine whether, coincident with the autolysis of tubercle bacilli occurring at incubator temperature, there was

also an increase in soluble tuberculosis antigen in the autolysate available for complement fixation tests, that is capable of uniting with tuberculosis antibody, human and bovine tubercle bacilli were suspended in sterile physiological salt solution and kept in the incubator at 37°C. At definite intervals the clear autolysate was tested for non-coagulable nitrogen content and titrated for its antigenic strength. The antigenic titre was obtained by determining the minimum amount necessary to give a complete binding of all the complement when using a definite amount of + + + + antituberculosis (human) serum. The Noguchi antihuman hemolytic system was used in these tests. The results are tabulated in Chart III.

	Nitrogen Curves	Antigen Curves							
Days	M G.N. PER C C	0.2 C C	0.1 C C	0.5 C C	0.01 C C	0.005 C C	0.001 C C	0.0005 C C	0.0001 C C
0	0.00	++++	++++	+++	−	−	−	−	−
1	0.00	++++	++++	+++	±	−	−	−	−
2	0.01	++++	++++	++±±	+++	±	−	−	−
3	0.02	++++	++++	++++	++++	+++	+ +	+	−
4	0.03	++++	++++	++++	++++	+++±	+++	+	±
6	0.05	++++	++++	++++	++++	++++	++++	+ +	+ +
8	0.06	++++	++++	++++	++++	++++	++++	+++	+ +
10	0.08	++++	++++	++++	++++	++++	++++	+++	+
13	0.15	++++	++++	++++	++++	++++	++++	+++	+ +

Chart III. Correlation of Autolysis and Antigen Formation

It is to be noted that coincident, although not absolutely parallel, with the increase in non-coagulable nitrogen liberated there also occurs an increase in the antigenic titre of the autolysate, so that an autolysate which only gave a titre of 0.1 cc. on the first day gradually and consistently increases to a titre of 0.001 cc. on the sixth day.

DETERMINATION OF INDIVIDUAL ENZYMES OF THE TUBERCLE BACILLUS

In the hope of being able to determine the presence of the various individual enzymes in the tubercle bacillus by the nephelometric method for determining enzymatic action recently

described by Kober and his colleagues (Graves and Kober, 1914; Kober, 1913; Kober and Graves, 1914), a series of experiments were carried out in which heavy emulsions of tubercle bacilli were mixed with definite amounts of various substrates (sodium caseinate for trypsin, acid casein for erepsin, acid edestin for pepsin, nucleic acid for nucleases, starch for diastase, sucrose for invertase, and urea for urease). In the majority of cases this method was found impractical, however, on account of the adsorption of the protein substrates by the tubercle bacilli, which made quantitative determinations impossible. In a number of instances this difficulty was, however, overcome by determining the presence of the enzymes in the autolysate where no adsorption occurs and checking their presence by other methods.

Methods

The methods used for quantitative nephelometric determination of the various substrates (casein, edestin and nucleic acid) were those described by Kober and his colleagues, the previously cited micro-method of Folin, modified by Bock and Benedict, for determining non-coagulable nitrogen, and the amino acid α nitrogen method of Harding and MacLean. The presence of urease was determined by the aeration method of Folin (Folin and MacCollum, 1912) and the presence of diastase and invertase by the amount of glucose liberated from starch and sucrose by the recent picramic acid method of Lewis and Benedict (1915) as used by Myers (1916) and Myers and Rose, (1916), for determining the presence of these enzymes in ptyalin and the presence of glucose, sucrose, dextrin and starch in foodstuffs. The quantitative Fehling method proved far too inaccurate and not delicate enough for this purpose.

Series I. Proteolytic enzyme acting in alkaline solution (trypsin-like enzyme)

Experiment 1. By its presence in the autolysate. (a) In a graduated 15 cc. centrifuge tube was emulsified 2 cc. of human tubercle bacilli and sufficient sterile physiological salt solution

was added to make a volume of 12 cc., and, as antiseptic, 2 cc. toluene and 1 cc. chloroform. After thoroughly mixing, this was placed in the incubator at 37°C. for twenty-four hours, centrifugated at the end of that time and the supernatant autolysate withdrawn, filtered through a hard filter paper and divided into two equal parts; one part heated for thirty minutes at 100°C., as control to destroy the enzymes, and the other half kept intact. To each was added 1 cc. 0.1 per cent sodium caseinate, sodium carbonate to make 0.3 per cent, 1 cc. toluene and 1 cc. chloroform. The tubes containing these mixtures were then incubated at 37°C., and a definite amount of the clear watery solution withdrawn at various intervals and compared, control and test, nephelometrically after precipitation with sulphosalicylic acid. The results obtained, figured in percentage of the heated control, were as follows:

	0	2 DAYS	4 DAYS	6 DAYS	12 DAYS
Percentage of original casein solution added...	100	82.2	79.6	78	63

The above figures indicate a definite splitting of the casein by the autolysate withdrawn at one day.

