ENERGY SUPPLY AND ENZYME ACTIVITY IN STRICT ANAEROBES. STUDIES ON PEPTIDASE ACTIVITY IN *CLOSTRIDIUM SPOROGENES*

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Interacting amino acids provide the energy for growth of the strict anaerobe *Cl. sporogenes*[1,2,3], diphosphopyridine nucleotide being an intermediate carrier in these reactions[4,5]. The question arose as to whether the presence of interacting amino acids would provide energy, under anaerobic conditions, for protein synthesis in suspensions of non-proliferating *Cl. sporogenes*. It was observed[7] that an apparent synthesis of peptidase, or enhancement of peptidase activity, took place in resting cells of *Cl. sporogenes* in presence of energy-yielding substrates. Further experimental work revealed that factors, other than direct synthesis of the enzyme, which result in apparent increased enzyme activity, operate as a consequence of the presence of energy-yielding substrates. The present communication is concerned with a description of this experimental work and its results.

EXPERIMENTAL METHODS

Nutrient media

Stock cultures of *Cl. sporogenes* were maintained on Brewer's meat with bi-weekly transfers to fresh media. A newly inoculated tube of media was heated at 80° C for 10 minutes prior to incubation at 38° C.

One ml of a suspension of organisms from the Brewer's meat was added to one litre of medium of the following composition:

15	g	Bacto-casitone
1	g	Sodium thioglycollate
1.25	g	Sodium chloride
1.5	g	Potassium chloride
0.75	g	Cysteine hydrochloride
1	mg	D-biotin
1	litre	Water

This medium was adjusted to pH 7.0 before autoclaving.

Preparation of bacterial suspensions

Washed cells of *Cl. sporogenes*, which were prepared in bulk and stored under frozen conditions, have been used for the majority of our experiments. Two litres of the medium described above were inoculated with two ml of a seven-hour culture of *Cl. sporogenes* grown on Brewer's meat medium and incubated at 38° C. Four to six litres of a 14–17 hours' culture were centrifuged on a De Laval centrifuge and the cells were washed twice in 0.15 *M* KCl in a Sorvall high-speed centrifuge at 20,000 *g*. The packed, washed, cells were divided equally and placed in test tubes, each of which contained the cells from one litre of medium. They were stored in the deep-freeze up to two weeks without appreciable loss of activity. When cells grown on a glucose medium were required for experimental work, 15 g glucose were added per litre of the basic medium, and the cells from four litres of culture medium were divided into six equal parts and stored in the same way.

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The frozen cells in one tube were suspended in five to seven ml of 0.15 *M* KCl. One ml of the thick suspension whose dry weight was approximately 30 mg was used in each incubation experiment.

Incubations

All incubations were carried out in the standard Warburg manometric apparatus at 37° C. In anaerobic experiments, 0.028 *M* $NaHCO_3$ was used as buffer at a final pH of 7.4, with a mixture of 93% nitrogen and 7% carbon dioxide as the gas phase. In aerobic experiments, 0.02 *M* sodium phosphate buffer was used at pH 7.4 and air formed the gas phase. Carbon dioxide, formed in aerobic experiments, was absorbed by 0.2 ml 20% KOH placed on filter paper in the centre well.

Solutions of amino acids, or other substrates, were placed in the side tubes of the manometric vessels and tipped into the main vessel, containing the suspension of washed *Cl. sporogenes* in the buffer solutions, after gassing and equilibration. The total volume of fluid in the Warburg flask was 3.2 ml.

After incubation of the suspension of *Cl. sporogenes* with energy-yielding substrates, the vessel contents were transferred to centrifuge tubes, the vessels rinsed with 10 ml 0.15 *M* KCl and the combined vessel contents and rinse solution were centrifuged. The packed cells from each reaction vessel were resuspended in 3 ml 0.15 *M* KCl, and 1 ml was used for measurement of peptidase activity. Optical density measurements were generally carried out on an aliquot of the resuspended cells, although no evidence of growth was obtained under these experimental conditions. The resuspended cells were added to vessels kept in an ice bath. When cell-free preparations were used for assay of peptidase activity, they were prepared from the cells which had previously been incubated under specified conditions.

Bacterial extracts

These were prepared by making use of the Hughes bacterial crusher[8], full details being given in our earlier paper[4].

