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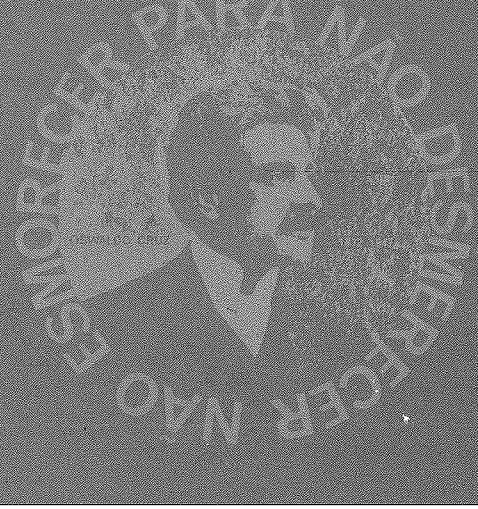

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Effect of drug resistance in growth and virulence of Shigella and invasive E. coli strains

Neusa Pereira S. Almada*
& Luiz R. Trabulsi*

Summary

The virulence and growth capacity of *Shigella flexneri* and invasive *E. coli* strains were studied before and after the development of drug resistance due to mutation or acquisition of R-factors. The presence of R-factors did not affect either virulence or growth exhibited by the strains studied. Chloramphenicol and kanamycin resistant mutants became avirulent and, in most cases, had their growth capacity impaired. Mutations to sulfadiazine resistance abolished virulence but were not related to alterations of growth. Nalidixic acid resistant mutants did not have their virulence or growth capacity affected.
Streptomycin resistant mutants, with one exception, showed attenuation or loss of virulence and increase in the length of lag phase.

Resumo

Efeito da resistência a drogas no crescimento e virulência de amostras de Shigella e E. coli invasor

Foram estudadas a virulência e capacidade de crescimento de amostras de *Shigella flexneri* e *E. coli* invasor antes e depois do desenvolvimento de resistência a drogas por mutação ou aquisição de fatores R. A presença de fatores R não teve efeito na virulência ou no crescimento das amostras estudadas. Mutantes resistentes a cloranfenicol e canamicina tornaram-se avirulentas e na maioria dos casos, apresentaram diminuição na capacidade de crescimento. Mutações para resistência a sulfadiazina aboliram a virulência das amostras mas sem apresentar relação com alterações de crescimento. Mutantes resistentes ao ácido nalidíxico não apresentaram alterações de virulência ou crescimento. Mutantes resistentes a estreptomicina, com uma exceção, mostraram atenuação ou perda de virulência e aumento na duração da fase lag.

Introduction

Drug resistance in bacteria is due to mutations or acquisition of resistance genes located on R-factors. Mutations are a wide spread mechanism and usually confer resistance to one drug, R-factors can bear resistance determinants to one or more drugs and have been described in Gram positive and Gram negative strains (6, 9). Since mutations can be followed by alterations in several bacterial properties (18) and the presence of R-factors could imply in an increase of the biossynthetic work involved in growth and cell division, the development of drug resistance could affect growth and virulence of pathogenic bacteria. These effects have been well observed with drug resistant mutants of *Mycobacterium tuberculosis* (5, 7) but the literature on other bacterial species is somewhat controversial (2, 4, 8, 11, 13, 15, 20, 23).

In this paper we describe the effect of drug resistance due to mutations or presence of R-factors in growth capacity and virulence of *Shigella flexneri* and invasive *Escherichia coli* strains.

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Material and Methods

Strains — *S. flexneri* 4a (151-66), *E. coli* O28a 28c, *E. coli* O136 and *E. coli* O144 were obtained from our bacterial collection. These strains were invasive according to Serény test and were sensitive to sulfadiazine, streptomycin, tetracycline, chloramphenicol, kanamycin and ampicillin. *S. flexneri* 4a (151-66) was selected because it is the only *Shigella* serotype which can utilize acetate as carbon source. The ability to grow on sodium acetate medium (21) was required for selection in mattings with other strains which could not utilize this carbon source. *S. flexneri* (O97-65), *S. flexneri* (294-70) and three strains of *E. coli* K12 (J53/N3; J53/RP4 and J53/R144) were also obtained from our collection and were choosed as R-factors donors since each one carries a different plasmid.

Mutants — Mutants were derived from *S. flexneri* 4a (151-66) and invasive *E. coli* serogroups O28a 28c, O136 and O144. Selection of mutants was done by the method described by Bryson & Szybalski (3).

R-factor bearing strains — *S. flexneri* 4a (151-66) was used as recipient strain in all matting experiments. Two strains of *S. flexneri* (O97-65 and 294-70) and three strains of *E. coli* K12 (J53/N3; J53/RP4 and J53/R144) were used as R-factor donors. Five transconjugants were obtained, each one carrying a different R-factor (Table 1). Selection was done in sodium acetate medium, with the appropriate drugs for each case.

Growth capacity — It was evaluated carrying out growth curves of all transconjugants, mutants and original strains in Brain Heart Infusion Broth — BHI (Difco), at 37°C, with aeration. Growth was measured in a nephelometer (Evans Electroselenium Ltd) for as long as 10 hours. The curves were iniciated adding 0.01ml of an overnight BHI culture to 10ml of the same medium.

Virulence test — The Serény test (17) was used to check virulence of the strains and the procedure was the one described by Trabulsi (22). Each strain was inoculated in three guinea pig eyes and the inoculum consisted of 0.1ml of a saline suspension of about 10^9 bacteria/ml. The guinea pigs were observed up to one week after the inoculation, and material was collected from the inoculated eye everyday during this period. When bacteria were recovered from the eye they were identified and confirmed to be the one that was inoculated.

Results

Growth capacity — When the several resistant mutants were compared to the original strains, some differences in growth capacity were observed, depending upon the drug to which the mutants were resistant.

Streptomycin resistant mutants showed an increase in the length of lag phase and sometimes an increase also in generation time. Nalidixic acid resistant mutants presented no differences in growth capacity when compared to the original strain. In four out of six mutants, de-

Table 1 — Resistance patterns of *S. flexneri* 4a (151-66) and the transconjugants obtained

Strain	Drugs					
	Su	Sm	Tc	Cm	Km	Ap
S. flexneri 4a (151-66)	10	5	1	1	1	1
S. flexneri 4a (151-66)/Ra	1000	200	200	200	1	1
S. flexneri 4a (151-66)/Rb	1000	200	200	200	1	1
S. flexneri 4a (151-66)N3	1000	200	100	1	1	1
S. flexneri 4a (151-66)RP4	10	5	100	1	200	1000
S. flexneri 4a (151-66)R144	10	5	10	1	500	1

Ra — R factor from *S. flexneri* (294-70)
Rb — R factor from *S. flexneri* (097-65)
N3 — R factor codding for resistance to Su, Sm, Tc belonging to group N of compatibility
RP4 — R factor codding for resistance to Tc, Km, Ap belonging to group P of compatibility
R144 — R factor codding for resistance to Tc and Km belonging to group Iα of compatibility
Su — sulfadiazine
Sm — streptomycin
Tc — tetracycline
Cm — chloramphenicol
Km — kanamycin
Ap — ampicillin

velopment of kanamycin resistance was followed by increase in length of lag phase and generation time. One mutant showed just an increase of lag phase and the other one no alteration in growth capacity. The chloramphenicol resistant mutant had its growth capacity markedly reduced, with a large increase in both generation time and length of lag phase. Four out of the six sulfadiazine resistant mutants showed increase in length of lag phase but the other two had no alteration in growth capacity.

All transconjugants showed no alteration in growth capacity, with generation time and length of lag phase identicals to the original strain.

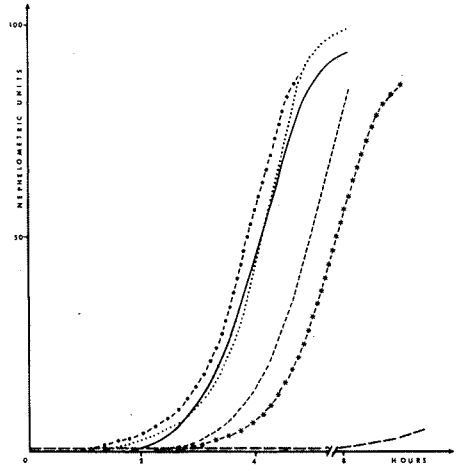
Fig. 1 shows growth curves of *S. flexneri* 4a (151-66) and some of its derived strains.

Virulence — All the original strains *E. coli* O28a 28c, *E. coli* O136, *E. coli* O144 and *S. flexneri* 4a (151-66) were virulent according to Serény test, causing kerato-conjunctivitis in the guinea pig eye.

Shigella flexneri mutants resistant to chloramphenicol and kanamycin lost their virulence. Streptomycin resistant mutants obtained from the same strain showed, with one exception, attenuation or loss of virulence. All but one nalidixic acid resistant mutants derived from *S. flexneri* kept the virulence unaffected.

All drug resistant mutants obtained from invasive *E. coli* strains became avirulent.

Figure 1 — Growth curves of *S. flexneri* 4a (151-66) and some of its derived strains



——————— original strain

. . . . . transconjugant bearing a plasmid from strain 294-70

–o–o–o–o–o– mutant resistent to 100μg/ml of nalidixic acid

– – – – – – – mutant resistent to 10μg/ml of kanamycin

— — — — mutant resistent to 20μg/ml of chloramphenicol

— *–*–* — mutant resistent to 50μg/ml of streptomycin

All transconjugants obtained from *S. flexneri* kept their virulence unaffected.

Table 2 summarizes the results of Serény test of all drug resistant mutants.

Table 2 — Resistance level and virulence of the mutants obtained

Original strain	Drug	Resistance μg/ml	N.º of mutants	Virulence
S. flexneri 4a (151-66)	Streptomycin	1000	1	lost
		500	1	lost
		50	1	attenuated
		20	1	lost
		20	1	unaffected
	Chloramphenicol	20	1	lost
	Kanamycin	20	1	lost
		10	1	lost
	Nalidixic acid	100	1	lost
		100	1	unaffected
		20	3	unaffected
E. coli O28a 28c	Kanamycin	20	2	lost
E. coli O136	Sulfadiazine	1000	2	lost
	Streptomycin	1000	3	lost
E. coli O144	Sulfadiazine	1000	2	lost
		200	2	lost
	Kanamycin	10	1	lost

Discussion

Growth capacity and drug resistance — Growth capacity of *S. flexneri* 4a (151-66) was not affected by introduction of different R-factors, but the development of drug resistance due to mutation was followed, in most cases by an impairment of growth capacity, with exception of nalidixic acid resistant mutants which showed no alterations of growth. Our observations on the effect of R-factors in growth capacity are in agreement with the report of Watanabe (24) with *E. coli* strains, where the presence of R-factors did not affect growth and with the observation of Watanabe (23) that R-factors could be introduced in *Salmonella typhimurium* strains without alterations of growth.