(b) The above experiment 1a was repeated but the autolysate was withdrawn after twelve days in the incubator at 37°C. The results obtained with the autolysate as compared to the heated control were:

	0	3 DAYS	6 DAYS	9 DAYS	12 DAYS
Percentage of original casein solution added...	100	80	74.4	73	71.4

These figures indicate a definite hydrolysis of the casein by the autolysate withdrawn at twelve days.

(c) In order to check this point more fully a more complete experiment was performed. Human tubercle bacilli, 2 cc. by volume, were suspended in physiological salt solution to make a total volume of 12 cc., and 2 cc. toluene and 1 cc. chloroform added. The mixture was incubated for three days, centrifu-

gated and about 10 cc. of autolysate withdrawn. This was then replaced by physiological salt solution and the entire tube incubated another three days. The same process was again repeated at nine and twelve days. The autolysate after the various intervals of three, six, nine and twelve days, was divided into two equal portions (1 portion to be heated for control and the other to be retained intact). To each of these was added

TABLE 1

Period of determination after adding substrate

AGE OF AUTOLYSATE		THREE DAYS	SIX DAYS	NINE DAYS
		per cent	*per cent*	*per cent*
Three days	T*	109	94	68
	H	125	125	121
Six days	T	83	87	84
	H	97	106	107
Nine days	T	89	83	87
	H	97	100	103
Twelve days	T	91	86	87
	H	98	97	98

* T designating the test for enzyme and being the percentage of casein recovered as compared with a standard containing the amount of casein originally added; H, designating the percentage of casein in the heated control as compared with the same standard containing the amount of casein originally added. The data are given in this form to rule out any conflicting turbidity or sources of error which may have been occasioned by the autolysate itself.

When these experiments were performed it was not realized that there were any conflicting turbidities formed or other sources of error and so a test sample was not withdrawn immediately, it being taken for granted that the heated control would suffice for this purpose.

1 cc. 0.1 per cent sodium caseinate, enough sodium carbonate to make 0.3 per cent, 1 cc. toluene and 1 cc. chloroform. This mixture was incubated at 37°C., and a definite amount withdrawn after definite intervals of incubation (three, six and nine days) and after precipitation with sulphosalicylic acid tested nephelometrically for casein content. The results are given in table 1.

These results indicate a definite splitting of the casein by the autolysate withdrawn at the third day of autolysis, but subsequently the enzymes have either been entirely washed away from the bacilli or have been so diluted by the additional salt solution added that they are incapable of exerting any action upon the casein.

Experiment II. By the non-coagulable nitrogen liberated in alkaline solution. Tubercle bacilli, 4 cc. of a heavy emulsion, were placed in 15 cc. graduated centrifuge tubes (2 cc. in each). One as control was heated to kill the bacilli, and to both was added 10 cc. physiological salt solution, sufficient sodium carbonate to make 0.3 per cent, 2 cc. toluene and 1 cc. chloroform. The tubes were then placed in the incubator at 37°C., after withdrawing 1 cc. as a control test, and 1 cc. of the clear supernatant solution was withdrawn after centrifugation daily, for eight days. The non-coagulable nitrogen was determined as before by the Folin micro-method.

	DAYS									RESIDUAL NITROGEN
	0	1	2	3	4	5	6	7	8	
										mgm.
Test	0.12	0.12	0.13	0.13	0.14	0.14	0.15	0.16	0.17	4.26
Heated control	0.11	0.11	0.11	0.10	0.11	0.12	0.12	0.11	0.12	4.8

The figures are given in milligrams of nitrogen per cubic centimeter

These figures indicate that there is present in tubercle bacilli an enzyme capable of decomposing the nitrogenous constituents of the bacillary bodies in alkaline solution.

Experiment III. By the amino acid α nitrogen liberated in alkaline solution. The above experiment (II) was repeated in all details except that the solution was freed from coagulable proteins by means of fifteen volumes of methyl alcohol, the alcohol evaporated off from the filtrate, the residue taken up by 2 cc. distilled water and this tested quantitatively for amino acid α nitrogen content. The following results were obtained, the figures being given in milligrams amino acids using asparagin as the standard.

