LVII. DEHYDROGENASES OF THE FILTRABLE ORGANISM OF *AGALACTIA*.

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THE detection of dehydrogenases in *Agalactia* has been reported in a previous paper [Pirie and Holmes, 1933] where the great instability of the enzymes has also been demonstrated. The experiments reported in this paper were undertaken in order to see how many substrates the organism could oxidise. *Agalactia* and *Pleuropneumonia* are the smallest organisms so far shown to possess enzymes, and it seemed possible that the number of enzymes might be very restricted and their kinetics rather different from those of ordinary bacterial enzymes. For the sake of simplicity *Agalactia* will be described as a virus in this paper in spite of the paper by Ledingham [1933] in which many different forms of the organism are described. This pleomorphism does not entirely differentiate *Agalactia* from viruses since Bedson [1933] has described a life cycle for the virus of psittacosis which involves many different and some large forms. While admitting that the exact classification of *Agalactia* is still in doubt the fact of its filtrability enables it to be considered as a very small organism.

The experimental procedure has been described fully in the previous paper. *Agalactia* has been grown on Hartley's broth *plus* 5–10 % horse-serum at 37° for 3 days. In order to obtain the virus free from the medium the culture was centrifuged at 3500 r.p.m. for one hour and the sediment was drained and rubbed up in saline. The volume of saline was usually 1/10th that of the original culture. The enzymes of this suspension could then be investigated. The oxygen uptake and the methylene blue reduction times in the presence of different substrates were measured. In every experiment a saline control and also a control of the virus *plus* some substrate which was known already to be oxidised were included since occasionally apparently well-grown cultures failed to show any dehydrogenases whatever. Table I shows the results obtained using the methylene blue technique. These indicate that a considerable number of substrates are oxidised by *Agalactia* if the substrate concentration is high enough. *Agalactia* oxidises very few compounds if their final concentration is less than $M/10$. Lactate, adenylic acid, adenosine, fructose and hexosediphosphate are the only substrates which are oxidised in low concentration. In this respect *Agalactia* resembles *Esch. coli* since Quastel and Whetham [1925] found that the dehydrogenases of the resting bacterium varied enormously in their affinity for their substrate and relatively few enzymes attacked substrates in low concentrations. The significance of enzymes which do not attack a substrate unless it is in high concentration is obscure. It seems possible that it is a question of incomplete specificity of enzymes so that each dehydrogenase has more than one substrate but has enormously varying affinities for the different substrates which it may attack. Table II shows the effect of variation of substrate concentration on the velocity of reduction of methylene blue.

Table I. *Dehydrogenases of Agalactia.*

Each Thunberg tube contained 1.0 ml. virus suspension, 1.0 ml. substrate, 1.0 ml. *M*/10 phosphate buffer of p_H 7.4 and 0.2 ml. 1/3000 methylene blue. The final substrate concentrations are given.

Substrate	Red. time in mins.	Substrate	Red. time in mins.
Broth	11, 6	Formate <i>M</i> /6	60
Serum	10	Succinate <i>M</i> /6	39
Lactate <i>M</i> /20	7, 11	Malate <i>M</i> /6	12
Glucose <i>M</i> /6	31	Oxalate <i>M</i> /6	∞
Fructose <i>M</i> /6	14	Gluconate <i>M</i> /6	∞
Galactose <i>M</i> /6	18	Glutamate <i>M</i> /12	56
Lactose <i>M</i> /6	24	Aspartate <i>M</i> /12	40
Adenylic acid <i>M</i> /300	17	Alanine <i>M</i> /6	48
Adenosine <i>M</i> /300	24	Citrate <i>M</i> /6	42
Adenine (saturated)	∞	Hexosediphosphate <i>M</i> /300	25
Xanthine	∞	Hexosemonophosphate	∞
Saline	∞		

Table II. *Effect of variation of substrate concentration on the reduction time of methylene blue.*

Conditions the same as in Table I.

Substrate	Conc.	Red. time	Substrate	Conc.	Red. time
Glucose	1/3 saturated	30	Lactose	<i>M</i> /6	38
	<i>M</i> /6	32		<i>M</i> /30	∞
	<i>M</i> /30	∞	Galactose	<i>M</i> /6	39
Fructose	<i>M</i> /3	14		<i>M</i> /30	60
	<i>M</i> /30	28		<i>M</i> /100	∞
	<i>M</i> /300	23	Succinate	<i>M</i> /6	46
Lactate	<i>M</i> /30	24		<i>M</i> /30	∞
	<i>M</i> /300	20	Aspartate	<i>M</i> /12	43
	<i>M</i> /1500	85		<i>M</i> /60	120

