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THE EFFECT OF AMMONIUM ION AND UREA UPON THE ACID PRODUCTION OF LACTOBACILLI

by

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The influence of ammonium ion and urea upon the glucose breakdown by oral micro-organisms has already been intensively investigated. *Kesel et al.* (1947) claimed to have shown that a combination of 3 per cent urea and 5 per cent dibasic ammonium phosphate was inhibitory to lactic acid production in saliva-glucose mixtures. Lactic acid production was accelerated in the presence of 3 per cent urea alone after 4 hours of incubation but 5 per cent dibasic ammonium phosphate alone was inhibitory. *Jenkins & Wright* (1951) stated that the ammonium ion had no specific inhibiting property but claimed that a mixture of 1.2 per cent urea and 2 per cent dibasic ammonium phosphate was strongly inhibitory to acid production in saliva-glucose mixtures. In concentrations of about 8 per cent, urea strongly inhibited acid production in saliva-glucose mixtures but 16 per cent urea was required for the inhibition to be complete. *Stephan* (1943) and *Muntz & Miller* (1943) found that 50 per cent urea was strongly inhibitory to acid production in dental plaque material *in vitro*.

Pearlman (1951), using the *Warburg* manometric technique found that the ammonium ion had no specific effect on the glycolysis of lactobacilli except a possible stimulation in very low concentrations. Urea was effectively inhibitory towards glycolysis only when its concentration approached 4 M. Since preliminary results obtained in this laboratory concerning the action of the ammonium ion did not confirm *Pearlman*'s results, a more extensive investigation was performed using the titration method

elaborated by the present author. In this paper, "ammonium ion concentration" means total concentration of NH_4^+ and NH_3 at pH 6.5.

MATERIAL AND METHODS

Two strains of lactobacilli were used (La 5, Frostell and Lactobacillus casei 4646, American Type Culture Collection). They were kept on *Rogosa*'s solid medium (*Rogosa et al.* 1951). Suspensions of active organisms were prepared in the following way: Material was taken from a plate to a tube containing *Rogosa*'s medium without agar and subcultivated for 24 hour periods two or three times in the same medium. When the organisms were actively growing the contents of a tube were transferred to a bottle containing 1 liter of *Rogosa*'s medium without agar and incubated for 16 hours at 37° C. under continuous vigorous shaking. The bottle was tightly closed with rubber stoppers; the amount of air in the flask amounted to about 5 per cent of the total volume. The cells were washed twice with 0.01 M phosphate buffer (pH 6.96), the wet weight was determined (*Frostell* 1957), and the organisms were finally suspended in 10—30 ml of the same buffer at a concentration of about 200 mg wet weight per ml. The pH of the suspension was set at 6.5. The suspensions were stored at 4—6° C.

Experiments were performed at constant pH and anaerobic conditions according to the titration method described elsewhere (*Frostell* 1959 b). The experiments were performed in the stable phase, i.e. the suspensions were at least 20 hours old (*Frostell* 1957). The pH was kept constant by the addition of 0.01 N NaOH. Samples were taken *in duplo* or *in triplo* for lactate determination before and after some of the experiments. Lactate determination was performed according to the method of *Barker & Summerson* (*Umbreit et al.* 1957). It was found that the presence of 1 M of ammonium ion in a pure glucose solution did not significantly influence the determination of the glucose concentration. Nitrogen gas free from oxygen as indicated by the *Haldane* method was passed over the suspension before and during the experiment as described elsewhere (*Frostell* 1959 a).

Thus it was possible to determine the acid production on the basis of the amount of alkali required to keep the pH constant,

and to determine the lactate production from the difference of the lactate concentration of the suspension before and after the experimental period. This period was kept at between 10 and 20 minutes. The error of the determination of the acid production was calculated from the controls at ± 6.7 per cent (variation coefficient). The error of the lactate determination (mean of three determinations) was determined at ± 5.0 per cent (variation coefficient).