	DAYS							
	0	1	2	3	5	7	10	30
Test	0.05	0.06	0.06	0.07	0.08	0.09	0.12	0.14
Heated control	0.06	0.06	0.07	0.05	0.05	0.05	0.06	0.05

This experiment indicates that there is an enzyme in tubercle bacilli capable of splitting off into simple form the amino acid building stones of the bacillary bodies.

Summary. The evidence obtained as to the action of the enzymes of the tubercle bacilli (autolysate) upon casein in alkaline solution and upon the bacillary bodies themselves in alkaline solution seem to indicate that this micro-organism possesses a proteolytic enzyme resembling trypsin in action.

Series II. Proteolytic enzyme acting in acid solution (pepsin-like enzyme)

Experiment I. By its presence in the autolysate. The nephelometric method in which edestin was used was not found to be serviceable for this purpose since physiological salt solution partially precipitated the edestin, and if distilled water was used as a solvent and for preparing the autolysate substances producing a turbidity were obtained from the tubercle bacilli.

Experiment II. By the non-coagulable nitrogen liberated in acid solution. The differences obtained in the non-coagulable nitrogen figures by the Folin micro-method, colorimetrically, were not sufficient to warrant drawing any conclusions.

Experiment III. By the amino acids liberated in acid solution. When using suspensions of bacilli (2 cc. bacillary sediment), killed by toluene (2 cc.) and chloroform (1 cc.) the solution being made 0.2 per cent acid with hydrochloric acid and tested at definite intervals as in series I, experiment III, for amino acid α nitrogen liberated, the results obtained were as follows:

	DAYS							
	0	1	2	3	5	7	10	30
Test	0.04	0.05	0.07	0.07	0.07	0.08	0.08	0.08
Heated control	0.05	0.06	0.05	0.06	0.05		0.05	0.04

Summary. Tubercle bacilli possess a pepsin-like enzyme which, though feeble in action, is capable of liberating amino acid α nitrogen from the bacillary bodies in the presence of 0.2 per cent hydrochloric acid.

Series III. Erepsin-like enzymes

Attempts were made to determine the presence of erepsin-like enzymes by using casein in acid solution and testing nephelometrically, as recommended by Kober, by using both the bacillary emulsion and autolysate therefrom, but both attempts failed on account of turbidities produced in the solutions by the bacillary emulsion and autolysate even before adding the precipitant. It was finally decided to test for erepsin-like enzymes by preparing a peptone from Witte's peptone which was completely precipitated by ten volumes of methyl alcohol so that no perceptible trace was dissolved therefrom by means of methyl alcohol, and using as criterion the splitting of this peptone in acid solution, testing for such splitting by means of Harding and MacLean's quantitative amino acid α nitrogen test.

Four cubic centimeters of tubercle bacilli were diluted with sterile physiological salt solution to 10 cc., 2 cc. toluene and 1 cc. chloroform were added, and the mixture, after shaking thoroughly, was placed in the incubator at 37°C. for twenty-four hours, after which the centrifugated supernatant liquid was filtered through a sterile hard filter paper and divided into two equal portions. One portion (5 cc.) was heated thirty minutes at 100°C., while the other portion (5 cc.) remained unheated. To each was added sufficient sterile physiological salt solution to make 10 cc., sufficient hydrochloric acid to make 0.2 per cent, 2 cc. toluene, 1 cc. chloroform and 1 cc. 0.25 per cent pure peptone solution (completely precipitable by ten volumes of methyl alcohol). From each of these tubes a control of 0.5 cc. was withdrawn and to this ten volumes of methyl alcohol was added, the whole centrifugated at 3000 revolutions until perfectly clear and the supernatant clear methyl alcohol analyzed quantitatively for amino acid content. At definite intervals

after incubation 0.5 cc. amounts were withdrawn and also analyzed for methyl alcohol soluble amino acid α nitrogen content. In making the analyses the amount of amino acids was always compared with a fresh solution of asparagin as standard (0.0317 mgm. amino acid α nitrogen as asparagin) and the figures given are in milligram amino acid α nitrogen as derived from this standard.