After incubation with the substrates, the packed, washed cells were crushed and 60 mg dry weight of cells were extracted in a final volume of 6 ml 1% sodium thioglycollate solution. The extract was centrifuged at 20,000 *g* at —2° C, and 1 ml extract corresponding to 10 mg dry weight of cells (as with intact cells) was used for estimation of peptidase activity.

Estimation of peptidase activity

Triglycine and diglycine were used as substrates for estimations of peptidase activity. The incubation mixture for triglycine hydrolysis consisted of 0.028 *M* $NaHCO_3$, pH 7.4, and a quantity of triglycine, usualy 0.0066 *M*, in a final volume of 3.2 ml. These incubations were carried out for 1 hour in a Warburg manometric vessel in a gas phase of 93% N_2 and 7% CO_2 at 37° C. The vessels were then removed from the bath and immersed in an ice bath. Aliquots were taken for colorimetric analysis of glycine by ninhydrin after separation by paper chromatography, as described in our earlier paper[4]. Control values on the organism incubated in the absence of triglycine were always determined. Peptidase activity was also estimated by a manometric modification of a formol titration which is fully described elsewhere[9]. When using this manometric technique for determining the extent of triglycine hydrolysis, the cells were removed by centrifuging and two ml of the clear supernatant were used for analysis.

Ferrous iron estimation

Ferrous iron was determined colorimetrically, using *αα'*dipyridyl as reactant. The red colour produced by *αα'*dipyridyl in presence of ferrous iron was measured at 525 mμ in a Fisher colorimeter. A standard calibration curve was prepared. The method is sensitive and accurate to ± 0.5 μg ferrous ions in 1 ml sample.

RESULTS

Effects with amino acids as substrates

Incubation of a suspension of *Cl. sporogenes*, in the absence of substrates, for 1–3 hours in a mixture of 93% nitrogen and 7% carbon dioxide results in a considerable decrease of the rate of triglycine hydrolysis compared with organisms which had no preliminary incubation. Typical results are shown in Fig. 1. If the incubation takes place, however, in presence of a pair of interacting amino acids, such as alanine and proline, no such diminution of activity occurs. In fact, a gradual rise in the peptidase

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activity is observed over a period of 3 hours (see Fig. 1). Experiments with glycyltyrosine, glycyltryptophane, diglycine and triglycine as peptidase substrates, indicate that, in the presence of alanine and proline, peptidase activity increases during incubation in contrast to the fall of activity that occurs in the absence of the amino acids.

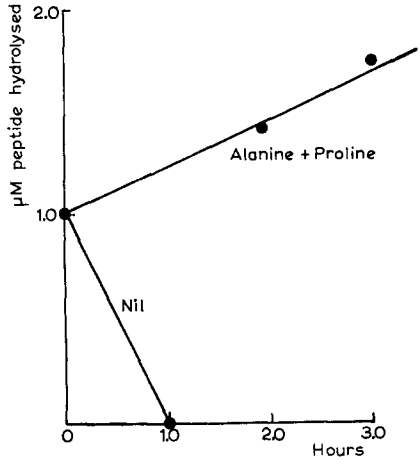

Fig. 1. Ordinate: Peptidase activity expressed in terms of μM peptide hydrolysed. Abscissa: Hours of incubation of *Cl. sporogenes* in presence of substrates (alanine and proline) or in absence of substrates.

Replacement of alanine by valine as the hydrogen donator amino acid results in a similar enhancement of peptidase activity (Table I). The presence of threonine alone also produces the same effect (Table I). Threonine is decomposed by this organism in the absence of a second amino acid and thus may replace a mixture of alanine and proline as energy source. However, the presence of amino acids which are not metabolised

TABLE I

INFLUENCE OF INCUBATION OF *Cl. sporogenes* WITH VARIOUS AMINO ACIDS ON PEPTIDASE ACTIVITY

Expt. No.	*Addition to initial incubation medium*	*Glycine produced during subsequent incubation with triglycine* μg
1	Nil	Nil*
	DL-Alanine	48
	L-Proline	Nil
	DL-Alanine + L-proline	264
2	Nil	104
	DL-Threonine	256
3	Nil	68
	L-Proline	80
	DL-Valine	96
	DL-Valine + L-proline	184
4	Nil	32
	L-Glutamate	56
	L-Glutamate + L-proline	32

Organism; grown in absence of glucose; 30 mg
Concentration of amino acids in initial incubation = 0.1 M (L-isomer)
Buffer = (during both incubations) 0.028 M $NaHCO_3$; pH 7.4
Gas phase (both incubations) = 93 % N_2 and 7 % CO_2
Triglycine = 0.01 M
Time; initial incubation = 2 h, 45 min
incubation with triglycine = 60 min
Glycine estimated colorimetrically with ninhydrin.