The relationship between drug resistance mutation and difficulty of growth that we observed with drugs such as chloramphenicol, kanamycin and streptomycin was also refered by several authors. Watanabe (24) observed that mutants resistant to kanamycin, tetracycline, chloramphenicol and streptomycin grew slower that the original strains, but resistance to sulfadiazine and nalidixic acid had no effect on growth. Several authors (1, 4, 7, 11, 13, 16) reported that mutations to drug resistance caused an impairment of growth capacity in strains of *E. coli, Salmonella, Mycobacterium tuberculosis, Streptococcus* and *Staphylococcus aureus*, showing that this effect can be observed in a wide range of organisms. There are however some papers reporting that penicillinase producing *Staphylococcus* strains showed a better ability to grow on synthetic and semisynthetic media than the non penicillinase producing (sensitive) strains (2, 8).

Virulence and resistance — Virulence of *S. flexneri* was not affected by introduction of R-factors, but development of drug resistance due to mutation caused attenuation or loss of virulence in most cases except with nalidixic acid resistant mutants which kept the original virulence unaffected. Invasive *E. coli* strains lost virulence after mutation to drug resistance. The experimental method used to test virulence was the development of kerato-conjuctival infection of guinea pig (Serény test) which is based upon the ability of the strain to invade and multiply in the epithelial cells of cornea and conjunctiva. Since these strains have to invade epithelial cells of intestinal mucosa to cause disease, the assay to check virulence is directly related to the real model of pathogenicity.

Osada & col. (14) studied the virulence of rifampicin resistant mutants of *Shigella* and enteropathogenic *E. coli* using three different methods to test their ability to cause disease: infection of L-cells, kerato-conjunctival infection of guinea pig and lethal toxicity to mice. They observed that in vitro resistant mutants partially or entirelly lacked the ability to penetrate L-cells or produce kerato-conjunctivitis. These observations corroborate our results with the Serény test for virulence of drug resistant mutants. On the other hand, the same authors observed that mutants obtained in vivo were invasive to L-cells although they had lost the ability to cause kerato-conjunctivitis and that lethal toxicity in mice could not be correlated with the other two assays. With respect to presence of R-factors and virulence the literature shows some controverse. Our findings that R-factors did not interfere with virulence are in agreement with the work of Parant & col. (15) with *Klebsiella pneumoniae* and Watanabe (23) with *Salmonella typhimurium*. Other authors report loss or attenuation of virulence after introduction of R-factors in strains of *Salmonella* (10, 19, 20) and *S. aureus* (12).

One factor that should not be neglected and could be involved in the explanation of the different results obtained is how the test used to check virulence of the strains was performed and the different species that were used.

Growth capacity and virulence — According to our data, the acquisition of R-factors had no effect on virulence or growth capacity. However, mutation to drug resistance was followed, in several cases, by decrease of growth rate and virulence. This fact was more evident with chloramphenicol, kanamycin and streptomycin resistant mutants. Nalidixic acid resistant mutants were not affected in their growth capacity or virulence. These results could explain why mutants resistant to some drugs are hardly ever isolated from clinical materials and reinforces the fact that R-factors constitute the major problem in drug therapy, since its presence does not affect bacterial survival in the absence of drugs and contributes to positive selection of resistant bacteria in the presence of appropriate drugs

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Occurrence of group A and nongroup A beta-hemolytic streptococci in human infections in Rio de Janeiro

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Summary

One hundred and sixty one strains of beta-hemolytic streptococci were isolated from patients with suspected streptococcal infection or complications in Rio de Janeiro and classified into Lancefield groups. Group A strains were more than twice as prevalent (69.5%) as strains of groups B, C, F and G (28,6%). Beta-hemolytic streptococci were present as frequently in the throat and nose as in skin lesions and simultaneous carriage was observed in 17 pyoderma patients. The prevalence rate for group A streptococci was 68.8% in skin lesions. Nongroup A strains were predominant over group A streptococci in throat and the prevalence rate for group G streptococci in the throat (6.7%) was similar to that for group A (6.9%). This suggests that the group G strains might be as pathogenic as the group A strains.

Resumo

Ocorrência de estreptococos beta hemolíticos do grupo A e de outros grupos sorológicos em infecções humanas no Rio de Janeiro

Cento e sessenta e uma amostras de estreptococos beta hemolítico foram isoladas de pacientes, com suspeita de infecções estreptocócicas ou de suas complicações, na área urbana da cidade do Rio de Janeiro e classificadas nos grupos sorológicos de Lancefield. As amostras do grupo A apresentaram uma prevalência (69,5%) superior ao dobro daquela dos grupos B, C, F e G (28,6%). Os estreptococos beta hemolíticos foram encontrados na garganta e no nariz com uma freqüência semelhante à verificada nas lesões de pele e a presença simultânea de dois grupos sorológicos foi observada em 17 pacientes com piodermite. A taxa de prevalência dos estreptococos do grupo A alcançou 68,8%, em lesões de pele. Na garganta, as amostras dos outros grupos sorológicos predominaram sobre as do grupo A e a taxa de prevalência para os estreptococos do grupo G (6,7%) foi semelhante àquela do grupo A (6,9%). Este fato sugere que as amostras do grupo G podem ser tão patogênicas quanto às estirpes do grupo A.

Introduction

Lancefield groups A, B, C and G of beta-hemolytic streptococci are frequently implicated in human disease and group A streptococcus is the most important as the agent responsible for most of the streptococcal infections (26). It is this organism that is commonly isolated from the pharynx and skin lesions of patients with bacterial pharyngitis and pyoderma respectively. Both acute rheumatic fever and glomerulonephritis can occur following group A streptococcal infections of the upper respiratory tract (24) and streptococcal impetigo associated with acute glomerulonephritis occurs with considerable frequency (24).

One of the most striking changes in the epidemiology of rheumatic fever and rheumatic

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heart disease is their emergence as a major problem in developing tropical and semi-tropical countries (20). Long considered a disease of temperate climates, rheumatic fever has, in recent years, been increasingly recognized as one of the leading causes of disabling heart disease in many developing countries in the tropics (12). With this shift in prevalence (or recognized prevalence) from colder to warmer countries, rheumatic fever and rheumatic heart disease constitute a significant form of heart disease in children and adults in these countries (1, 11) and have now joined the ranks of the classical tropical diseases. Acute nephritis occurring as repeated large epidemics in relationship to skin infections, has also been reported in tropical countries where impetigo is a common disease year around (13). Thus both of the delayed nonsuppurative complications of group A streptococcal infections abound in developing tropical countries, posing serious health problems.

Clinical reports on the occurrence of rheumatic fever and rheumatic heart disease in Brazil are numerous and suggest a high incidence and prevalence of these complications of streptococcal infections (14, 16, 21). Acute nephritis is still a problem of great magnitude in Brazil (7, 22). Although these reports of serious complications suggest that streptococcal infections occur with high frequency and are among the diseases of foremost importance in Brazil (4), the information available to date is limited both epidemiologically and bacteriologically. In their pionneer studies of the occurrence of streptococci among school children, Solé-Vernin & Castro (18) demonstrated the importance of various serological groups of beta-hemolytic streptococci in the infections of the upper respiratory tract and of the skin.

Identification of human strains of beta-hemolytic streptococci and screening for group A strains is therefore of great value in management of patients and studies of the epidemiology of nonsuppurative complications of streptococcal infections. With this in the back of our mind, we started to develop a laboratory and a program of microbiological research on streptococci and streptococcal diseases in the Institute of Microbiology of the Federal University of Rio de Janeiro. We report here the laboratory identifications and origins of beta-hemolytic streptococci isolated from children and adults with suspected streptococcal infections or complications. The children were seen at the Infectious Disease Section of a municipal hospital and at the cardiologic and dermatologic outpatient clinics of the Department of Pediatrics, Federal University of Rio de Janeiro. The remaining subjects were adults who presented cutaneous lesions or complained of a sore throat. The populations of patients belonged to the northern area of the city of Rio de Janeiro.

Material and Methods

Clinical material — Strains of beta-hemolytic streptococci were collected from September 1978 until September 1979. Pediatric patients were selected by the clinical collaborators on the basis of symptoms and signs compatible with a diagnosis of streptococcal infection or complications. Epidemiological features included scarlet fever, streptococcal pharyngitis and documented cases of rheumatic fever, streptococcal impetigo and glomerulonephritis. Three hundred and six children met these criteria. One hundred and twenty seven adults with suspected streptococcal infection were also included in the study. Cultures were obtained from each individual of throat and nose and skin lesions (if present). Every individual was cultured only once. Four vaginal specimens were obtained at 2-month intervals from a nonpregrant woman with vaginitis and one culture of the cerebrospinal fluid of a child with acute meningitis was processed.

Bacteriologic studies — Plates containing tryptose blood agar base (Difco Laboratories, Detroit, Michigan, USA) and 5% sheep blood were inoculated by swabbing and streaking as recommended by Wannamaker (23). The plates were examined after incubation for 18hr at 35°C and hemolytic activity was noted as beta. Single (three) colonies of beta-hemolytic streptococci were picked from each positive plate and grown for 18hr at 35°C in 4ml of Todd-Hewith broth (Difco) containing 5% sheep blood. Plates were routinely examined after incubation for 48hr. Two of the 3 subcultures were tested for bacitracin sensitivity for the presumptive identification of group A streptococci by using the disk technique (8) and commercial disks (BBL, Division of Beckton, Dickinson and Company, Cockeysville, Maryland, USA). Any zone of inhibition of growth extending beyond the edge of the disk was considered positive as suggested by Maxted (8). If only one of the streptococcal isolates from each positive cultu-

re was sensitive to bacitracin, both isolates were grouped serologically.

At a later stage of the survey, streptococcal strains were isolated as follows: three beta-hemolytic colonies were picked from the positive plate, subcultured in the same blood-broth tube and serologically grouped by testing the acid extract against the six specific grouping antisera to ascertain that the 3 colonies were from the same Lancefield group. From this time onwards this method was used to isolate streptococci.

Serological grouping of streptococci — Grouping of the 161 streptococci isolates was carried out according to the nitrous acid procedure described by El Kholy & col. (3) and the streptococci used for the preparation of nitrous acid extracts were from 5ml broth cultures or from the growth of one-quarter of the plate used for the bacitracin sensitivity test.* Nitrous acid extracts were prepared directly from the growth of the primary plate if enough beta-hemolytic colonies were present in the culture examined and if overgrowth by nonstreptococcal bacteria did not occur*. Of the 161 strains, 111 were also grouped by the method of Lancefield (5).