Alternatively it suggests that a co-enzyme is necessary for the reaction or that an intermediate compound, not the actual substrate added, is the true substrate for the enzyme. The effect of the addition of the co-enzyme for liver glucose dehydrogenase, described by Mann [1932], on the velocity of glucose oxidation by *Agalactia* was investigated. It was found that the co-enzyme preparation itself was rapidly oxidised and that no acceleration of glucose oxidation could be shown. The reduction time of a mixture of glucose and co-enzyme was approximately the mean of the reduction times for the separate substrates as though only one enzyme were concerned. The same is true for mixtures of fructose or galactose with the co-enzyme or with adenylic acid. Since *Agalactia* dehydrogenates adenosine but has no effect on adenine it is presumably the carbohydrate part of the molecule that is attacked. There seems to be only one enzyme in *Agalactia* which oxidises all the different carbohydrates since a mixture of any two of these is oxidised at the mean rate of oxidation of the two separately. It is possible that the co-enzyme of glucose dehydrogenase also contains a carbohydrate radical since it is oxidised by the same enzyme (Table II).

The addition of phosphate buffer to the mixture of virus and substrate in the vacuum tube often greatly decreased the reduction time. This was at first thought to be a specific effect of phosphate since borate and sulphate had no accelerating effect. Quastel and Whetham [1924] state that replacement of phosphate by borate had no effect on the activity of succinic dehydrogenase of *Esch. coli*. But it seems more probable that the accelerating action of phosphate

Table III. *Unity of carbohydrate dehydrogenases of Agalactia.*Final conc. of total sugars $M/4$. Reduction times in minutes.

		Calc. mean			Calc. mean			Calc. mean
Glucose	31	—	Fructose	14	—	Lactose	24	—
+ Fructose	16	22	+ Glucose	16	22	+ Glucose	24	27
+ Lactose	24	27	+ Lactose	16	19	+ Fructose	16	19
+ Galactose	21	24	+ Galactose	14	16	+ Galactose	21	21
Galactose	18	—	Fructose	33	—	Glucose	32	—
+ Fructose	14	16	+ Co-enzyme	28	28	+ Co-enzyme	26	27
+ Glucose	21	24	Fructose	43	—	Glucose	32	—
+ Lactose	21	21	+ Adenylic acid	24	32	+ Adenylic acid	25	27

on the enzymes of *Agalactia* is a p_H effect, since some of the dehydrogenases have a sharp optimum p_H of 7.4. In vacuum tubes containing no buffer the p_H rises during evacuation and therefore the enzyme may not be acting under optimum conditions. It was impossible to be certain of this point since experiments with citrate and glycylglycine as buffers were inconclusive. Those enzymes which seemed most affected by phosphate were also the most unstable and were often destroyed after the suspension of *Agalactia* had stood on the bench for half an hour. In order to test the relative effects of different buffers all vacuum tubes had to be evacuated simultaneously. Since it seemed possible that phosphate had either a stabilising or stimulating action on some of the enzymes of *Agalactia* it was thought that it might have a similar action on the organism itself. The sediments from equal volumes of culture were suspended in equal volumes of $M/10$ phosphate buffer of p_H 7.4 or in physiological saline of the same p_H . The suspensions were kept on the bench and the activity of some dehydrogenases was determined at intervals. It was found that the glucose and succinic dehydrogenases were destroyed more rapidly in phosphate than in saline whereas the lactic dehydrogenase was equally stable in either solution. Elford and Galloway [1933] found that the virus of louping-ill was less stable, as judged by its pathogenicity, in phosphate solutions than in saline and it would be tempting to consider that this case also is one of enzyme destruction.

The dehydrogenases of *Agalactia* varied considerably in their relative activities. Many cultures were investigated under standard conditions before this variability was accepted as being a real occurrence not due to chance changes in technique. The ratio of the glucose and lactic dehydrogenases was particularly investigated. First of all the ratio of the two enzymes at different stages of growth of the culture was estimated. 30 ml. samples were removed from cultures on the 2nd, 3rd and 4th days of growth and centrifuged for one hour, and the lactate and glucose dehydrogenase activity was estimated in the usual way. A saline control was also put up, the virus sediment being rubbed up in 3.0 ml. saline. If an active glucose dehydrogenase was present the ratio of the enzymes was usually 2-3 to 1 at all stages of growth, but if a very feeble one only was detectable this seemed to be at its maximum on the 3rd day of growth when the ratio might be 7:1.