EXPERIMENTS AND RESULTS

Influence of the ammonium ion

Six series of experiments were performed with La 5 and three with L 4646. The following solutions were pipetted into the reaction bottle: (A) 2 ml of phosphate buffer (0.01 M, pH 6.96); (B) Different amounts of NH_4Cl, 0.01 N NaOH and physiological saline so as to obtain a pH of 6.5 and a final volume of about 10 ml; (C) 0.5 or 1.0 ml of bacterial suspension, depending on the activity of the organisms. Since it was known that phosphate is inhibitory to lactate production (*Pearlman* 1951) the pH was adjusted by NaOH. For the higher concentrations of NH_4^+ a few ml of 0.1 N NaOH was used, the precise adjustment of the pH being performed by the addition of 0.01 N NaOH. The sodium concentration was usually between 0.002 and 0.006 M. For the highest concentrations of NH_4^+ the sodium concentration was about 0.05 M. It was found that these concentrations did not significantly affect the acid production of the organisms. The final volumes of the suspensions varied between 8 and 15 ml but in most instances were about 10 ml. As was shown by many control experiments the differences in total volume did not significantly influence the results.

Nitrogen gas was let in for approximately 12 minutes. When the temperature was 37.5 ± 0.2°C., 1.0 ml of a glucose solution (2.5 g/50 ml) was added.

In one series of experiments a concentrated solution of ammonia was used, this being neutralized by hydrochloric acid. The volumes in this series of experiments were greater, the volume of the highest ammonium ion concentration being 23 ml, while the others were between 10 and 15 ml.

Ammonium ion concentration was set at different values be-

Table I

Influence of the Ammonium Ion on the Acid Production of Lactobacilli

Strain	Molar conc. of NH_4^+	Acid production in per cent of the mean of the controls	Symbol in Fig. 1	Lactate production in per cent of the mean of the controls
La 5	0	101		100
	0	99		—
	0.06	97		111
	0.16	98	+	109
	0.26	149		147
	0.33	153		157
La 5	0	100		100
	0.32	184	●	150
	0.33	175		171
	0.61	61		72
La 5	0	100		
	2.85	0		
La 5	0	100		
	0.14	108	■	
	0.21	98		
La 5	0	101		
	0	99		
	0.30	140		
	0.53	163	□	
	0.76	148		
	0.98	124		
	1.15	102		
	1.31	58		
La 5	0	100		
	0.21	155	⊙	
L 4646	0	102		
	0	98		
	0.18	118		
	0.39	120		
	0.84	158		
	1.17	127		
	2.77	0		
	0	102	not included in the mean	
L 4646	0	100		
	0.34	125		
L 4646	0	100		103
	0	100		97
	0.47	165		118
	0.47	109		83
	0.47	142		143
	0	109	not included in the mean	

Table II

Influence of the Potassium Ion on the Acid Production of Lactobacilli

Strain	Molar conc. of K^+	Acid production in per cent of the mean of the controls	Symbol in Fig. 1
La 5	0	102	
	0	98	
	0.10	84	▲
	0.20	98	
	0.30	83	
	0.39	71	
La 5	0	107	
	0	97	
	0	96	
	0.20	75	
	0.20	76	

tween 0 and 2.85 M. If it was suspected that the activity of the suspension had changed during the experimental day a third control experiment was performed at the end of the series of experiments. This control was not incorporated in the mean of the controls. Usually, two or three control experiments without ammonium were run in each of the series.

For comparison, experiments were performed with different concentrations of NaCl and KCl.

The activity was calculated in ml of 0.01 N NaOH per minute required to keep the pH constant or in micro-moles of lactate produced per minute. The activity of the control or of the mean of the controls was called 100 per cent and the activity found in the different experiments was expressed in per cent of the control.