* There is considerable variation between the peptidase activities of different preparations.

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in the absence of a second amino acid, or of pairs of amino acids that do not interact, does not increase the peptidase activity of *Cl. sporogenes*. Thus, incubation of the organism with alanine, valine and proline separately does not show any significant effect on peptidase activity. Neither does incubation with a mixture of glutamate and proline, a non-interacting pair of amino acids, have any effect on peptidase hydrolysis of the organism (Table I).

Effects with glucose as substrate

Incubation of *Cl. sporogenes* with glucose, or a mixture of glucose and proline, under anaerobic conditions, results in enhanced peptidase activity (Table II). Whenever glucose was present as a substrate, cells grown on a casein medium supplemented with 1.5% glucose were used. These cells generally have a lower initial peptidase activity than those grown in absence of glucose, and the peptidase activity found after incubation of the cells with energy-yielding substrates is much greater than that found with the non-glucose-grown cells. The interaction of alanine and proline also leads to an appreciable increase in peptidase activity with these cells (Table II) although the rate of alanine-proline interaction is lower than with non-glucose-grown cells.

Not only is the peptidase activity of the resting cells increased by exposure to energy-yielding substrates, but the peptidase activity in extracts of these cells is similarly increased. Thus, if the resting cells are incubated in a glucose-proline mixture and the cells then crushed, the peptidase activity of the cell extract is much greater than that of the extract of cells incubated in the absence of energy-yielding substrates (Table II). This result shows that the effect of the energy-yielding reaction on peptidase activity of *Cl. sporogenes* cannot be explained by an alteration of cell permeability to the peptide under investigation.

Effects with pyruvate as substrate

Surprisingly, the presence of pyruvate, which breaks down anaerobically in presence of *Cl. sporogenes*, does not enhance the peptidase activity appreciably (Table III), although the gas evolution during the preliminary incubation period is of the same order

TABLE II

EFFECTS OF INCUBATION OF *Cl. sporogenes* WITH GLUCOSE AND AMINO ACIDS ON PEPTIDASE ACTIVITY

Additions to initial incubation medium	*Triglycine hydrolysed after incubation period* *μmoles*
Intact cells	
Nil (initial activity; no incubation)	0.6
Nil	Nil
DL-Alanine	0.07
DL-Alanine + L-proline	2.4
Glucose	1.9
Glucose + L-proline	4.0
Extract	
Nil	3.5
Glucose + L-proline	8.6

Organism; grown in presence of glucose; 30 mg
Concentration of amino acids in medium = 0.1 *M* (L-isomer)
Glucose = 0.05 *M*
Buffer = 0.028 *M* $NaHCO_3$, pH 7.4 in both incubations
Gas = 93% N_2 and 7% CO_2 in both incubations
Time = 2 h, 45 min
Concentration of triglycine = 0.01 *M*
Extract prepared after incubation, as described in text.

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of magnitude as that with a mixture of alanine and proline. However, if proline is added to the pyruvate the resulting peptidase activity is of the same order of magnitude as that obtained with a mixture of alanine and proline.

TABLE III

EFFECTS OF INCUBATION OF *Cl. sporogenes* WITH PYRUVATE, GLUCOSE AND AMINO ACIDS ON PEPTIDASE ACTIVITY

Expt. No.	*Addition to initial incubation medium*	*Triglycine hydrolysed after incubation period µmoles*
1	Nil (Initial activity; no incubation)	0.6
	DL-Alanine	0.4
	DL-Alanine + L-proline	1.2
	L-Proline	0.5
	Pyruvate	0.7
	Pyruvate + L-proline	1.1
2	Nil (Initial activity; no incubation)	0.06
	Nil	Nil
	Pyruvate	0.25
	Glucose	2.6
	DL-Alanine + L-proline	2.7

Expt. 1: Non-glucose-grown organism; 30 mg
Expt. 2: Glucose-grown organism; 30 mg.
Concentration of amino acids and pyruvate = 0.1 *M* (as L-isomer)
Concentration of glucose = 0.05 *M*
Concentration of triglycine = 0.0066 *M*
Buffer = 0.028 *M* $NaHCO_3$; pH 7.4
Gas = 93% N_2 and 7% CO_2
Initial incubation time = 2 h, 45 min
Incubation time with triglycine = 60 min.

