INVESTIGATIONS ON THE DEHYDROGENATING PROPERTIES OF CERTAIN PATHOGENIC OBLIGATE ANAEROBES

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I. THE DEHYDROGENATING FERMENTS OF CLOSTRIDIUM BOTULINUM, C. PARABOTULINUM AND C. WELCHII

The metabolism of the obligate anaerobes has in recent years been the subject of several thorough investigations. Most notable are the papers by Stickland (1934, 1935a, b, c), Woods (1935b), Woods and Clifton (1937, 1938), Barker (1938), Elberg and Meyer (1939) and Clifton (1939, 1940a, b, 1942). The dehydrogenating properties of *C. sporogenes* have been especially studied by Kocholaty and Hoogerheide (1938). We felt that a study of the dehydrogenating ferments of certain pathogenic obligate anaerobes would be of interest. For this purpose we chose *C. botulinum*, *C. parabotulinum* and *C. welchii*, since they differ markedly both in their biochemical and serological characteristics and in the type of toxin which they produce.

Method

Preparation of bacterial suspensions. The cultures used were obtained from the Robert Koch Institute in Berlin in 1933, and since then maintained in our laboratory. Their biochemical behaviour and toxicity were in each case typical.

The bacteria were grown on the following medium:

Liebig meat extract	3.0 gm.
Peptone	10.0 gm.
NaCl	5.0 gm.
KH_2PO_4	2.0 gm.
Ascorbic acid	0.25 gm.
Rabbit serum	20.0 ml.
Aqua dest	1000 ml.
pH	7.2–7.4

The serum was added in order to secure as heavy a growth as possible. The cultures were grown aerobically at 37°C., since the addition of 0.25 per thousand neutralized ascorbic acid permits the growth of strict anaerobes in the presence of air (Kligler and Guggenheim, 1938). After 40–45 hours incubation the cultures were centrifuged, the sediments washed with 0.85% NaCl solution and suspended in saline. Each 100 ml. of culture yielded 5 ml. of bacterial suspension. It is sufficient to wash the organisms once; as a matter of fact, as Kocholaty and Hoogerheide (1938) have shown, repeated washing may destroy the sensitive ferments. Microscopic examinations of the suspensions performed from time to time, proved that they consisted chiefly of vegetative cells, spores being extremely rare.

Detection of hydrogen donators. The method used was the Braun and Woerdehoff (1933) modification of the well known Thunberg procedure. Cresyl-blue was employed as acceptor. Each tube contained: 1.0 ml. phosphate buffer solution at pH 7.4; 0.5 ml. 1/5000 cresyl-blue; 0.5 ml. bacterial suspension; and 1.0 ml. of a 1/20 molar solution of the substance tested. In the case of substances of low solubility (tyrosine, cystine and uric acid), saturated solutions were used. Where necessary the donator solutions were neutralized with NaOH.

Immediately after a tube was prepared, it was well shaken, overlayed with 4–5 cm. of melted vaseline, cooled in ice water and placed in a 37°C. water bath. We observed that even under the most exact experimental conditions the dehydrogenase content of a bacterial suspension was subject to significant variations. Consequently, each substrate was tested four to five times with each of the organisms. In consideration of these variations we do not feel justified in classifying the results according to "relative velocities of dehydrogenation," as suggested by Stickland (1934) and Kocholaty and Hoogerheide (1938), but find it preferable to indicate the extent of fermentative activity as follows: +++ = complete dehydrogenation after 0–30 minutes; ++ = complete dehydrogenation after 30–180 minutes; + = complete dehydrogenation after more than 180 minutes; 0 = no dehydrogenation; in other words, no activation of the substrate.

All experimental series in which the control tube without donator showed dehydrogenation within 6 hours were rejected; actually, this occurred rarely.

Results

The results are given in table 1.

It is noteworthy that there are hardly any differences among *C. botulinum*, *C. parabotulinum* and *C. welchii*, although these bacteria are sharply distinguished from one another in their other biochemical properties and in the nature of the toxin formed by them.

The great majority of the substances tested were dehydrogenated. Of the monocarbonic acids, lactic and pyruvic acids, and of the dicarbonic acids, succinic, malic, glutaric, fumaric and maleic, were dehydrogenated; the only tricarbonic acid tested, citric acid, was found not to be dehydrogenated. Other substances which were not dehydrogenated were methanol, erythritol, arabinose, glycine and cystine. Urea itself was not dehydrogenated, although its derivatives, creatine and creatinine, were dehydrogenated by all strains and guanidine by *C. botulinum* alone.

A comparison of our results with those of Stickland (1934) and of Kocholaty and Hoogerheide (1938) is possible only to a limited extent, because these authors worked with *C. sporogenes*, and used other culture media. Kocholaty and Hoogerheide (1938) pointed out the significance of variations in culture media for the formation of dehydrogenating ferments by *C. sporogenes*. Nevertheless, their results as well as ours demonstrate the pronounced donator activities of d-alanine, leucine, valine, phenylalanine and pyruvic acid.