	DAYS						
	0	1	2	3	5	7	10
Erepsin test	0.015	0.023	0.024	0.024	0.026	0.026	0.032
Heated control	0.019	0.023	0.022	0.022	0.022	0.019	0.021

Summary. The autolysate from tubercle bacilli possesses an enzyme capable, in 0.2 per cent hydrochloric acid solution, of splitting a completely methyl alcohol precipitable peptone prepared from Witte's peptone into simple amino acid α nitrogen compounds, not precipitable by this means.

Series IV. Nuclease (nucleic acid splitting enzyme)

Experiment I. Nephelometrically. To the autolysate (twenty-four hours in incubator under toluene) obtained from a heavy suspension of tubercle bacilli (2 cc. bacillary residue) in sterile physiological salt solution, was added (to a test and heated control) 1 cc. 0.1 per cent nucleic acid making a total of 6 cc., and 2 cc. toluene and 1 cc. chloroform. The nucleic acid was determined quantitatively by the nephelometric method of Kober. In this preliminary test the heated control was used as the standard and the results obtained in percentage as compared to heated control, were; immediately—100 per cent nucleic acid; three days—80 per cent nucleic acid; and six days 77.5 per cent nucleic acid.

This experiment was then repeated more elaborately in that both the heated control and test were compared with a freshly prepared standard of nucleic acid each time. The standard

contained 0.05 cc. of 0.1 per cent yeast nucleic acid in distilled water and was considered as 100 per cent. The total volume in this experiment was 10 cc. instead of 6 cc. as above and to this was added 1 cc. 0.1 per cent freshly prepared yeast nucleic acid solution. The results obtained, in percentage of the standard nucleic acid solution, were as follows:

	DAYS						
	0	1	3	4	5	7	10
Test	74.3*	56.6	56.0	52.8	51.3	49.1	50.5
Heated control	60.3†	59.8	60.0	60.2	60.6	59.7	58.4

* The turbidity produced in the test solution was greater than that accounted for by the nucleic acid added. No explanation has been found for this except the fact that certain substances may have been liberated by the autolysis of the tubercle bacilli which combine with the precipitant to form a turbidity.

† The turbidity of the heated control after the albumin precipitant was added was also greater than that accounted for by the nucleic acid added but less than that of the unheated test.

Summary. The autolysate from tubercle bacilli possesses an enzyme capable of splitting nucleic acid into simpler form.

Experiment II. In an attempt to corroborate the findings of experiment I by amino acid determinations, the following experiment was performed and the results are given for what they may be worth.

Two cubic centimeter amounts of bacillary residue were prepared in four tubes. Sufficient hydrochloric acid was added in one tube to make the final strength 0.1 per cent; to the second tube sufficient hydrochloric acid to make the final strength 0.1 per cent and 3 cc. 0.1 per cent acid caseinate; the third was kept as a neutral control; and to the fourth was added 3 cc. 0.1 per cent nucleic acid. All were diluted to 10 cc. with physiological salt solution, 2 cc. toluene and 1 cc. chloroform was added and the tubes incubated at 37°C. At various intervals definite amounts of the solution were withdrawn and tested for amino acid α nitrogen content.

	INTERVALS					
	0	36 HOURS	4 DAYS	6 DAYS	8 DAYS	11 DAYS
	mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
1. HCl to 0.1 per cent	0.020	0.026	0.027	0.031	0.039	0.042
2. HCl to 0.1 per cent and 0.1 per cent acid caseinate	0.022	0.039	0.043	0.048	0.051	0.058
3. Neutral control	0.018	0.027	0.036	0.046	0.053	0.065
4. 3 cc. 0.1 per cent nucleic acid	0.024	0.025	0.028	0.034	0.041	0.043

Series V. Urease

The presence of urease in tubercle bacilli was determined by making an emulsion of human bacilli in physiological salt solution, 3 cc. bacillary residue made up to 32 cc. in each of two tubes, and adding 50 mgm. of urea and 5 cc. toluene to a heated control and a test. After withdrawal of a 3 cc. control (filtering through hard filter paper and using 2 cc. for analysis) the tubes were incubated at 37°C., and samples taken out for analyses at various intervals. The amount of urea decomposed was determined by the Folin aeration method. The enzyme action was stopped by the addition of 1 cc. saturated sodium carbonate to 2 cc. of the filtrate. The results obtained are expressed as milligrams of nitrogen in 2 cc. of filtrate.

	PERIOD OF WITHDRAWAL OF SOLUTIONS					
	0	18 HOURS	2 DAYS	3 DAYS	5 DAYS	8 DAYS
Urease test	0.020	0.060	0.100	0.135	0.134	0.132
Heated control	0.015	0.013	0.014	0.016	0.010	0.012

Summary. Tubercle bacilli possess an enzyme capable of decomposing urea.