Effects with triglycine as substrate

The rate of peptide hydrolysis subsequent to the preliminary incubation period is not affected by the presence of the peptide (triglycine) during this incubation period. The results given in Table IV clearly show that pre-incubation with triglycine alone, or in the presence of a mixture of glucose and proline, does not affect the peptidase activity found in the absence of the tripeptide. However, it should be borne in mind that the triglycine undergoes some hydrolysis by the thick suspension of cells (30 mg) used in the preliminary incubation, and the concentration of the peptide during the course of incubation may fall too rapidly to exercise a significant effect.

TABLE IV

EFFECTS OF INCUBATION OF *Cl. sporogenes* WITH TRIGLYCINE ON SUBSEQUENT PEPTIDASE ACTIVITY

Additions to initial incubation medium	*Triglycine hydrolysed after preliminary incubation µmoles*
Nil (Initial activity; no incubation)	2.0
Nil	0.4
Triglycine	0.4
Glucose + L-proline	8.6
Glucose + L-proline + triglycine	8.8

Glucose-grown organism used
Concentration of L-proline = 0.1 *M*
Concentration of glucose = 0.05 *M*
Concentration of triglycine
= 0.012 *M* (in preliminary incubation)
= 0.0066 *M* (in subsequent incubation)
Buffer = 0.028 *M* $NaHCO_3$, pH 7.4. Gas = 93% N_2 and 7% CO_2
Time; Preliminary incubation = 2 h
Incubation with peptide = 1 h

Effects of aerobic conditions on peptidase activity

Replacement of the nitrogen-carbon dioxide atmosphere by air during the initial incubation prevents the appearance of increased peptidase activity. Typical results are

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shown in Table V. Although oxygen uptake takes place in the presence of alanine, or of a mixture of alanine and proline, the peptidase activity is approximately the same as that found in the anaerobic control where *Cl. sporogenes* cells are incubated in the absence of amino acids. Similarly, little peptidase activity is found after aerobic incubation with glucose as substrate, compared with that found under anaerobic conditions (Table V). Earlier results[4] have pointed to the harmful effect of oxygen on the enzymes catalysing amino acid interactions in *Cl. sporogenes*. The present results indicate additional toxic effects of oxygen on enzymic activity in strict anaerobes (see also MASCHMANN[14]).

TABLE V

EFFECTS OF INCUBATION OF *Cl. sporogenes* UNDER AEROBIC CONDITIONS ON PEPTIDASE ACTIVITY

Expt. No.	*Initial incubation*		*Triglycine hydrolysed after initial incubation µmoles*
	Addition to medium	*Gas phase and time of incubation*	
1	DL-Alanine	air; 150 min	Nil
	DL-Alanine + L-proline	air; 150 min	Nil
	DL-Alanine + L-proline	(No incubation)	0.81
	DL-Alanine	93% Nitrogen and 7% carbon dioxide 150 min	0.5
	DL-Alanine + L-proline	93% Nitrogen and 7% carbon dioxide 150 min	1.4
2	Nil	air; 90 min	Nil
	Glucose	air; 90 min	0.63
	Nil	93% Nitrogen and 7% carbon dioxide 90 min	0.4
	Glucose	93% Nitrogen and 7% carbon dioxide 90 min	4.5

Expt. 1: Non-glucose-grown organism
Expt. 2: Glucose-grown organism
Concentration of amino acids = 0.1 *M* (L-isomer)
Concentration of glucose = 0.05 *M*
Triglycine = 0.0066 *M*
Buffer: Aerobic = 0.02 *M* sodium phosphate, pH 7.4
Anaerobic = 0.028 *M* $NaHCO_3$, pH 7.4
For peptide hydrolysis: 0.028 *M* $NaHCO_3$ buffer and 93% N_2 and 7% CO_2 as gas phase; incubation period = 60 min

Effects with ferricyanide on peptidase activity

The results obtained when ferricyanide is present in the preliminary anaerobic incubation medium are essentially the same as those found under aerobic conditions and indicate that replacement of the natural acceptor amino acid by ferricyanide does not result in enhanced peptidase activity (Table VI). Addition of ferricyanide to a glucose system also largely prevents the appearance of the increased activity associated with pre-incubation with glucose. It should be noted that ferricyanide does not affect appreciably the rate of peptidase hydrolysis *per se* and, therefore, the absence of activity cannot be due to an inhibition of the peptidase system by ferricyanide. For example, addition of 0.2 ml 10% potassium ferricyanide to a suspension of *Cl. sporogenes* reduced the peptidase activity (with triglycine) by only 14%.