The results are presented in Tables I—III and in Fig. 1. Acid production in the presence of the ammonium ion was about equal to the controls at concentrations of 0—0.2 M. In the range 0.2 to about 1.2 M the acid production was stimulated in most experiments. Maximum stimulation was found at 0.3—0.8 M. At concentrations over 1.2 M the acid production was inhibited. The

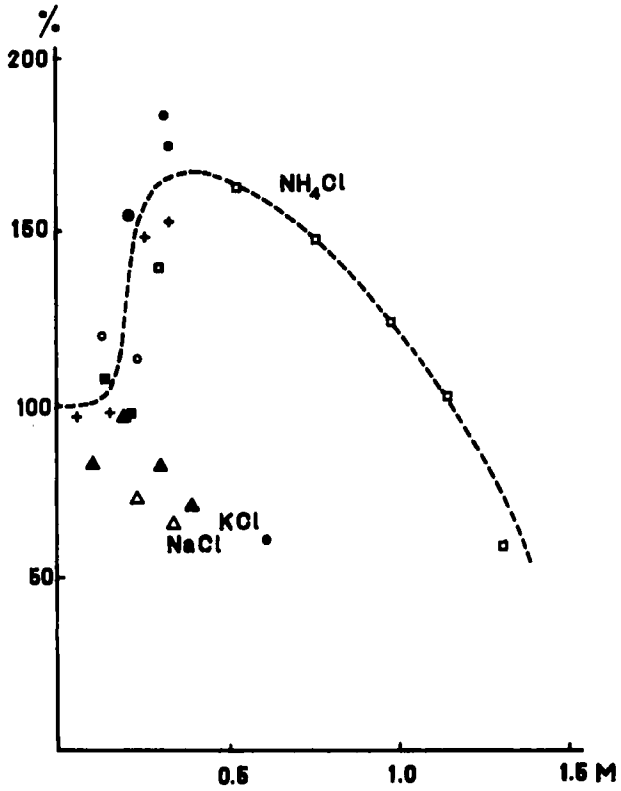

Fig. 1. *Influence of Various Concentrations of Ammonium, Sodium and Potassium on the Acid Production of Lactobacilli (La 5)*

The different symbols refer to the results of the experiments tabulated in Tables I, II and III. The dotted line indicating the influence of ammonium ion is drawn schematically.

Table III

Influence of the Sodium Ion on the Acid Production of Lactobacilli

Strain	Molar conc. of Na^+	Acid production in per cent of the control	Symbol in Fig. 1
La 5	0	100	
	0.23	73	△
	0.34	65	

results were confirmed by the results of the lactate determinations.

No stimulation was found with NaCl and KCl. Acid production was partially inhibited at 0.3 M with these salts.

A fourth series of experiments with L 4646 will be reported in the subsequent section.

Influence of urea

Three series of experiments were performed with La 5. The total volume was kept between 7.0 and 8.9 ml at the start of the experiments. The urea concentration was set at different values between 0 and 3.3 M.

The results are given in Table IV. At urea concentrations between 0 and about 1.0 M acid production was approximately equal to that of the controls. At higher concentrations acid production was inhibited. At an urea concentration of 3.3 M, acid production was 6.4 per cent of the mean of the controls. These

Table IV

Influence of Urea on the Acid Production of Lactobacilli

Strain	Molar conc. of urea	Acid production in per cent of the mean of the controls	Lactate production in per cent of the mean of the controls
La 5	0	90	84
	0	110	117
	1.04	110	87
	1.10	95	99
	2.10	87	77
	3.30	6	5
La 5	0	99	
	0	101	
	0.50	109	
	0.99	80	
	1.45	81	
	2.0	66	
La 5	0	100	
	0.99	88	

Table V

Influence of Ammonium chloride, Sodium chloride and Urea on the Acid Production of a Suspension of Lactobacilli

Strain	Substance tested	Molar conc. of substance	Acid production in per cent of the mean of the controls	Lactate production in per cent of the mean of the controls
L. 4646	—	0	106	92
	—	0	94	108
	NH_4Cl	0.25	81	38
		0.33	92	145
		0.33	107	105
		0.50	98	79
		0.67	21	0
	NaCl	0.37	8	0
	Urea	0.76	88	88
		1.32	16	12

results were confirmed by the results of the lactate determinations.