Streptococcal grouping antisera were purchased from Difco (groups A, B, C, D, F and G) or produced in our laboratories (groups A, B, C and G) in Rio de Janeiro and the precipitin reactions were carried out in capillary tubes according to the technique originally described by Lancefield (5).

Results

There was a greater recovery of beta-hemolytic streptococci from specimens received during the summer months and from specimens collected from the pediatric patients. The recovery of group A strains demonstrated seasonal variation with the greatest number isolated during the summer months. Recovery of nongroup A beta-hemolytic isolates however did not demonstrate seasonal variation. There were 158 males and 148 females in the children group and 34 males and 93 females in the adult group. The age and sex distribution of individuals with beta-hemolytic streptococcus in these groups are shown in Table 1.

Streptococcal groups — Altogether 306 children between the ages of 2 months and 14 years and 127 adults with pyoderma or suspected streptococcal infection of the upper respiratory tract and complicatins were investigated during the one-year period of this report. The distribution of streptococcal strains belonging to the individual groups of hemolytic streptococci were studied. One hundred and sixty one strains of beta-hemolytic streptococci were isolated. Serological grouping was successful in 158 (98%) of the streptococcal isolates. Of the 161 strains, 112 were group A, 32 group G, 10 group B, 3 group C and 1 group F (Table 2). Three strains were from other groups or nongroupable as they failed to show positive precipitin reactions despite repeated attempts with freshly prepared extracts and extraction of the C-polysaccharide with either hot-hydrochloric acid (5) or nitrous acid (3).

Clinical sources of streptococci — Streptococcal isolates were identified by laboratory and medical records with regards to patient and body site cultured for all individuals investigated. The sites of isolation of the streptococcal strains and the corresponding distribution of serogroups are summarized in Table 2. Group A streptococci were isolated from the skin lesions of 75(68,8%) of the 109 patients with impetigo. Simultaneous carriage was found in 17 pyoderma patients. These 17 individuals were carrying a group A strain in the skin lesion and a group A (8 patients), a group G (8 patients) or a group B (one patient) in the throat. Four of the isolates of group B streptococci were from the female genital cultures and two group B isolates were from cutaneous lesions. The prevalence rate for group G streptococci (6.7%) in the throat was similar to that for group A (6.9%). One patient was carrying a group A strain and a group G strain in an impetigenous lesion and one group A strain was isolated from the cerebrospinal fluid of a patient with acute meningitis. Beta-hemolytic streptococci (group A) were isolated from the throat of only one pyoderma patient when skin cultures were negative.

* Figueiredo, A.M.S.; Benchetrit, L.C.; Borges-Neto, A.A. & Oliveira, C.M. — Evaluation of a new method for grouping beta-hemolytic streptococci. Rev. Bras. Pesq. Med. Biol. (in press).

Table 1 — Age and sex distribution of individuals with beta-hemolytic streptococcus in the two study groups

Study group	Nº of males	Nº of females	Nº of individuals cultured	Age range (year)				
				0-5	6-14	15-20	21-35	35-50
Children	158	148	306	57(18.6)*	51(16.7)	0	0	0
Adults	34	93	127	0	0	6(4.7)	13(10.2)	8(6.3)
Total	192	241	433					

* Number of individuals with beta-hemolytic streptococcus (per cent)

Table 2 — Beta-hemolytic streptococci recovered from various body sites and their sensitivity to bacitracin*

Serological groups	Body sites					Total	Number of strains sensitive to bacitracin
	Skin Lesions	Throat	Nose	Vagina	CSF**		
A	75***	30	6	0	1	112(69.5)****	112
B	2	4	0	4	0	10(6.2)	0
C	1	2	0	0	0	3(1.9)	0
F	0	1	0	0	0	1(0.6)	0
G	1	29	2	0	0	32(19.9)	1
NG*****	0	3	0	0	0	3(1.9)	1
Total	79	69	8	4	1	161(100)	114

* In all, 433 patients here examined
** CFS = Cerebrospinal fluid
*** Number of strains
**** Number of strains (per cent)
***** NG = Nongroupable

Discussion

A review of the literature suggests that beta-hemolytic streptococci are relatively common pathogenic organisms in Brazil. A survey of carrier rates and antistreptolysin O titers among urban (Rio de Janeiro) school children (2, 6) was interpreted to indicate that 236,000 children between 6 and 12 years of age had streptococcal infection at the time the study was undertaken (15). In a similar and in-depth survey among rural school children of Ribeirão-Preto, 23% were found to harbor group A streptococci on throat culture, 9% group C and 8% group G (17). In a survey of university students in Fortaleza, Noronha & col. (9) found throat carrier rates of 11%, 9% and 2% for group A, B and C respectively.

We document here the occurrence of various serogroups of beta-hemolytic streptococci in pediatric and adult populations as part of an epidemiological survey of streptococcal infections in the urban area of Rio de Janeiro. The use of serological grouping on a routine basis for the identification of the streptococcal strains isolated during the period of study was found to be reproducible and convenient. One of the 32 group G strains and one of the nongroupable strains would have been identified as group A streptococci if bacitracin sensitivity had been the sole criterion. The prevalence for group G streptococci in the throat (6.7%) was similar to that for group A (6.9%) and suggests that group G strains migh be as pathogenic as group A strains. It is noteworthy that nongroup A streptococci (39 strains) predominated over group A (30 strains) in throat. This conclusion is in accord with previous observations of Castro (2) and Ogumbi (10) in Brazil and Nigeria respectively.

It seems apparent from this prospective study and from the literature that more studies need to be carried out to understand the epidemiology of streptococcal infections in Brazil. Any streptococcus isolated should be completely identified according to its Lancefield serologic group. Work is in progress to determine the current prevalent serologic types of groups B streptococci and T-types of group A strains isolated during this study.

Acknowledgments

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Thermal degradation products of sugars in alkaline pH.

I — Lethal effect of glucose-phosphate solutions on Escherichia coli

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Summary

Solutions of reducing sugars, sterilized by heat in the presence of alkaline phosphates, produce a lethal effect upon *Escherichia coli* cells. With the concentration used, this toxic effect is enhanced by previous UV-irradiation and the amplitude of the lethal effect is similar upon bacterial populations ranging from 10^8 to 10^4 cells per milliliter. The toxic substances developed within the autoclaved solution seem to be aldehyde-like, and their activity is oxigen-dependent. Previous incubation at 37°C, with aeration, alters the toxic solution activity and the amplitude of the toxic effect is temperature dependent.

Resumo

Produtos de termo-degradação de açúcares em pH alcalino. I — Efeito letal de soluções de glicose-fosfato em Escherichia coli

Soluções de açúcares redutores, esterilizadas a quente em pH alcalino, produzem efeito letal sobre células de *Escherichia coli*. Na concentração estudada, este efeito tóxico é aumentado por prévia irradiação com ultravioleta e sua amplitude é semelhante em populações bacterianas variando de 10^8 a 10^4 células por mililitro. As substâncias tóxicas que surgem na solução autoclavada parecem ser de natureza aldeídica e sua atividade dependente de oxigênio. A incubação prévia a 37°C, com aeração, altera a atividade da solução tóxica e a amplitude do efeito é dependente da temperatura de incubação.

Introduction

The heat sterilization of reducing sugars, in the presence of phosphates in alkaline pH, produces one or more substances that block bacterial multiplication (8). In concentrated alkalis, sugars caramelize and produce several decomposition products, as well as yellow and brown pigments (13). Baumgartner (6) reported that the pigments formed during degradation of glucose are not responsible for the toxic effect. Probably, the extensive isomerization and changes in location of the double bonds in glucose molecules are related to the production of toxic substance(s) (13).

A similar effect is obtained whenever agar is autoclaved in presence of phosphates in alkaline pH (9), leading to a "dark medium" (1).

It seems probable that aldehydes found in solutions of glucose autoclaved in alkaline pH are the main toxic products influencing the surviving fraction of bacteria. Amongst many of the identified and tested ones, reductone (enol-tartronic aldehyde) is the one that reproduces the lethal effect of autoclaved solutions of saccharides (agar or reducing sugars) in alkaline pH. The lethal effect of reductone, sugar-phosphates and agar-phosphates is equally inhibited by catalase activity (7).

In the presence of these substances, strong additional effects upon UV-irradiated repair-proficient *Escherichia coli* cell strains is observed (2).

This paper describes some aspects of the toxic effect of thermal degradation products of glucose in alkaline pH as well as the conditions

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for the effectiveness of these products in inactivating bacterial *Escherichia coli* cells.

Material and Methods

Bacterial strain — *Escherichia coli* K12S was used in all experiments, kindly supplied by Institute of Radium, Paris.

Culture media and growth — Cells were grown overnight in a shaking incubator (model R76, New Brunswick, N.J., USA) at 37°C, in M9 medium (3). A starting inoculum was then taken and diluted also in M9 medium to about 10^7 cells per ml and grown until mid-exponential phase (1-2 × 10^8cells/ml), followed by turbidimetric measurements. The cells were collected on a Millipore filter (0.45μm) pore size, Millipore Filter Corporation, USA), and resuspended in M9 salts buffer. For colony counting, samples were properly diluted in 0.15M NaCl solution and spread on the surface of nutrient agar plates, these being incubated a 37°C, at least during 16 hours.

Ultraviolet irradiation — UV-irradiation and dosimetry has been carried out as previously described (4). Cells were exposed to a UV dose enough to reduce to 10% the number of viable cells in culture (LD_{10}).

Preparation of glucose-phosphate toxic solution — The autoclavation, for 30 minutes, at 37°C of a solution containing 20% of D(+) glucose and 10% of dissodic phosphate leads to the production of the so-called glucose-phosphate toxic solution (GPT) (1). The solution was used immediately after reaching room temperature, about five minutes after autoclavation.

A control solution (GPC) was obtained by mixing two concentrated parts of the same components, autoclaved separately. In all experimental procedures, GPT and GPC were conveniently diluted for bacterial treatments. Potentiometric assays showed that the addition of either GPT or GPC was suitably buffered by M9 salts solution, the pH remaining unchanged.

Inactivation kinetics — UV-irradiated and unirradiated bacterial cells, in M9 salts buffer, were incubated with GPT solution and assayed for additional lethal effect by titulation of cell viability at different periods. Controls were performed with GPC solution.

Similar experiments were performed employing different aeration conditions and various incubation temperatures.

An analysis of GPT activity was done either by using pre-treated GPT (GPT pre-treated at 37°C for 0, 10, 20, 30, 40 and 60 minutes) or reutilization of an already incubated one, for 60 minutes, with bacterial suspension.