Series VI. Diastase and Invertase

The presence of diastase and invertase in the tubercle bacillus was tested by determining the amount of reducing sugar formed from starch and sucrose as performed by Meyers and Rose

using the Lewis and Benedict colorimetric picramic acid method. Among four tubes was divided equally 14 cc. of bacillary emulsion (2 cc. bacillary residue in each). Two of the tubes were heated for thirty minutes at 100°C., in a water bath to destroy the enzymes present. To each of the four tubes was added 2 cc. toluene and 1 cc. chloroform and sufficient sterile physiological salt solution to make a total volume of 10 cc. of emulsion. To two of the tubes, one heated and one unheated, was added 5 cc. of 2 per cent sucrose making a concentration of 1 per cent sucrose, and to the other two, one heated and one unheated, 5 cc. of freshly prepared arrowroot starch emulsion made by adding 2 grams of starch in suspension in cold water to about 75 cc. boiling water and diluting to 100 cc. after cooling.

After thorough mixing, the tubes were centrifugated and 1 cc. of the solution drawn off and tested for reducing sugar. The tubes were then incubated at 37°C., and 1 cc. withdrawn and tested at various intervals thereafter.[3] The results are given in the following table.

		TIME OF TEST IN DAYS					
		0	1	3	6	8	10
Starch....	Not heated	Trace (about 0.1 mgm.)	0.2	0.2	0.2	0.2	0.2
	Heated control	Trace (about 0.15 mgm.)	0.15	0.15	0.2	0.2	0.2
Sucrose...	Not heated	Trace (about 0.15 mgm.)	0.15	0.25	0.3	0.3	0.3
	Heated control	Trace (about 0.2 mgm.)	0.2	0.2	0.25	0.25	0.25

Summary. Tubercle bacilli do not possess an enzyme capable of hydrolyzing starch or sucrose in sufficient amount to be demonstrable by the method used.

[3] Control tests of 1 per cent starch and 1 per cent sucrose to which had been added chloroform and toluene revealed no difference from a blank test with the reagents alone. Varying amounts of glucose, 0.5, 0.4, 0.3, 0.2, and 0.1 mgm., in 1 cc. water to which had been added toluene and chloroform also revealed no influence of the antiseptics upon the accuracy of the test.

Series VII. Elastic and connective tissue digesting enzymes.

In order to demonstrate the presence or absence of elastic or connective tissue digesting enzymes in the tubercle bacilli, an indirect method of attack had to be resorted to. In the first place it was necessary to demonstrate just how much digestion was a result of the action of the enzymes upon the proteins already obtained from the bacilli and then using this as control, to determine whether there was really any digestive action upon the elastic or connective tissues. Since the amino acid α nitrogen method of Harding and McLean was the most delicate and accurate method available for the purpose it was used as the index of the digestion taking place during the course of the experiments.

Experiment I. Elastic tissue digesting enzyme. To 10 cc. of emulsified human tubercle bacilli was added 25 cc. sterile 0.9 per cent salt solution, 5 cc. chloroform, and 10 cc. toluene. This was placed in the incubator at 37°C. for twenty-four hours when it was centrifugated and 8 cc. of the supernatant autolysate was drawn off and filtered through a sterile hard filter paper. The filtrate was divided into three equal portions, one was heated for thirty minutes in boiling water to destroy the enzymes and the other two portions were kept unheated. All were now diluted to 5 cc. and to the heated and one of the unheated portions was added 0.5 gram of elastic tissue prepared from lamb lung.[4] A definite amount of the solution (0.5 cc. each time) was withdrawn immediately, one day, three days, five days, seven days, and ten days after incubation at 37°C. and used for determining the amino acid α nitrogen content. Similarly 8 cc. of autolysate were withdrawn at four days and eight days and tested as above for digestive action upon the elastic tissue. The results of the analyses are given in the following table, the

[4] The elastic tissue was prepared by finely grinding lamb's lung and treating it at room temperature, shaking frequently, with a large amount of 5 per cent potassium hydroxide and 10 per cent acetic acid alternately until the potassium hydroxide and acetic acid solutions used remained absolutely colorless and clear after twenty-four hours contact. The elastic tissue thus prepared was washed with water until absolutely free from acetic acid and was kept for use in distilled water with toluene and chloroform as preservatives.

figures being in milligrams of amino acid α nitrogen per 0.5 cc. using asparagin as standard.