Lactate production usually amounted to about 80 per cent of the total acid production in these experiments as well as in the ammonium ion experiments.

In one series of experiments the effect of ammonium ion and sodium ion was also tested. The results of these experiments are given in Table V. No stimulation of acid production by ammonium ion could be demonstrated in this series. The results indicate, however, that this suspension was very sensitive to the toxic effects of sodium chloride and urea. Acid production was not inhibited by 0.5 M of the ammonium salt, but 0.37 M of the sodium salt was sufficient to exert an almost complete inhibition.

DISCUSSION

The error of the titration method was determined from the controls. In many series of experiments it has been shown (*Frostell* 1959 a, 1959 b) that the acid or alkali production activity of micro-organisms may be determined with some degree of accuracy by the titration method. If, however, the effect of various substances in different concentrations is tested by this method there

will often be a great variation in results between experiments with different suspensions, the main reason probably being biological variation due to differences in the state of the organisms tested. The results of the experiments given in Table V, for example, indicate that the micro-organisms of this suspension are very sensitive to the toxic effects of all the substances tested. Thus the author believes that the fact that no stimulation of acid production by ammonium ion could be demonstrated in this series of experiments depends on an extreme sensitivity to the toxic effect of the same ion, which manifested itself with increasing strength at higher concentrations.

The results may be influenced by differences in the procedures leading up to the actual titration experiment, e.g. differences in the length of the time the organisms are exposed to the substance before the start of the experiment proper. This is especially true when concentrated solutions are tested. Variations in the results within the series performed with a certain suspension are believed to depend on such differences which are not entirely unavoidable. The result 61 % activity at 0.61 M belonging to the second series in Table I is puzzling. It may be that there is a balance between the toxic and the stimulating actions of the ammonium ion, and that this balance for unknown reasons sometimes may be displaced.

It must be kept in mind that when the titration method is used for determination of the influences of various substances on the activity of micro-organisms, the concentration of the substance will decrease during the experiment owing to the increase of volume caused by the addition of acid or base. This drawback might be avoided if the substance in question is added to the titration solution in the same concentration as in the reaction bottle. This, however, usually is unpractical, since it would be necessary to keep several different titrator solutions in the laboratory.

The author chose to keep the experimental period short in order to avoid a high dilution of the substance tested. For example, if at an initial concentration of 0.3 M the addition of base to the reaction bottle is 2.0 ml, the concentration of the substance will decrease to 0.25 M during the experiment provided the total volume was 10.0 ml at the start. These changes of the concentra-

tion are considered to be of minor importance when the activity is tested over a vast range of concentrations and when the biological variation is great.

Control experiments showed that the presence of 1 M of ammonium ion in pure glucose solutions did not influence the determination of the glucose concentration. However, in one of the series of experiments it was found that the colour of the tubes used for the spectrophotometric determination of the lactate content rapidly vanished in the samples containing large amounts of ammonia. For this reason one of the series of lactate determinations in suspensions of L 4646 was excluded because the values were much too low and scattered quite irregularly.

The results of the present study lend no support to the opinion that acid production by lactobacilli is inhibited in the presence of concentrations of ammonium ion or urea likely to occur in the oral cavity under normal physiological conditions. However, on the use of so-called ammoniated dentifrices high concentrations of those substances may occur in the human oral cavity.

The results of the study of the influence of the ammonium ion on the glycolysis of lactobacilli are of great interest when compared with the excellent study by *Pearlman* (1951). For obvious reasons it is impossible to illustrate the effect of a single ion on glycolysis in a single experiment since positively and negatively charged ions will always balance each other. The study by *Pearlman* in fact illustrates the effect of the phosphate ion balanced by ammonium ion or potassium ion. The effects of the positive ions are masked by the inhibition caused by the phosphate ion. However, *Pearlman*'s results indicate that the ammonium salt is less inhibitory than the potassium salt. At a concentration of about 0.3 M of each salt the activity of the ammoniated suspension is about 180 per cent of that of the suspension with potassium ion.