TABLE 1

Substrates which were found to be activated as donators by C. botulinum, C. parabotulinum and C. welchii

SUBSTRATE	C. BOTULINUM	C. PARABOTULINUM	C. WELCHII
Organic acids			
Formic acid	0	0	0
Acetic acid	0	0	0
Propionic acid	0	0	0
Butyric acid	0	0	0
Lactic acid	++	++	++
Pyruvic acid	++	++	++
Oxalic acid	0	0	0
Succinic acid	+	+	+
Malic acid	+	+	+
l-Tartaric acid	0	0	0
d-Tartaric acid	0	0	0
dl-Tartaric acid	0	0	0
Glutaric acid	+	++	++
Fumaric acid	+	+	+
Maleic acid	+	+	+
Citric acid	0	0	0
Alcohols			
Methyl alcohol	0	0	0
Ethyl alcohol	++	+++	+++
Glycerol	+	++	++
Erythritol	0	0	0
Adonitol	+	+	+
Mannitol	0	+	+
Sorbitol	+	+	+
Dulcitol	+	+	+
Inositol	+	+	+
Carbohydrates			
Xylose	0	+	+
Arabinose	0	0	0
Rhamnose	+	+	+
Glucose	+	+	+
Fructose	++	++	++
Galactose	++	+	+
Mannose	+	+	+
Sorbose	+	+	+
α-Methyl glucoside	+	+	+
Sucrose	+	+	+
Maltose	+	+	+
Lactose	+	+	+
Raffinose	++	+	+
Amino acids			
Glycine	0	0	0
d-Alanine	+++	+++	+++
dl-Alanine	+++	+++	+++
β-Alanine	+	+	+
Leucine	+++	+++	+++

TABLE 1—*Concluded*

SUBSTRATE	C. BOTULINUM	C. PARABOTULINUM	C. WELCHII
Amino acids—Continued			
Serine	++	++	++
Valine	+	++	++
Arginine	+	+	+
Aspartic acid	++	++	++
Glutamic acid	++	++	++
Cystine	0	0	0
Lysine	+	++	++
Asparagine	++	++	+
Phenylalanine	++	++	++
Tyrosine	+	+	+
Histidine	+	+	+
Tryptophane	+	+	+
Proline	+	+	+
Urea derivatives			
Urea	0	0	0
Creatine	+	+	+
Creatinine	++	++	++
Guanidine	+	0	0
Purines			
Uric acid	+	+	+
Amines			
Histamine	+	+	+

Summary

62 substrates, including organic acids, alcohols, carbohydrates, amino acids urea and its derivatives and other organic nitrogen compounds, were tested for their ability to be activated as hydrogen donators by *C. botulinum*, *C. parabotulinum* and *C. welchii*. The most active donators were lactic acid, pyruvic acid, glutaric acid, ethyl alcohol, glycerol, fructose, galactose, raffinose, d-alanine, dl-alanine, leucine, serine, valine, aspartic acid, glutamic acid, lysine, asparagine, phenylalanine and creatinine.

II. THE EFFECT OF POISONS ON THE DEHYDROGENATING FERMENTS OF C. BOTULINUM

It was shown above that the pathogenic obligate anaerobes were able to dehydrogenate a large number of different substrates. In order to obtain a deeper insight into the mechanism of this fermentative activity, we tested the influence of various poisons on the dehydrogenating mechanisms. Since the three anaerobes studied showed very little difference in their dehydrogenating properties, we confined ourselves to the investigation of only one strain, *C. botulinum.* Furthermore, only the most active dehydrogenases were selected for this purpose, i.e. those for lactic and pyruvic acid; ethyl alcohol and glycerol; fructose and raffinose; d-alanine, dl-alanine, leucine, serine, valine, asparagine, aspartic acid, glutamic acid and phenylalanine.

Method

Seven pharmacologically different poisons were tested: hydrocyanic acid, the poison for heavy metal catalysis (as KCN); ethyl urethane as a surface active narcotic; arsenic (As_2O_3); fluoride (as NaF); bivalent copper ($CuCl_2$), bivalent manganese ($MnCl_2$) and trivalent iron ($FeCl_3$) as heavy metals. The poisons were applied in varying concentrations, as indicated in the tables. The preparation of the bacterial suspensions and the dehydrogenating technique were the same as mentioned above. Cresyl blue served generally as acceptor; in the case of the rapidly reducing substances, d-alanine, dl-alanine and leucine, phenosafranine was used. Each tube contained: 1.0 ml. phosphate buffer solution at pH 7.4; 1.0 ml. M/20 donator solution; 0.2 ml. 1/2000 cresyl blue or 1/2000 phenosafranine; 0.3 ml. solution of the poison in suitable concentration; 0.5 ml. bacterial suspension.