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TABLE VI

EFFECTS OF INCUBATION OF *Cl. sporogenes* WITH FERRICYANIDE ON PEPTIDASE ACTIVITY

Expt. No.	*Additions to initial medium*	*Initial incubation time*	*Triglycine hydrolysed after initial incubation period µmoles*
1	Nil	135 min	1.6
	DL-Alanine	135 min	2.9
	$K_3Fe(CN)_6$	135 min	1.4
	DL-Alanine + $K_3Fe(CN)_6$	135 min	2.5
	DL-Alanine + L-proline	135 min	4.8
2	Initial activity	Nil	0.5
	Nil	90 min	0.4
	Glucose	90 min	3.0
	Glucose + $K_3Fe(CN)_6$	90 min	1.9

Glucose-grown organism
Concentration of amino acid = 0.1 *M* of L-isomer
Glucose = 0.05 *M*
$K_3Fe(CN)_6$ = 0.2 ml of 10% per vessel
Triglycine = 0.0066 *M*
Buffer = 0.028 *M* $NaHCO_3$, pH 7.4. Gas = 93% N_2 and 7% CO_2
Incubation time with peptide = 60 min

The results with ferricyanide suggest that the substitution of the "acceptor" amino acid by ferricyanide as an oxidant of DPNH leads to a fall, or divergence, of the energy needed for the enhanced peptidase activity of *Cl. sporogenes* (see also LEHNINGER[16]).

Effects of cell poisons and inhibitors on peptidase activity

Preliminary incubation of *Cl. sporogenes* with glucose and proline in presence of such substances as benzimidazole, polymyxin, desoxyribonuclease, penicillin, chloromycetin, pentothal, sodium azide, methionine sulfoxide and α-methylglutamate was without effect on subsequent peptidase activity. However, the presence of 0.02 *M* fluoride, which decreased the rate of glucose breakdown in the preliminary incubation period, resulted in a decrease of about 35% in the subsequent peptidase activity.

Effects of 2:4 dinitrophenol on peptidase activity

The presence of 2:4 dinitrophenol (DNP) at 10^{-3} *M* does not affect the rates of glucose-proline, or alanine-proline, interactions as measured by gas evolution and ammonia production, nor does it affect the rate of triglycine hydrolysis *per se*. However, if a mixture of glucose and proline or a mixture of alanine and proline are incubated in the presence of 10^{-3} *M* DNP, the subsequent enhancement of peptidase activity is greatly diminished. Typical results are presented in Table VII where it will be seen that over 80% inhibition is obtained in the presence of 10^{-3} *M* DNP. When 10^{-4} *M* DNP is used, only a negligible inhibition of peptidase activity is obtained. The lack of effect with 10^{-4} *M* DNP may be due to the fact that, during incubation with glucose or alanine together with proline, the DNP undergoes chemical change, the solution becoming colourless. On re-exposure to air, the solution becomes pink. With 10^{-4} *M* DNP, the solution becomes colourless after the first few minutes of incubation. It is likely, therefore, that inactivation of DNP occurs in the preliminary incubation period and, therefore, a relatively high initial concentration is necessary to maintain DNP activity throughout this incubation period.

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TABLE VII

EFFECTS OF INCUBATION OF *Cl. sporogenes* WITH 2:4 DINITROPHENOL (DNP) ON PEPTIDASE ACTIVITY

Expt. No.	*Addition to initial incubation medium*	*Gas evolved during preliminary incubation μmoles*	*Triglycine hydrolysed after preliminary incubation μmoles*
1	Nil	9.8	1.12
	DL-Alanine + L-proline	29.2	3.8
	DL-Alanine + L-proline + DNP	27.2	0
	Glucose + L-proline	55.0	9.0
	Glucose + L-proline + DNP	55.0	2.7
2	* Triglycine	—	12.6
	* Triglycine + DNP	—	13.0

Organism: grown on glucose medium
Concentration of amino acids = 0.1 *M* (L-isomer)
Concentration of glucose = 0.05 *M*
Concentration of DNP = 10^{-3} *M*
Concentration of triglycine = 0.0066 *M*
Buffer = 0.028 *M* $NaHCO_3$, pH 7.4; Gas = 93% N_2 and 7% CO_2
Time: Initial incubation = 150 min; Incubation with peptide = 60 min
* In this experiment, there was no preliminary incubation period.