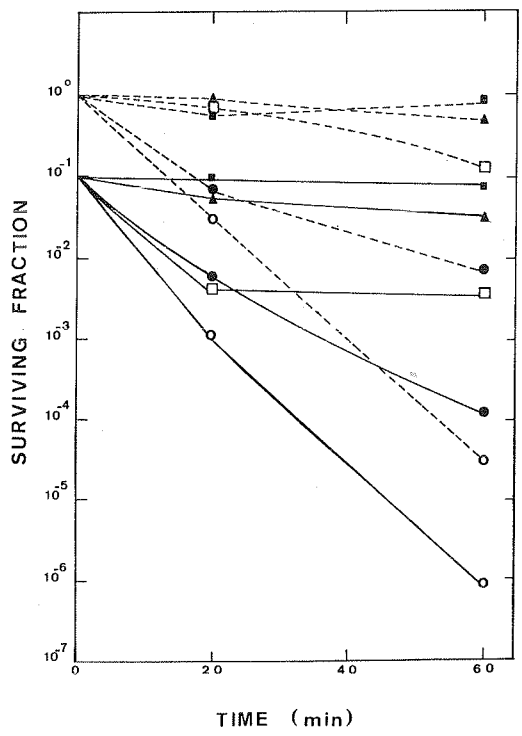
Effect of GPT upon different concentrations of viable cells was assayed by incubation of diluted pre-irradiated bacterial cultures.

Drugs — Culture media components were obtained from E. Merck Darmstadt and Difco Laboratories. D(+) glucose was from Reagen Ltda. and dissodic phosphate from E. Merck Darmstadt.

Results

Influence of different GPT concentrations upon bacterial survival — Inactivation rates by GPT are negligeable beyond 10^{-3} dilutions from the standard solution; GPT toxicity increases the greater the concentration utilized (Fig. 1) while

Figure 1 — Effect of different GPT concentrations upon *E. coli*

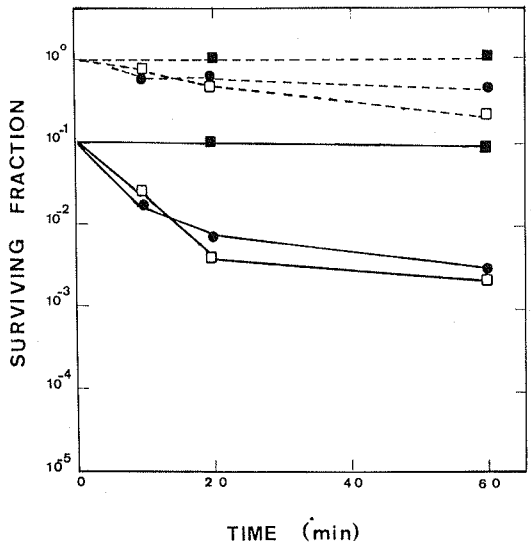


E. coli K12S cells grown in minimal medium until 1 to 2×10^8 cells/ml were filtered and resuspended in M9 salts buffer. Parts of the culture were submitted to the following treatments:
(—) UV-irradiation (LD_{10}) and incubation, in a water bath shaker, at 37°C, with GPT at the final dilutions
(°) undiluted; (•) 5×10^{-1}; (□) 1×10^{-1}; (▲) 1×10^{-2}; (■) 1×10^{-3} (--) Unirradiated cells treated in the same conditions listed above

GPC was actionless in all tested concentration ranges (results not shown).

Influence of different methods of incubation upon the killing effect of GPT — The intensity of GPT lethal effect in three aeration conditions is shown in Figure 2; the degree of aeration is important to the toxicity of GPT solution. Similar results (not shown) were obtained by varying the speed of shaking.

Figure 2 — GPT effect on *E. coli* under different aeration conditions



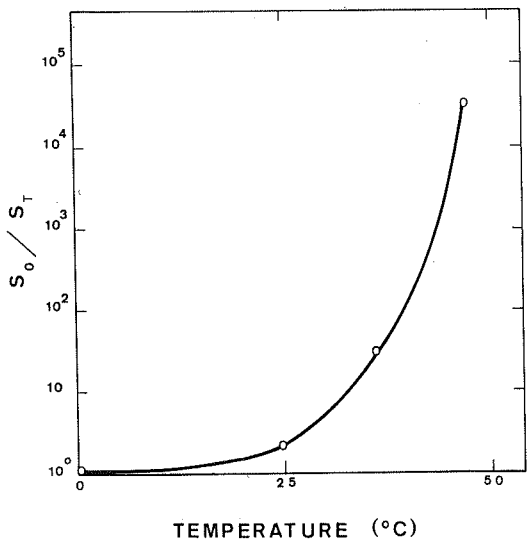
E. coli K12S cells, grown in minimal medium until exponential phase, were filtered, resuspended in M9 salts buffer and UV-irradiated with LD_{10}. A constant volume of 2ml from this culture was treated with GPT in: (■) small tubes (105mm × 15mm); (□) large tubes (200mm × 25mm), with aeration; (•) Erlenmeyer flasks (50ml of capacity) with shaking. Controls with unirradiated cells were performed (data not shown).

GPT lethal effect is temperature-dependent, being negligible at 0°C and increased until 48°C, the highest employed temperature (Fig. 3).

Loss of GPT toxicity — In all experimental conditions, when an additional lethal effect of GPT is observed, the shape of inactivation kinetics curves is similar. Inactivation rate is constant until 20 or 30 minutes of incubation decreasing in longer treatments, the inactivation reaching a plateau at about 60 minutes of incubation, and remained constant at least until 120 minutes (results not shown).

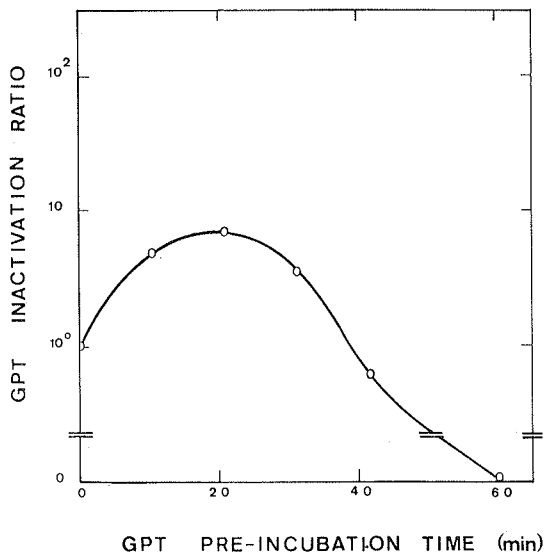
GPT solution activity is markedly modified by previous incubation, with shaking, in Erlenmeyer flasks at 37°C, from 0 to 60 minutes (Fig. 4).

Figure 3 — Inactivation efficiency of GPT submitted to different incubation temperatures



Cultures of *E. coli* K12S, grown in minimal medium and UV-irradiated with LD_{10} in M9 salts buffer were treated with GPT under the following incubation temperatures: 0°, 25°, 37° and 48°C. The inactivation kinetics were held as described in Material and Methods. The relations S_0/S_T were obtained considering S_0 the survival fraction after 60 minutes incubation at 0°C, and S_T the survival fraction after incubation at each tested temperature, during the same period.

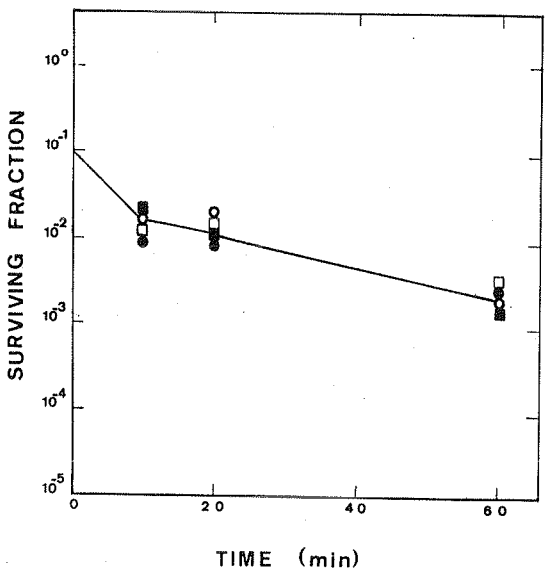
Figure 4 — Inactivation efficiency of GPT pre-treated at 37°C



E. coli K12S cells, grown in minimal medium, filtered and resuspended in M9 salts buffer were UV-irradiated and treated with GPT solutions that had already been incubated for different periods. The inactivation ratios were obtained by relation between the observed lethal effect after 60 minutes incubation with a recently autoclaved GPT and the observed one when GPT was previously incubated during the tested periods.

Relationship between GPT effectiveness and concentration of bacterial cultures — Inactivation kinetics of cell populations, in different bacterial concentrations, ranging from 10^8 to 10^4 cells/ml are similar, as shown in Figure 5. For the same GPT concentration, inactivation kinetic is not dependent on the total number of cells, i.e., inactivation rates are the same, regardless of the ratio between the number of toxic substance molecules (not known) and the total number of cells incubated. This can be verified by calculating the ratio between the mass of the solute in GPT and the total number of incubated cells, from the results in Figure 5.

Figure 5 — Effect of GPT on different bacterial concentrations



E. coli K12S cells, grown till mid-exponential phase, filtered and resuspended in M9 salts buffer were irradiated with LD_{10} and suitably diluted until (□) 10^7 cells/ml; (■) 10^6 cells/ml; (°) 10^5 cells/ml and (•) 10^4 cells/ml and then incubated at 37°C; inactivation kinetics being performed during 60 minutes.

Discussion

The conditions for GPT utilization were initially established; ten times diluted GPT has been employed throughout all experiments. This concentration was chosen on the basis of the differential effect obtained upon UV-irradiated cells after 20 minutes of incubation (for comparison, see Figure 1).

In the experimental procedures described in this paper, the main stable products of glucose degradation would be aldehydes and enediol structures. Aldehydes and similar compounds act upon cellular components as producers of genetic damage. Dicarbonyl compounds interact with nucleic acids or their components, possibly by two successive reactions, leading to damages that can be repaired by excision enzymes (5).

Reductone, one of the aldehydes in GPT solution, acts at the molecular level blocking macromolecular synthesis, cellular respiration and causing DNA damage (10).