The velocity of the reduction of the donators is expressed in arbitrary units related to the velocity of reduction of the donator concerned in the absence of any poisonous effect, which was considered 100. It should be noted, however, that the figures given in the tables are reproducible only to a certain degree, since, as mentioned above, the dehydrogenase content of the bacterial suspensions is subject to considerable variations. This also holds true for the sensitivity of the dehydrogenases to poisons. In other words, a given poison can have a stronger promoting and inhibiting effect on one bacterial suspension than on another. The effect of a given poison is reproducible only qualitatively but not quantitatively. Our figures are, therefore, to be considered as average results of 4–5 experiments.

Results

The results are given in tables 2 to 4.

The data shown in tables 2 to 4 bring out the following facts:

Potassium cyanide almost always exhibits an inhibiting effect; only the dehydrogenases of d-alanine, dl-alanine and leucine are not influenced by it.

Ethyl urethane inhibits the dehydrogenases for lactic acid and ethyl alcohol strongly, those for serine and aspartic acid weakly, while those for the remaining substances are unaffected.

Arsenic trioxide has a stimulating effect on all dehydrogenases.

Sodium fluoride inhibits the dehydrogenases for lactic acid strongly, those for pyruvic acid, ethyl alcohol, glycerol, fructose, raffinose, leucine, serine, valine, aspartic acid and glutamic acid less strongly, while those for alanine, asparagine and phenylalanine are unaffected.

Of the *heavy metals*, *copper* inhibits strongly in all cases. The effect is so marked, that a concentration of M/25000 suffices to prevent all action in the case of ethyl alcohol and glycerol dehydrogenases.

The behaviour of *manganese* and *iron* is somewhat complicated. In many cases the inhibiting effect depends on the concentration (for instance manganese in the case of lactic acid and glycerol and iron in the case of alanine and leucine). In other cases there is a distinct stimulating effect, which has a maximum at

medium concentrations (M/1000 and M/5000), and which becomes weaker or disappears at higher and lower concentrations (M/200 and M/25000); this is seen with manganese in the case of pyruvic acid, alanine and asparagine, and with

TABLE 2

The effect of potassium cyanide and of ethyl urethane on certain dehydrogenases of C. botulinum

SUBSTRATE	POTASSIUM CYANIDE					ETHYL URETHANE			
	M/100	M/500	M/2500	M/10000	M/50000	M/10	M/50	M/250	M/1000
Lactic acid	0	20	41	45	50	28	33	86	100
Pyruvic acid	0	45	56	87	100	100	100	100	100
Ethyl alcohol	0	0	30	53	85	0	0	19	26
Glycerol	10	27	46	75	83	100	100	100	100
Fructose	24	33	70	100	100	100	100	100	100
d-Alanine	100	100	100	100	100	100	100	100	100
dl-Alanine	100	100	100	100	100	100	100	100	100
Leucine	100	100	100	100	100	100	100	100	100
Serine	0	0	23	55	100	65	100	100	100
Valine	0	0	0	27	39	100	100	100	100
Asparagine	0	6	10	100	100	100	100	100	100
Aspartic acid	0	12	19	54	88	64	100	100	100
Glutamic acid	0	21	36	59	100	100	100	100	100
Phenylalanine	55	77	87	92	100	100	100	100	100

TABLE 3

The effect of arsenous acid and sodium fluoride on certain dehydrogenases of C. botulinum

SUBSTRATE	ARSENOUS ACID				SODIUM FLUORIDE			
	M/50	M/250	M/1000	M/5000	M/10	M/50	M/250	M/1000
Lactic acid	240	120	100	100	11	25	38	47
Pyruvic acid	350	100	100	100	20	34	64	93
Ethyl alcohol	600	330	165	145	21	29	47	100
Glycerol	545	180	100	100	33	50	70	75
Fructose	800	140	100	100	65	100	100	100
Raffinose	710	320	100	100	34	100	100	100
d-Alanine	310	220	160	160	100	100	100	100
dl-Alanine	230	130	130	100	100	100	100	100
Leucine	400	220	135	100	20	34	64	93
Serine	705	122	100	100	56	82	100	100
Valine	410	143	100	100	22	50	100	100
Asparagine	280	210	125	100	100	100	100	100
Aspartic acid	350	130	100	100	42	78	100	100
Glutamic acid	710	100	100	100	66	100	100	100
Phenylalanine	390	130	100	100	100	100	100	100

iron in the case of pyruvic acid, ethyl alcohol and glycerol. Finally, there are cases in which stimulation occurs at low and medium concentrations and inhibition at high concentrations; for instance, with manganese in the case of ethyl alcohol, serine and phenylalanine, and with iron in fructose, raffinose and valine.