The variability depended more noticeably on the medium. Different batches of Hartley's broth gave very different glucose dehydrogenases although the lactic dehydrogenase was constant. In order to see if the medium were the controlling factor in variability of the enzymes a culture grown on a broth which tended to diminish the glucose enzyme was transplanted to a "good"

broth. The enzyme activity increased considerably. After two cultures on this broth a subculture was made on to the "bad" broth and the enzymes were estimated again. There was a tendency for the glucose and other dehydrogenases to disappear in the first subculture, the lactic dehydrogenase being the only one left. After several generations the enzymes might return even on the bad medium. Variability therefore seems to be neither completely a matter of medium nor a matter of phase of growth. It is possible that different media

Table IV. *Variability of enzymes.*

(a) Comparison of enzymes on two broths.

Substrate	Type of broth		
	"Bad"	"Good"	"Bad"
Control	∞	101	∞
Glucose <i>M</i> /1	∞	48	∞
Lactate <i>M</i> /10	14	28	12
Formate <i>M</i> /2	182	39	∞
Fructose <i>M</i> /10	180	—	—

(b) Glucose and lactate dehydrogenases of successive cultures on different broths.

Substrate	Type of broth			
	"Good"	"Bad"	"Bad"	"Bad"
Saline	200	28	101	138
Glucose <i>M</i> /1	36	28	100	70
Lactate <i>M</i> /10	26	6	8	10

Conditions are the same as in previous tables. Reduction times are given in minutes.

influence the preponderance of one form of the organism over any other [Ledingham, 1933]. Microscopical preparations of the cultures by Ledingham's method for the detection of the different forms was not carried out, but bacterial sterility was tested by inoculation into broth and by Gram staining of the sediment from the centrifuged culture.

A more detailed study of the lactic dehydrogenase of *Agalactia* was made since this enzyme seemed to be the most active and stable one possessed by the virus. Quastel found that the lactic dehydrogenase of *Esch. coli* was inhibited by other acids containing the groups $-\text{CHOH}.\text{COOH}$ or $-\text{CO}.\text{COOH}$. The effect of such acids on the activity of the lactic enzyme of *Agalactia* was investigated in order to see if this was of the same type as the enzyme contained in *Esch. coli*. The solution of the inhibitor was added to the virus before the lactate in order that it might be adsorbed at the surface non-competitively. The time taken by the virus to reduce methylene blue in the presence of lactate, the inhibiting acid and a mixture of the two was then determined. Solutions of oxalate and gluconate inhibited lactic dehydrogenase almost completely while glycollic and glyceric acids had slight inhibitory actions. Glucose, citrate, tartrate and malate had no effect. Gluconate or oxalate was added to the medium in which *Agalactia* was grown in order to see if any depressing action on the growth of the organism could be observed. Lactic dehydrogenase was so much the most active of the enzymes so far detected in *Agalactia* that it seemed possible that if this enzyme were inhibited the growth of the organism might be impaired. It was found that the addition of 1.0 % oxalate to the medium entirely prevented growth of *Agalactia*. Oxalate in lower concentrations permitted some growth, while concentrations of gluconate up to and including 1.0 % retarded but did not inhibit the growth of *Agalactia*. Subcultivation on

to normal medium gave normal growth. The addition of broth had a slight inhibitory effect on lactic dehydrogenase showing that under normal conditions of culture the enzyme cannot be optimally active. It seems unlikely that the concentration of any inhibitor occurring naturally will be great enough to inhibit growth.

The oxygen uptake of *Agalactia* in the presence of different substrates was measured in Barcroft differential manometers. Lactate, broth, serum, fructose and citrate were regularly oxidised while aspartate, alanine and succinate were irregularly attacked. In general it seemed that those substances which were dehydrogenated regularly in the presence of methylene blue could also be oxidised by oxygen while those which were only occasionally hydrogen donors to methylene blue were also only occasionally oxidised in air. The oxygen uptake of *Agalactia* in presence of broth was linear for the whole course of the experiment while the uptake in the presence of all other substrates fell off usually during the second hour. It was found that in presence of broth the enzymes remained active, while without it they became inactive during the shaking. The destruction of the enzymes was determined by testing the reducing capacity of the organism in the presence of fresh broth or lactate at the end of the experiment. *Agalactia* which has been shaken in the manometer with broth still decolorises methylene blue rapidly whereas after shaking in the presence of any other substrate the reduction of methylene blue does not occur. This protective effect of broth is unexplained.

SUMMARY.

A more comprehensive survey of the dehydrogenases and oxidising enzymes of *Agalactia* has been made. The most active dehydrogenases are those attacking lactate, adenylic acid, adenosine, fructose and hexosediphosphate. Other dehydrogenases need very high substrate concentrations. The relative strengths of the dehydrogenases vary from culture to culture. This variability does not seem to depend entirely on the medium or on the phase of growth at which the enzymes are estimated. The lactate enzyme is similar to the lactic dehydrogenase of *Esch. coli* in that it is strongly inhibited by gluconate and oxalate.

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