The lethal activity of GPT is dependent upon the enchancement of medium aeration, which is brought about by the free area exposed to atmospheric air and also by the intensity of shaking. This strongly suggests that the lethal effect of GPT is linked to an activation by O_2 or even the development of at least a toxic substance within the incubation tubes, catalized by O_2. It has long been shown that, under several experimental conditions, when oxidizing molecules are available, aldehydes can form reactive hydroxi-alkyl peroxides and/or free radicals (5). If we already have peroxide radicals in water solutions, in air or O_2 saturated solutions of nucleic acid derivatives, H_2O_2 will be produced in addition to any organic hydroperoxide formed by the reaction of O_2 with solute radicals (12). Superoxide ions (O_2^-) play an important role in the development of peroxide and hydroxil radicals, through a chain of oxidation-reduction reactions (11).

The relevance of O_2 to GPT toxic effect is clear from the results shown in Figure 2.

It can be suggested that the substances developed into GPT behave as peroxides or as peroxide inducers in intracellular medium.

There are several ways of explaining this phenomenon, the first being that it is caused by interference in oxidation-reduction processes. Alternatively, the substance(s) itself could suffer oxidation, depending on O_2 levels, at last losing its activity by total oxidation.

These conclusions are reinforced by the analysis of results shown in Figure 4. From zero until 20 minutes of incubation, with shaking, we can observe an increase in effectiveness, which later decreases, becoming null at 60 minutes; this strengthens the idea of an activation (or generation) of a toxic substance. The loss of activity of GPT after 60 minutes of incubation is independent of the presence of bacterial cells, which suggests that molecular reactions occur within GPT solutions (results not shown).

The effect of GPT is modified by the temperature of incubation, being greater the higher

the temperature level. The effect could be justified by different rates of GPT activation or DNA (or other macromolecules) induced damage at each tested temperature, or by the heat interference in DNA recovery processes, or by more than one phenomena (14). The highest assayed temperature has been 48°C, which in itself causes little lethal effect upon untreated bacteria. Heating the preparation above 48°C would raise the lethal potential of any induced damage (14).

The action of GPT on cells at different concentrations shows that, in the chosen dilution, the same inactivation kinetic is obtained with different ratios between GPT and total number of cells (Fig. 5). It seems that a small unknown number of molecules is able to cause a toxic effect which is enough to act upon bacterial concentrations ranging from 10^8 to 10^4cells/ml.

Acknowledgment

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Fungos toxicogênicos associados a cereais. 1 — Levantamento da micoflora associada a milho, trigo e arroz

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Resumo

O presente trabalho teve por objetivo um levantamento qualitativo de fungos associados a milho, trigo e arroz, por meio de amostragens procedentes de várias regiões do Brasil. Nesse levantamento foram mais frequentemente isoladas espécies dos generos *Fusarium, Aspergillus* e *Diplodia* e, com menor freqüência espécies dos gêneros *Penicillium, Cephalosporium, Curvularia, Trichoderma* e *Mycelia sterilia.*

Summary

Toxigenic fungi associated to grains. 1 — Survey of micoflore associated to corn, wheat and rice

The present paper describes a qualitative survey of fungi associated with corn, wheat and rice, based on samples which were collected from different regions in Brazil. During the survey of fungi associated with cereals the majority were of the genera *Fusarium, Aspergillus* and *Diplodia* and a smaller percentage belonged to the genera *Penicillium, Chephalosporium, Curvularia, Thichoderma,* and *Mycelia sterilia.*

Introdução

Desde a mais remota antiguidade, é conhecida a capacidade dos fungos produzirem toxinas as quais, se ingeridas pelo homem ou animais, poderão provocar sintomatologia bem característica, causando inclusive a morte. Assim tanto na antiga Roma como na idade Média, são citados envenenamentos por cogumelos comestíveis e, ainda mais significativos, são os relatos de intoxicações coletivas de pequenas povoações pelas toxinas de *Claviceps purpurea,* agente do Esporão do Centeio.

Recentemente, as micotoxinas despertaram um renovado interesse (2, 8, 9). De um lado, a descoberta de alucinógenos, produzidos por fungos e, de outro, os problemas ligados à alimentação animal, especialmente os provocados pela aflatoxina. Essa micotoxina é responsável por perdas significativas na avicultura e pecuária, devido ao uso constante de tortas oleaginosas na alimentação animal (1, 5, 19).

A partir do isolamento e caracterização das aflatoxinas é que se evidenciou a importância de fungos contaminantes de sementes, no desenvolvimento de processos patológicos em animais e sua possível implicação em patologia humana, com indícios de ação hepatocarcinogênica (4, 6, 12).

Por esses problemas surgidos na alimentação humana e animal, foram conduzidas pesquisas sobre a produção de toxinas por fungos associadas a cereais utilizados na alimentação. Esses trabalhos indicaram que numerosos fungos pós-colheita, dependendo de condições favoráveis, poderão produzir outras toxinas, além das aflatoxinas, igualmente tóxicas ao homem e animais, com diferentes quadros sintomatológicos (7, 13, 14, 15, 21).

Atualmente, aceita-se que outras espécies de fungos, além das do gênero *Aspergillus* e *Penicillium*, possam produzir micotoxinas, embora somente poucas tenham sido estudadas. No Brasil, poucos são os estudos sobre micotoxinas e os existentes enfatizam apenas as aflatoxinas (1, 10, 17, 18, 19).

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O presente trabalho teve como objetivo fazer um levantamento qualitativo de fungos associados aos cereais. Posteriormente, será verificada a possível ocorrência desses fungos toxicogênicos, através de ensaios biológicos com ratos albinos.

Material e Métodos

Os cereais utilizados foram milho, trigo e arroz, em amostragem procedente de vários estados do Brasil. Após chegarem ao laboratório, as amostras foram etiquetadas e protocoladas. Os grãos de milho, trigo e arroz foram desinfetados superficialmente, durante o período de um a dois minutos, com solução aquosa de cloro ativo, obtida mediante a mistura de uma parte de solução comercial de hipoclorito de sódio contendo 5% de cloro ativo e três partes de água estéril. Em seguida, as sementes foram transferidas assepticamente para placas de petri esterilizadas, contendo meio de agar-água (20 gramas de agar em 1.000ml de água destilada) no total de cinco sementes por placa e cinco placas por amostra. Essas placas foram mantidas por cinco dias, em estufa regulada, a 28°C. Após a verificação do estado de pureza das culturas, foi feita a transferência para tubos de ensaio, contendo o mesmo meio de cultura. Para a identificação dos isolados, as características macro e microscópica foram observadas e comparadas com as descritas por Barnett (3).

Resultados e Discussão

Os resultados do levantamento qualitativo de fungos que ocorrem em grãos de milho, trigo e arroz aparecem na listagem a seguir.

Procedência	Hospedeiro	Variedade	Classificação	
			Gênero	N.º de isolados
Nepomuceno (MG)	Milho	Opaco 11	*Diplodia* sp	1
			Fusarium spp (1, 2)*	3
Água Santa (SP)	Milho	Agroceres-62	*Diplodia* sp	3
			Fusarium spp (1, 2, 3)*	16
			Verticillium sp	2
Ribeirão Preto (SP)	Milho	C-507	*Cephalosporium* sp	1
		C-501	*Fusarium* spp (1, 2)*	6
Guaira (SP)	Milho	Piranão	*Fusarium* spp (1, 2)*	14
Piracicaba (SP)	Milho	Agroceres-64		
		Sintético-10	*Fusarium* spp (1, 2, 3)*	9
		CMS-XM-603-604	*Cephalosporium* sp	1
Santa Cruz das Palmeiras (SP)	Milho	Opaco 11	*Fusarium* spp (1, 2)*	8
			Aspergillius sp (4)*	1
Jacarezinho (Pr)	Milho	TGe.		
		Opaco 2	*Fusarium* spp (1, 2, 3)*	25
			Aspergillius spp (4, 5, 6, 7)*	23
			Trichoderma sp	1
			Penicillium sp	1
			Diplodia sp	1
Santo Antonio Platina (Pr)	Milho	Ag 28	*Fusarium* spp (1, 2, 3)*	5
			Aspergillius spp (4, 5)*	7
			Diplodia sp	3
Anhenbi (SP)		Flint Composto	*Fusarium* spp (1, 3)*	4
		Dentado Composto		
			Aspergillius sp (4)*	2
			Trichoderma sp	1
Tatuí (SP)	Milho	IAC-Hind 7974	*Fusarium* sp (1)*	1
Manduraí	Milho	7974 Híbrido		
		IAC Maya	*Fusarium** sp (1)	2
		Opaco 2		
Campinas (SP)	Milho	Sintético 10	*Fusarium* spp (1, 3)*	2
Passo Fundo (RS)	Trigo	PF 6946 (NT_5)		
		Horto 22902	*Fusarium* sp (2)*	10
		IAS 54-22892	*Aspergillius** sp	2
		IAS 59 22899	*Penicillium* sp	1
Cachoeirinha (RS)	Arroz	Caloro	*Fusarium* sp (2)*	6
			Curvularia sp	3
			Micelio sterilia sp	3
Alegrete (RS)	Arroz	Agulha		
		bico torto	*Fusarium* sp (1)*	1
Santa Vitória do Palmar (RS)	Arroz	Caloro	*Fusarium* sp (1)*	1
Mostarda (RS)	Arroz	Bluebelle	*Micelia sterilia* sp	2
Santo Antonio Patrulha (RS)	Arroz	Bluebelle	*Micelia sterilia* sp	3

* Foram agrupados os isolados com características morfológicas semelhantes (provavelmente sendo da mesma espécie). Os números ao lado dos gêneros representam os diferentes agrupamentos.

Existem vários métodos, comumente empregados para o isolamento de fungos associados às sementes. Entre eles, foi escolhido o da transferência do material para meio de ágar-água, o qual permitiu o desenvolvimento dos fungos com mínima contaminação bacteriana. Além disso, o método ofereceu certas vantagens, como a de possibilitar o isolamento de fungos, tanto esporulantes como não esporulantes, além de ser simples, rápido e pouco oneroso.

A desinfecção externa das sementes possibilitou eliminação de contaminantes externos, provenientes de poeira e partículas de solo, bem como eventuais fungos do ar, aos quais as sementes ficam expostas desde a colheita até o manuseio em laboratório. Essa técnica já foi utilizada com resultados satisfatórios por outros autores (11).

Os isolados foram separados em grupos, conforme mostra a listagem, por apresentarem características semelhantes, sendo cada grupo provavelmente representativo de uma espécie. Não foram classificados até espécie devido à dificuldade e controvérsia que ocorrem na taxonomia do gênero *Fusarium* (20, 24).

As espécies representativas do grupo *Fusarium* sp. (1) foram as que apareceram com maior freqüência e de acordo com suas características morfológicas tudo indica que esse grupo seja constituído provavelmente por *Fusarium moniliforme*, as do grupo *Fusarium* (2) provavelmente *F. graminearum* e a espécie do grupo *Fusarium* (3) não foi possível identificar.