It is evident that above a certain concentration all metals exert an inhibitory effect. In the case of copper, the threshold is so low that a strong inhibition was present even in the lowest concentrations employed by us. Iron and manganese have a higher threshold, which is different for each substrate; in lower concentrations these two heavy metals are either without effect (e.g., leucine is not affected by manganese in dilutions greater than M/1000 or by iron in dilutions greater than M/5000), or actually have a stimulating effect (e.g. manganese in the case of asparagine or iron in the case of glycerol, fructose and raffinose).

It should again be stressed that the effect of the bacterial suspensions in different experiments was not always the same; the degree of inhibition or stimulation by a given concentration of the salt varied in the different experiments. The results represent average values.

TABLE 4

The effect of copper, manganese and iron on certain dehydrogenases of C. botulinum

SUBSTRATE	$CuCl_2$				$MnCl_2$				$FeCl_3$			
	M/200	M/1000	M/5000	M/25000	M/200	M/1000	M/5000	M/25000	M/200	M/1000	M/5000	M/25000
Lactic acid	0	0	0	10	10	14	20	25	90	160	175	100
Pyruvic acid	0	0	0	13	160	175	205	200	180	300	380	110
Ethyl alcohol	0	0	0	0	90	130	145	100	110	165	140	130
Glycerol	0	0	0	0	30	46	55	73	140	250	380	202
Fructose	0	0	0	12	15	53	100	100	20	256	135	100
Raffinose	0	0	0	30	34	48	58	63	56	280	255	125
d-Alanine	0	0	0	12	100	110	120	100	0	33	66	73
dl-Alanine	0	0	0	14	100	130	145	100	11	50	70	92
Leucine	0	0	0	3	80	87	100	100	53	69	80	100
Serine	0	0	0	18	32	129	145	100	65	170	130	116
Valine	0	0	0	21	12	21	85	100	23	100	183	157
Asparagine	0	0	6	10	166	360	360	280	12	26	60	80
Aspartic acid	0	0	0	15	20	48	54	74	44	89	200	155
Glutamic acid	0	0	0	17	59	81	100	100	36	78	135	122
Phenylalanine	0	0	0	63	71	113	130	117	12	120	170	100

All dehydrogenases, with the exception of that for leucine, can be stimulated by either manganese or iron. The significance of heavy metal catalysis for the action of dehydrogenases is obvious and this is confirmed by their high sensitivity to KCN.

Certain of the poisons studied by us have already been investigated with *C. sporogenes.* Kocholaty and Hoogerheide (1938) studied the sensitivity of the dehydrogenases for d-alanine, pyruvic acid and ethyl alcohol to HCN, As_2O_3, $CuSO_4$ and NaF. Their results differ from ours. They found that HCN and As_2O_3 acted in the same way in all circumstances, i.e. inhibiting towards ethyl alcohol, stimulating towards d-alanine and without effect on pyruvic acid; that $CuSO_4$ always inhibited completely, even in concentrations of M/5000, and that NaF was without effect even in m/5 solution. These results do not correspond with those which we obtained for *C. botulinum.*

The poisoning effect of KCN, urethane and NaF on the dehydrogenating processes of other bacteria have also been investigated. Bernheim, Bernheim and Webster (1935) and Webster and Bernheim (1936) found, that KCN completely inhibited the amino acid dehydrogenases of *Proteus vulgaris* and *Pseudomonas aeruginosa*, and that NaF (1%) and urethane (1%) had no effect in the case of *P. aeruginosa*. According to Barron (1936), the lactic acid dehydrogenase of gonococcus is inhibited by KCN and urethane but not by sodium fluoride, while the pyruvic acid dehydrogenase is inhibited by NaF as well as by KCN and urethane.

From the above it will be clear that the corresponding dehydrogenase reacts differently when originating in different bacteria, and that different dehydrogenases of the same bacterium react differently to the various poisons. We may, therefore, conclude that the bacterial dehydrogenases possess a differentiated structure and specificity.

Summary

The effect of *potassium cyanide*, *urethane*, *arsenic*, *fluoride*, *copper*, *manganese* and *iron* on dehydrogenases of *Clostridium botulinum* for 15 substrates was investigated. HCN generally inhibits; arsenic always stimulates; urethane and fluoride inhibit in some cases, and are without effect in others. All heavy metals investigated inhibit when in high concentrations. Copper inhibits in concentrations as low as M/25000. In the case of manganese and iron the threshold of the inhibiting action lies at higher concentrations, the specific concentration varying with each substrate. Below the given threshold manganese shows a stimulating effect in 7 cases and iron in 11. The significance of catalysis by heavy metals in the inhibitive effect exerted by cyanide is emphasized.

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