The results with DNP provide further evidence that the enhanced peptidase activity, found when *Cl. sporogenes* is incubated anaerobically with glucose or interacting amino acids, is dependent on the energy released by these reactions.

Effects of incubation of Cl. sporogenes *with ferrous ions on peptidase activity*

The loss of peptidase activity that takes place when suspensions of *Cl. sporogenes* are incubated anaerobically at 37° in the absence of energy-yielding substrates (see Tables I, II, IV) may also be prevented by the addition of small quantities of ferrous ions in presence of sodium thioglycollate. This reactivation takes place whether the ferrous ions are added to the cells during the initial incubation period or during the subsequent period of incubation with triglycine for estimation of peptidase activity, thioglycollate being used throughout. Typical results are given in Table VIII.

Suspensions of *Cl. sporogenes* incubated in presence of a mixture of glucose and proline for two hours at 37° hydrolyse 4.5 μmoles of triglycine in 1 hour, but on the addition of approximately 6 μg/ml of ferrous iron this hydrolytic activity is increased to 6.7 μmoles per hour.

Cells incubated in the absence of substrate or iron show little hydrolysis (1.1 μmoles of triglycine in 1 hour). However, upon addition of 6 μg/ml of ferrous ions, the activity is largely restored with an hydrolysis rate of 5.3 μmoles/hour.

It is important to emphasise that thioglycollate (or presumably another –SH compound) must be present with the ferrous iron to effect the enhancement of peptidase activity. The presence of ferrous ions alone exerts no appreciable effect on peptidase activity. This may be due to its conversion to the ferric ion. Moreover, the presence of sodium thioglycollate alone exerts no effect on peptidase activity. The combination of ferrous ions and –SH compound seems to be essential for optimal peptidase activity in *Cl. sporogenes*[14].

These results show that there is no loss of peptidase from *Cl. sporogenes* during incubation in the absence of energy-yielding substrates. The enzyme is present but in-

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TABLE VIII

EFFECTS OF INCUBATION OF *Cl. sporogenes* WITH FERROUS IRON, ETC. ON PEPTIDASE ACTIVITY

Additions to initial incubation medium (2 hours incubation)	*Additions to peptide hydrolysing system*	*Triglycine hydrolysed in 1 hr after preliminary incubation µmoles*
Initial activity (no incubation or additions)	(a) Nil	Nil
	(b) Fe^{++}	7.2
Nil	(a) Nil	1.1
	(b) Fe^{++}	5.3
Fe^{++}	(a) Fe^{++}	4.8
Glucose + proline	(a) Nil	4.5
	(b) Fe^{++}	4.5
Glucose + proline + Fe^{++}	(a) Nil	5.3
Glucose + proline + Fe^{++}	(b) Fe^{++}	7.0

Organism grown in glucose medium
Cells suspended in 1% sodium thioglycollate solution throughout.
Buffer = 0.028 *M* $NaHCO_3$; pH, 7.4. Gas = 93% N_2 and 7% CO_2
Fe^{++} = 20 µg/3.2 ml; Glucose = 0.05 *M*; L-proline = 0.1 *M*; Triglycine = 0.0066 *M*

active, activity being restored on addition of excess ferrous ions and thioglycollate, or maintained by energy-yielding substrates without further additions of ferrous ion or thiol compounds.

Effects of heated cell-free extracts of Cl. sporogenes *on peptidase activity*

A cell-free extract of *Cl. sporogenes* prepared from cells incubated for two hours at 37° in absence of substrates has little or no peptidase activity. However, the addition of a *heated* extract, particularly if the heating is carried out in an acid medium, reactivates the peptidase. The degree of reactivation is directly proportional to its content of ferrous iron. The effects of the addition of various concentrations of ferrous ions on the peptidase activity of extracts of *Cl. sporogenes* are given in Fig. 2. The stimulation of activity obtained by 1 ml of heated extract is equivalent to the addition of 3.5 µg Fe^{++}. On analysis with $\alpha\alpha'$dipyridyl the extract was found to contain 2.6 µg Fe^{++} per ml. It should be mentioned that all the extracts are prepared in a thioglycollate medium, and therefore the effect is doubtless due to the combined presence of ferrous ion and thiol compound.