Essas espécies do gênero *Fusarium* estão distribuídas na natureza, crescendo como saprófitas em matéria orgânica em decomposição e como parasitas de várias partes da planta. Podem causar podridões em raízes, caules e da espiga do milho e a sarna ou queima do topo em trigo, aveia, cevada, em muitas regiões do mundo, de modo que o consumo, por animais domésticos, de grãos ou rações contaminadas por *Fusarium*, é quase inevitável. As espécies dos gêneros *Aspergillus* e *Pernicillium* também são polífagas, podendo crescer nos substratos mais variados, em milho, arroz, mandioca e muitas outras espécies vegetais, de uso freqüente na alimentação humana e animal.

No levantamento qualitativo feito em milho, trigo e arroz, coletados em diversas regiões do Brasil, os fungos mais freqüentemente isolados foram espécies dos gêneros *Fusarium* (1), *Aspergillus* (4) e *Diplodia,* que representaram mais da metade do total de isolados, fato esse já observado por Wilcoxson (23) e Mohamed (16). Outros isolados, como espécies do gênero *Penicillium Cephalosporium, Curvularia* e *Mycelia sterilia*, foram menos freqüentes.

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Enzymatic properties of cellulase C_x from a local isolate of Aspergillus niger R-1237

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Summary

The enzymatic properties of cellulase C_x (B-1-4-glucan 4-glucano hydrolase, 3.2.1.4) was studied. The enzyme was obtained from a local isolate of *Aspergillus niger* R-1237. The reaction followed first order kinetic, as $v = \frac{1}{t} \cdot 1n \frac{a}{a-x}$. The apparent temperature optimum fell at about 60°C. The enthalpy of activation of the ES-complex was calculated as about 2600cal/mole. The Arrhenius equation is valid, and the energy of activation of the forward reaction (E) was calculated to be 12600cal/mole. The standard free energy change (delta G) and the standard entropy change (delta G) were found to be −102.7cal/mole and +39.3cal/mole/degree at 50°C. The values of thermodynamics quantities at other temperatures ranged from 30°C to 60°C were also studied. The effect of temperatures on the two parameters, i.e. V_{max} and K_m values was discussed.

Resumo

Propriedades da celulase C_x (B-1-4-glucano 4-glucano hydrolase, 3.2.1.4.) obtida de Aspergillus niger R-1237

Reação cinética como $v = \frac{1}{t} \cdot 1n \frac{a}{a-x}$; temperatura ótima, 60°C; entalpia de ativação do complexo ES, 2600cal/mole; equação de Arrhenius válida e energia de ativação da reação (E), 12600cal/mole; delta G e delta S, −102,7cal/mole e +39,3cal/mole, a 50°C. Foram também estudados: valores termodinâmicos na variação de 30 a 60°C e os valores V_{max} e K_m.

Introduction

In recent years, cellulase C_x (B-1,4-glucan-4-glucano hydrolase, 3.2.1.4) has assumed considerable industrial importance, because of its useful application in many industries such as in the extraction of green tea components, soybean or coconut protein, sweet potato or corn starch and agar, the production of unicellular vegetables, vineagar from citrus pulp, the removal of the soybean seed coat, for increasing the tesile strength of paper. A potential future application of cellulase is the preparation of glucose from cellulosic wastes, this helping the alleviation of pollution problems. This use plus the unusual properties of this enzyme prompted us to include cellulase in the investigations of our laboratories. Japanese workers (10), have reported the properties of cellulase from *A. niger*. As their strain was unavailable and the action of cellulase somewhat novel, we felt it necessary to examine the enzyme from our culture to see if it was identical to that reported. One of our strains namely *A. niger* R-1237 had the highest activity in the initial assay and was chosen for detailed study.

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Material and Methods

Culture — A preliminary screening program for high cellulase producing molds grows in submerged culture, indicated that *A. niger* R-1237 was one of the most active. The organism was one of the most active. The organism was maintained on yeast malt agar slants based on that of Haynes & col. (9), and transferred at 4 weeks interval.

Culture conditions — Culture of *A. niger* R-1237, employed in this study was grown in 250ml baffled Erlyenmeyer flasks, containing 50ml of medium (8). The medium contained proteosepeptone 0.4%, sodium nitrate 0.1%, KH_2PO_4 0.5%, KCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05% and $FeSO_4$ 0.001%, Corn cobs 1%, and pH of 6.0.

A suspension of the spores from yeast malt agar slant was prepared and the medium was inoculated under aseptic conditions with 0.5ml of the spore suspensions. The flasks were incubated at 30°C on an incubated rotary shaker 150rpm. for 5 days.

Enzyme production — After the incubation period, the inutilized cellulosic materials and the mycellium were removed by centrifuging at 10000g for 15 minutes. The clear light yellow supernatant was concentrated under vacuum at 40°C to one-fifth. The clear concentrated enzyme fluid was brought to 30% saturation with ammonium sulfate by slow addition of the salt and simultaneous stirring. After keeping the mixture at 5°C overnight, the precipitate was removed by centrifugation and discarded. The supernatant was then brought to 80% saturation with ammonium sulfate and allowed to stand overnight at 5°C. The precipitate was collected and redissolved in a minimum amount of deionized water and dialysed against distilled water for 24 hours. Further purification was made by Amberlite IRA-400 and IRC-50.

Reaction order — The enzyme in citrate buffer at pH 5.0 was rapidly brought to 55°C in a waterbath regulated to 0.05°C and stirred continuously. Aliquots were removed at 10 minutes intervals, immediatly cooled in a freeze mixture to 10°C, and the activity was determined (4).

Enzyme assay — Cellulase C_x was determined by the method of Denison (4) as follows: 0.45ml of 1% carboxymethil cellulose (CMC) in sodium citrate buffer, pH 5.0 at a temperature of 55°C was added to 0.05ml aliquot of diluted enzyme sample. The mixture was then incubated for 15 minutes at the same temperature in a controlled temperature waterbath. Immediatly after removing the enzyme substrate mixture from the incubated waterbath, 0.5ml of 3,5-dinitrosalysilic acid (DNS) reagent was added. The mixture was then heated in a boiling waterbath for 5 minute and cooled to room temperature. Deionized water was added to make a 5ml volume. Absorbance of the sample was determined with Carl-Zeiss spectrophotometer PMQ II at 540nm. D-glucose concentrations were obtained from a standard preparation (0.05 to 1.0mg/ml). One unit is the amount of enzyme which under the test conditions liberates one microgram of reducing groups per minute calculated as D-glucose.

Results

To examine the effect of temperature on the activity of the enzyme, the hydrolysis of carboxymethyl cellulose (CMC) catalyzed by R-1237 cellulase was carried out at pH 5.0, and determined by measuring the reducing power of the reaction mixture after the incubation period. Cellulase from R-1237 has optimum activity at 60°C (Figure 1). Contrary observations have, however been made by Ryoko Ikeda & col. (10), using their isolate of *A. niger.* They found that the optimum temperature for hydrolysis was 50°C at pH 2.5.

From Figure 2, it can be seen that there are two important points regarding first order reaction: (i) the rate constant k, can be determined from the ratio of two concentrations determined at two times, (ii) the half-life of the reaction is a constant and does not depend on the initial concentration.

Figure 1 — Effect of temperature on cellulase activity

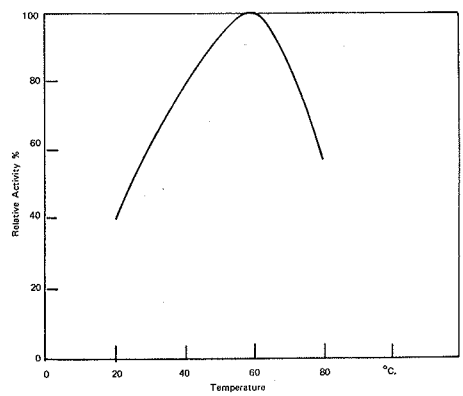
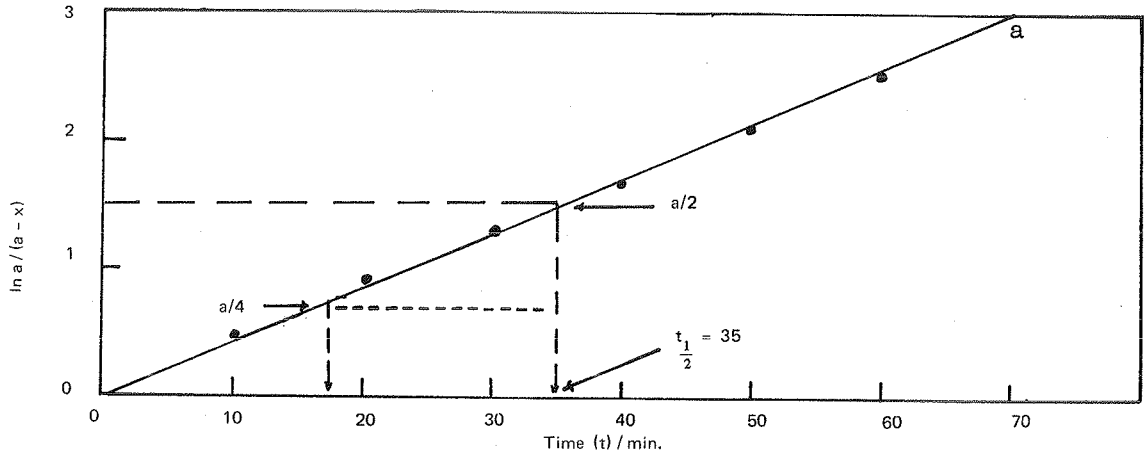


Figure 2 — Schematic plot of a first order kinetics



Since $t = t_{\frac{1}{2}}$ (when $x = \frac{a}{2}$)

Substitution in: $1n\ (\frac{a}{a-x}) = kt$, gives

$kt_{\frac{1}{2}} = 1n\ 2$ and $t_{\frac{1}{2}} = 0.693/k$

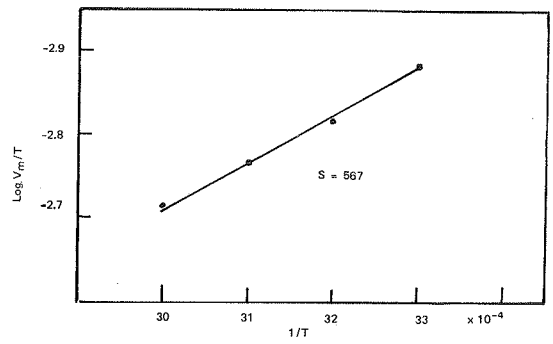
The rate constant was determined in this experiment to be 0.0198, provided a reaction follows first order kinetics, the time for 50% of the reaction to be completed cannot be altered by changing the concentration of the reactant. This finding of the first order reaction for R-1237 cellulase was rather expected, as very many reactions are first order in each reactant (1, 13). In these case, it is often possible to carry out the reaction under pseudo-first order conditions overall by keeping every reactant except one in large excess. Thus, in many practical situations, the problem of determining rate constants can be reduced to the problem of determining the rate constant for a first order kinetics.