AGE OF AUTOLYSATE		DAYS					
		0	1	3	5	7	10
One day	T	0.014	0.013	0.015	0.019	0.020	0.029
	NHC	0.013	0.014	0.015	0.018	0.018	0.028
	HC	0.015	0.013	0.014	0.015	0.015	0.014
Four days	T	0.016	0.024	0.028	0.031	0.035	0.036
	NHC	0.017	0.026	0.027	0.027	0.030	0.035
	HC	0.021	0.021	0.020	0.023	0.022	0.021
Eight days	T	0.017	0.019	0.019	0.021	0.021	0.020
	NHC	0.018	0.021	0.018	0.019	0.019	0.019
	HC	0.020	0.019	0.020	0.021	0.019	0.020

T, Test, containing autolysate and elastic tissue.
NHC, Non-heated control, containing autolysate alone.
HC, Heated control, containing autolysate (heated) and elastic tissue.

Experiment II. Connective tissue disintegrating enzyme. This experiment was done exactly as in Experiment I with the exception that the 0.5 gram elastic tissue was replaced by 0.5 gram connective tissue prepared from the capsule of large tubercles.[5] The results of analyses given as the amount of amino acid α nitrogen in milligrams per 0.5 cc. of solution are tabulated in the following table.

[5] In order to prepare the connective tissue for this experiment rabbits were injected subcutaneously in the back with fat free tubercle bacilli of which 0.05 gram was used for each site of injection. In order to make a uniform suspension a 10 per cent starch paste was prepared with sterile boiling water and to every 5 cc. was added 0.05 gram tubercle bacilli. The bacilli were well mixed with the starch paste by stirring and the entire amount injected subcutaneously while warm by means of a Murphy glycerine syringe or other pressure syringe. After two to three months a large firm tubercle had formed at the site of injection and was removed. After separating all muscle, blood, and caseous material from the thick connective tissue capsule of the tubercle, the latter was well washed with physiological salt solution, then ground up fine in a meat grinder and washed 12 times with salt solution or until no more turbidity appeared in the salt solution upon vigorous shaking. The connective tissue was then heated for one-half hour at 90°C. to destroy the enzymes and again washed with salt solution. The connective tissue thus prepared was preserved for use in salt solution with chloroform and toluene.

AGE OF AUTOLYSATE		DAYS					
		0	1	3	5	7	10
One day	T	0.012	0.016	0.015	0.014	0.019	0.022
	NHC	0.012	0.011	0.013	0.019	0.021	0.021
	HC	0.011	0.012	0.013	0.012	0.013	0.013
Four days	T	0.019	0.021	0.021	0.024	0.024	0.031
	NHC	0.021	0.020	0.022	0.027	0.028	0.029
	HC	0.020	0.020	0.019	0.019	0.021	0.019
Eight days	T	0.016	0.017	0.017	0.019	0.019	0.019
	NHC	0.019	0.020	0.023	0.024	0.025	0.024
	HC	0.018	0.018	0.019	0.018	0.019	0.019

T, Test, containing autolysate and tubercle connective tissue.
NHC, Non-heated control, containing autolysate alone.
HC, Heated control, containing autolysate (heated) and connective tissue.

Summary. As a result of the above experiments it may be stated that evidence of the presence of a connective tissue, or elastic tissue, disintegrating enzyme in the tubercle bacillus was not obtained, at least by the methods used for this purpose.

COMPLETE SUMMARY

1. Tubercle bacilli of both the human and bovine varieties possess autolytic enzymes, as indicated by the non-coagulable nitrogen and amino acid α nitrogen liberated at incubator temperature after the bacilli have been killed by toluene and chloroform.

2. The bacilli themselves, or autolysates therefrom, also possess a trypsin-like enzyme capable of splitting proteins in alkaline solution, an erepsin-like enzyme capable of decomposing peptone in acid solution, a weak pepsin-like enzyme capable of splitting proteins in acid solution, a nuclease capable of splitting nucleic acid and a urease capable of decomposing urea.

3. The tubercle bacilli, or autolysates therefrom, do not possess enzymes capable of hydrolyzing starch or inverting sucrose, demonstrable by the delicate Lewis and Benedict picramic acid method.

4. Autolysates from tubercle bacilli do not possess enzymes capable of digesting elastic tissue prepared from lamb lung, or connective tissue prepared from tubercles, at least, as indicated by the methods used for demonstrating these enzymes.

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