When the ammonium ion is balanced by the chloride ion, as in the present experiments, it is clearly seen that certain concentrations of the ammonium ion had a specific stimulating action on the acid production of the strains of lactobacilli used. Since only two strains of lactobacilli have been studied it is impossible to draw more general conclusions from this investigation concerning the action of the ammonium ion on the glycolysis of

other lactobacilli or other types of organisms. Further studies with other strains of lactobacilli and other organisms are necessary.

The effect of the phosphate ion on the glycolysis of lactobacilli has been studied by the titration method and results closely resembling those of *Pearlman* were obtained.

A stimulating action of the ammonium ion on the glycolysis and transphosphorylation processes in dialyzed extracts of macerated yeast cells was found by *Ohlmeyer & Ochoa* (1937) and by *Muntz* (1947). The stimulation, however, occurred at low concentrations of ammonium ion (0.0002—0.02 M).

SUMMARY

Acid production experiments were performed with two oral strains of lactobacilli according to the titration method elaborated by the author in order to study the possible effects of different concentrations of ammonium ion and urea upon the acid production from glucose. No effect of ammonium ion concentrations between 0 and about 0.2 M was found. Acid and lactate production were usually stimulated at concentrations between approximately 0.2 and 1.2 M. Concentrations of ammonium ion over 1.2 M were inhibitory. Urea concentrations less than 1.0 M were not significantly inhibitory to acid production or lactate production. At concentrations over 1.0 M inhibition occurred, but even at concentrations of urea of 3.3 M some activity was present.

RÉSUMÉ

ACTION DES IONS AMMONIUM ET DE L'URÉE SUR LA PRODUCTION D'ACIDE PAR LES BACILLES LACTIQUES

Pour étudier l'action possible de différentes concentrations d'ammonium et d'urée sur la production d'acide à partir du glucose par les bacilles lactiques, l'auteur, selon la méthode de titrage qu'il a mise au point, a fait des expériences de production d'acide, qu'il rapporte ici. Aucune influence de la concentration d'ion ammonium entre 0 et 0,2 M. Les productions d'acide et de lactate sont généralement stimulées par des concentrations comprises entre 0,2 et 1,2 M. Par contre, les concentrations d'ion ammonium supérieures à 1,2 M sont inhibitrices. Les con-

centrations d'urée inférieures à 1,2 M n'ont pas d'action inhibitrice significative sur la production d'acide ou de lactate. Au delà, il y a inhibition, mais même à une concentration d'urée de 3,3 M, il subsiste quelque activité.

ZUSAMMENFASSUNG

ÜBER DIE EINWIRKUNG VON AMMONIUMIONEN UND HARNSTOFF AUF DIE SÄUREPRODUKTION VON LAKTOBAZILLEN

Säureproduktionsversuche wurden nach der von dem Verfasser ausgearbeiteten Titrationsmethode mit zwei oralen Stämmen von Laktobazillen durchgeführt. Die Absicht war die Effekt von verschiedenen Konzentrationen von Ammoniumion oder Harnstoff auf die Säureproduktion und die Laktatproduktion dieser Organismen zu studieren. Ammoniumkoncentrationen zwischen 0 und 0,2 M hatten keine Einwirkung auf die Säureproduktion. Säureproduktion und Laktatproduktion wurden meistens von Ammoniumkonzentrationen von ungefähr 0,2 bis 1,2 M stimuliert. Höhere Konzentrationen hatten eine hemmende Einwirkung. Die Säureproduktion wurde meisten nicht durch Harnstoffkonzentrationen unter 1,0 M beeinflusst. Höhere Konzentrationen von Harnstoff übten eine Hemmung aus. Noch bei Konzentrationen von 3,3 M wurde eine gewisse Aktivität demonstriert.

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