According to Eyring (7), the rate constant V_m is given by:

$$1n\frac{V_m}{T} = \text{delta}\frac{H}{RT} + (1n\frac{R}{Nh} + \text{delta}\frac{S}{R})$$

where R is the gas constant, T the absolute temperature, N Avogadro's number, h Planck's constant. Thus a plot of $\log (\frac{V_m}{T})$ against (T^{-1}), revealed a straight line with a slope, delta $\frac{H}{2.303R}$, which provides a value of the enthalpy of activation of the ES-complex (Figure 3). From Figure 3, the enthalpy of activation of the ES-complex was calculated as about 2600cal/mole.

Figure 3 — Effect of temperature on $\log\frac{V_m}{T}$



The Arrhenius equation, $k = Ae^{\frac{-E}{RT}}$ predicts that when the log of the specific reaction rate k is plotted against the reciprocal of the absolute temperature T, a straight line relation should obtain (Figure 4). The slope of the line multiplied by 4.58 is the energy of activation of the forward reaction E under the experimental conditions. The heat of the forward reaction (delta E), accompanied by the process has been calculated from the slope of the plot to be 12600 cal/mole. The standard free energy change of the reaction (delta G) is given by the equilibrium constant of Gibbs-Helmholtz equation, delta G = $^{-}$RT 1n k.

The standard entropy change (delta S) of the enzyme can be obtained from the following equation, delta $S = \frac{(E^{-}G)}{T}$. The values of both delta G and delta S are clearly shown in Table 1.

Figure 4 — Effect of temperature on the heat activity of cellulase (delta E = 12600 cal/mole)

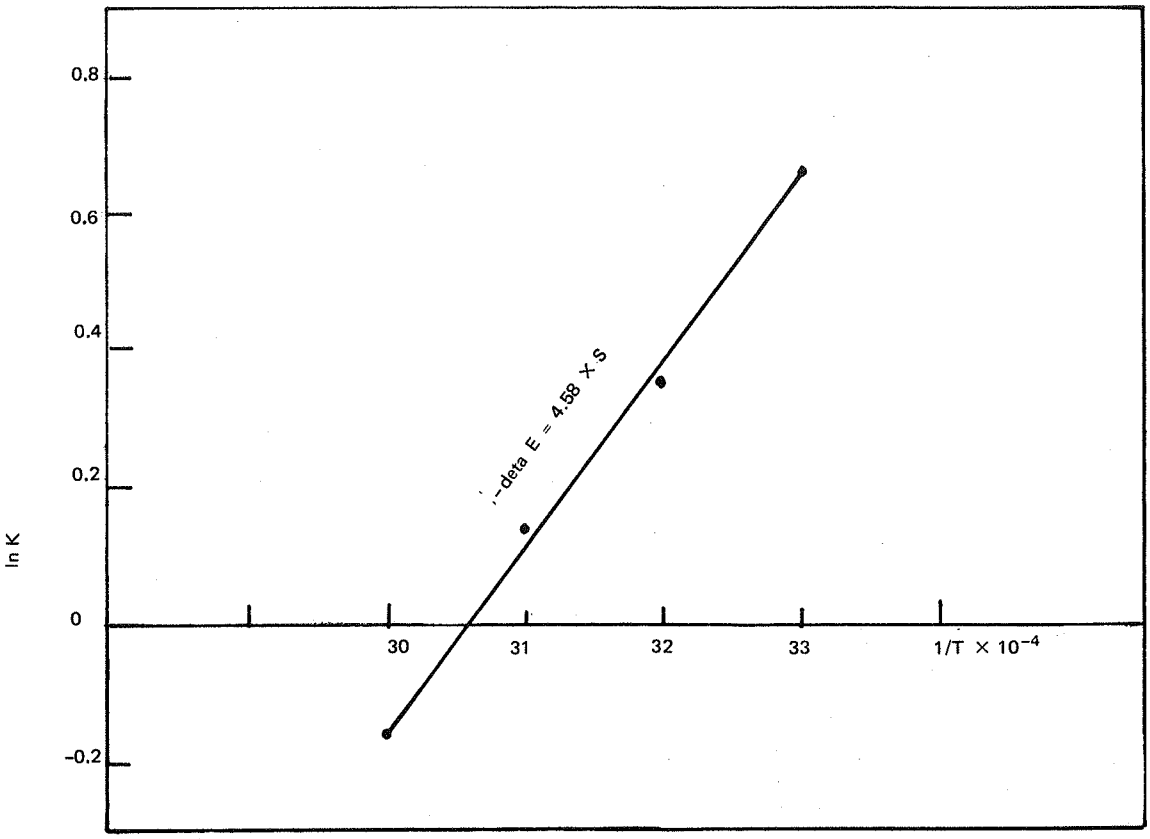


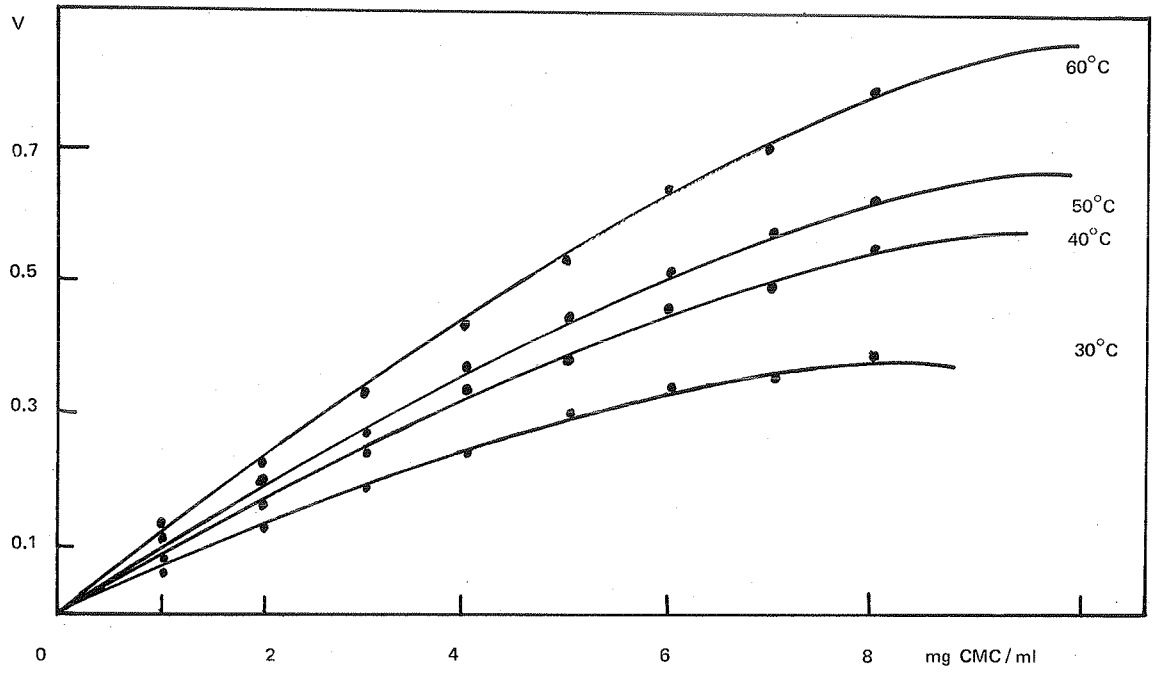
Table 1 — Thermodynamic quantities of cellulase

Temp. °C	$\frac{1}{T} \times 10^{-4}$	1n k	delta E cal/mole	delta G cal/mole	delta S cal/mole/degree
30	33	+ 0.66	12600	− 397.4	+ 42.9
40	32	+ 0.32	12600	− 199.0	+ 40.9
50	31	+ 0.16	12600	− 102.7	+ 39.3
60	30	− 0.16	12600	+ 105.9	− 37.5

From Table 1, it is clearly shown that the standard free energy (delta G) change for the conversion of substrate into product is negative at temperatures below 60°C, i.e. positive in the reverse direction. In this case, the concentration of product is low comparing with substrate. This indicates that the enzyme attacks CMC very slowly with the decrease of temperature. This finding is rather expected, as the major problems in obtaining high glucose concentrations from CMC are the susceptibility of the substrate, the enzyme in the system and the incubation temperature for CMC conversion to glucose.

V_m and K_m are two important kinetic parameters that are very useful for describing the properties of an enzyme-catalized reaction. The following equation: $v = \frac{VS}{K_m + S}$ is the steady-state rate equation for a homogenous reaction involving a recycling catalyst. At high values of S, $v = V_m$ and under these conditions the reaction velocity is constant and independent of S and hence is an example of zero order kinetics. When S is higher than 100 K_m the deviation from zero order kinetics is less than 1%, even when S is higher than 10 k_m, the deviation from zero order kinetics is only 9%. If S is more higher than K_m, the equation becomes $v = \frac{VS}{K_m}$, and the reaction obeys first order kinetics with $\frac{V}{K_m}$ as the rate constant. When S is between 0.1 K_m and 10 K_m, the reaction is intermediate between zero and first order. The two kinetic parameters V and K_m define the shape of the curve. The properties of this curve may be seen from Figure 5. Unique values of V and K_m are then obtained by drawing several such lines as in Figures 6, 7, and 8, and estimating the accurate value of both parameters by intersections that have been created. Thus the

Figure 5 — Effect of substrate concentration on cellulase activity at different temperature



experimental v and s observations (Figure 5) are entered in the determination of V and K_m parameters.

Various methods for plotting kinetic data for the calculation of V and K_m have been used. Figure 6 shows that the values of V and K_m are obtained by drawing several lines and estimating the values of both parameters indicated by the intersections that have been created. In this sense, the values of V and K_m are 1.19 and 7.6mg/ml respectively. The method of Lineweaver & Burk (11) in which 1/v is plotted versus 1/s has the advantage that the variable v and s are separated. Dixon (5) has pointed out that the negative reciprocal of the intercept of such a plot on the 1/s axis is equal to the K_m (Fi-

Figure 6 — Plot of v againsts according to the Michaelis-Menten equation

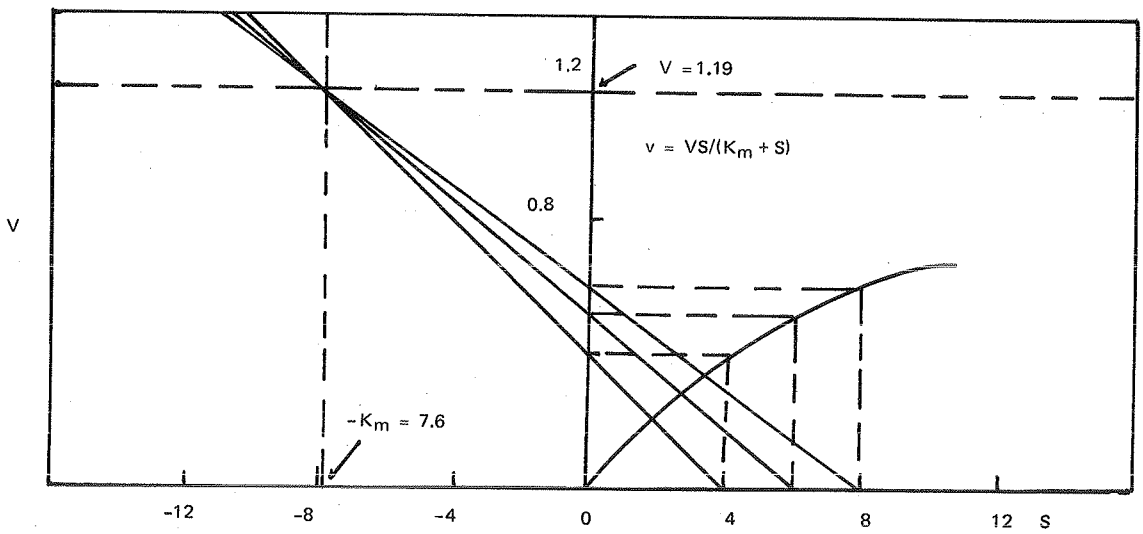
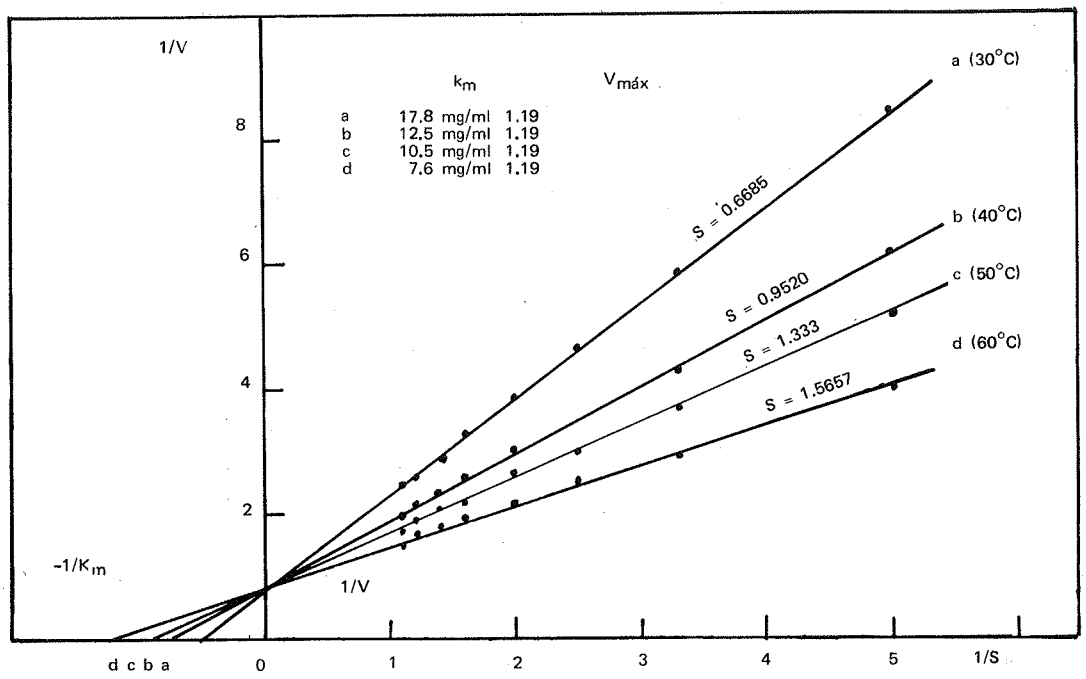


Figure 7 — Lineweaver-Burk plot for the calculation of the K_m for CMC in the cellulase assay at different temperatures



gure 7). The two parameters may also be calculated (6) from plots of v versus v/s (Figure 8). The values of the slops and intercepts in these various plots are summarized in Table 2.

From the above mentioned results, the effect of temperature on enzyme-catalyzed reactions is introduced by the irreversible Michaelis & Menten (12) reaction as generalized by Briggs & Haldane (2): $E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_1} E + P.$

Figure 8 — Calculation of the K_m for CMC in the cellulase assay at different temperatures

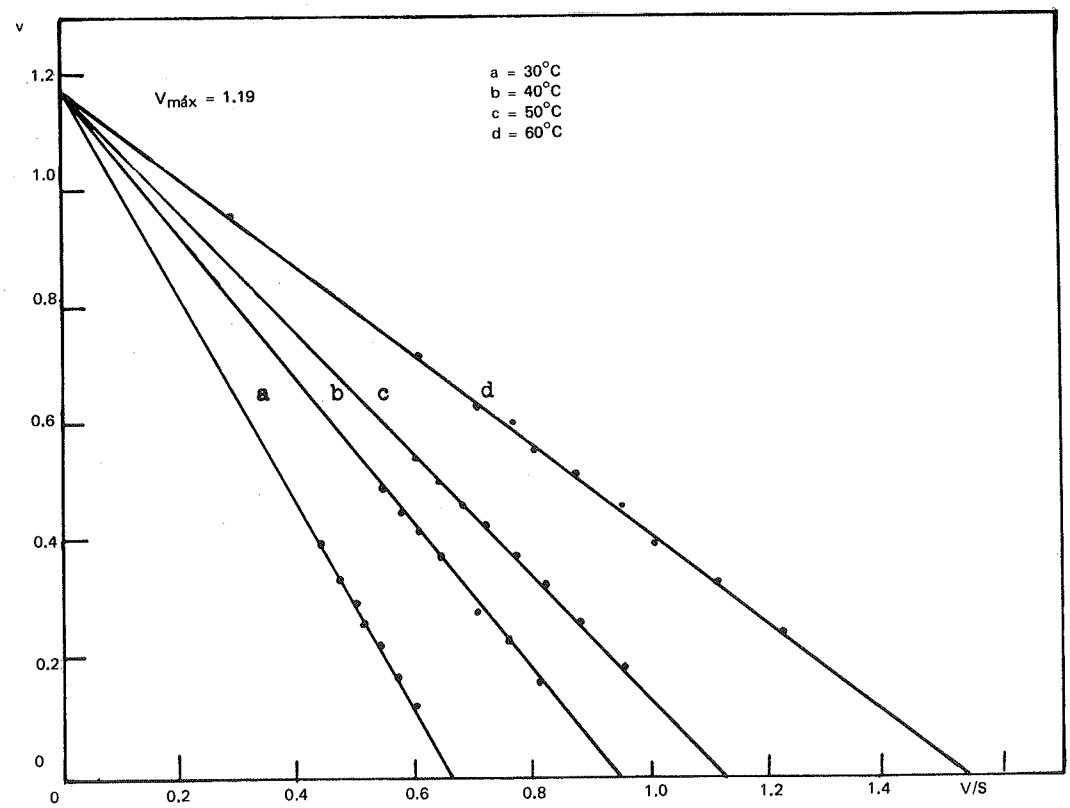


Table 2 — Different techniques for calculating V and K_m

Plot	Temperature °C	Slope	Intercepts Ord.	Intercepts Abs.	V	K_m
$\frac{1}{v}$ versus $\frac{1}{s}$	30	$\frac{K_m}{V}$	$\frac{1}{V}$	$\frac{-1}{K_m}$	1.19	17.8
	40				1.19	12.5
	50				1.19	10.5
	60				1.19	7.6
v versus $\frac{v}{s}$	30	$-K_m$	V	$\frac{V}{K_m}$	1.19	17.8
	40				1.19	12.5
	50				1.19	10.5
	60				1.19	7.6

The K_m values are calculated as 17.8, 12.5, 10.5 and 7.6mg/ml using CMC as substrate at temperatures of 30, 40, 50 and 60°C respectively. The V is 1.19 at all levels of temperatures.

As increase in temperature will affect this reaction by changing the rate constant for each step. The net effect of the changes in k_1 and k_2, however, is represented by the change in K_m. The change in k_3 gives the change in V (value of the measured maximum velocity). Thus the effect of increasing temperature on the enzyme itself is to denature it, and this is an important effect in practice. This conclusion is true, as the increasing temperature affect only the K_m, while the V value is unchangable.

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Errata

Artigo "Ocorrência de uma variante de Salmonella typhimurium que fermenta a lactose tardiamente". Rev. de Microbiol., 10(3):103-105, 1979.

Onde se lê:

Tabela 1 — Freqüência de isolamento da variante lactose tardia de *Salmonella typhimurium,* das fezes de crianças com e sem diarréia, entre fevereiro e novembro de 1977

Crianças	Nº	*Salmonella*					
		typhimurium lactose tardia		*typhimurium* e outras lactoses		Total	
		Nº	%	Nº	%	Nº	%
Com diarréia	249	21	8,4	7	2,8	28	11,2
Sem diarréia	100	2	2,0	1	1,0	3	3,0

Leia-se:

Tabela 1 — Freqüência de isolamento da variante lactose tardia de *Salmonella typhimurium,* das fezes de crianças com e sem diarréia, entre fevereiro e novembro de 1977

Crianças	Nº	*Salmonella*					
		typhimurium lactose tardia		*typhimurium* e outras lactose —		Total	
		Nº	%	Nº	%	Nº	%
Com diarréia	249	21	8,4	7	2,8	28	11,2
Sem diarréia	100	2	2,0	1	1,0	3	3,0

Artigo "Epizootia provocada por um bacilo difteróide em serpentes mantidas em biotério". Rev. de Microbiol., 10 (4):139-142, 1979.

Onde se lê:
Recentemente, um surto de uma nova moléstia infecto-contagiosa, dę caráter epizoótico, até então não descrita, acometeu cerca de 70% das serpentes dos gêneros *Bothrops* Linnaeus, 1758 e *Crotalus* Wagler, 1824, mantidas no mesmo biotério. Esta doença, se traduzia pela formação de u'a massa caseosa, de coloração branco-amarelada, de odor fétido, e que invadia pre lesando as glândulas veneríferas, resultando na morte de 25% dos ofídeos comprometidos.

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