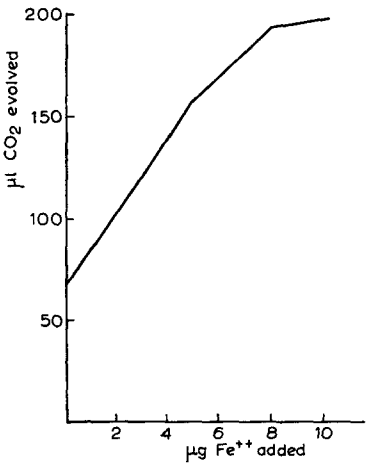

Fig. 2. Ordinate: Peptidase activity expressed in terms of µl CO_2 evolved in the estimation of peptidase by the manometric formol technique[9]. Abscissa: µg Fe^{++} added.

Effect of manganese ions

Manganese ions are also effective in stimulating the peptidase activity, although not to the extent obtained with ferrous ions (see Table IX). The stimulations obtained

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TABLE IX

EFFECTS OF FERROUS AND MANGANESE IONS (IN PRESENCE OF THIOGLYCOLLATE) ON PEPTIDASE ACTIVITY OF EXTRACTS OF *Cl. sporogenes*

Additions to peptide hydrolysing system	*Triglycine hydrolysed in one hour µmoles*
Extract	2.1
Extract + heated extract	4.5
Extract + Fe^{++}	7.4
Extract + Mn^{++}	5.8
Extract + Fe^{++} + Mn^{++}	8.0
Extract + heated extract + Fe^{++}	6.9
Extract + heated extract + Mn^{++}	6.3
Extract + heated extract + Mn^{++} + Fe^{++}	7.5

Organism grown in glucose medium. Extract, in presence of 1% sodium thioglycollate, prepared after 2 hours' incubation of cells in the absence of substrate. The heated extract was prepared by making the extract acid with *N* HCl, heating and then neutralising before use. 1 ml extract was added per vessel (total vol. = 3.2 ml).
Buffer = 0.028 *M* $NaHCO_3$. Gas = 93% N_2 and 7% CO_2
Triglycine = 0.0066 *M*; Fe^{++} = 20 µg/3.2 ml; Mn^{++} = 20 µg/3.2 ml

with ferrous ions, manganese ions and heated extract are not additive. This fact points to the possibility that both iron and manganese, as well as the heated extract, reactivate the peptidase in *Cl. sporogenes* by a similar mechanism.

Effects of various metallic ions on peptidase activity of Cl. sporogones.

The presence of zinc, copper, cobalt or magnesium ions is without stimulatory effect on peptidase activity, but zinc ions are slightly inhibitory. Molybdenum ions have a stimulatory activity on the enzyme.

Effects of versene and αα'dipyridyl

The fact that the activity of a resting cell preparation of *Cl. sporogenes* or a cell-free extract of this organism, is dependent on the presence of a metal ion for activity is illustrated by the results of experiments with versene and αα'dipyridyl. Data given in Table X show that the presence of a small quantity of αα'dipyridyl greatly diminishes the peptidase activity. The addition of versene to a cell-free extract of *Cl. sporogenes*, activated by the addition of heated extract, also results in virtual abolition of activity.

Mechanism of inactivation of the peptidase

It was thought possible that the loss of peptidase activity from *Cl. sporogenes* in the absence of energy-yielding substrates might be due to the leakage of ferrous ions. Careful examination, however, with αα'dipyridyl, showed no differences in iron leakage from, or of iron content of, *Cl. sporogenes* incubated under various conditions.

Attempts were also made to determine possible loss of manganese ions from *Cl. sporogenes* by the benzidine test, and of molybdate ions by the reaction with αα'-dipyridyl and stannous chloride. The tests showed no indication of the presence of these ions either in the cells or in the solutions in which the cells were suspended.

In the absence of any experimental evidence that ferrous ion leakage is prevented

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TABLE X

EFFECTS OF VERSENE AND $\alpha\alpha'$DIPYRIDYL ON PEPTIDASE ACTIVITY OF EXTRACTS OF *Cl. sporogenes*

Expt. No.	*Additions to peptide hydrolysing system*	*Triglycine hydrolysed in one hour µmoles*
1	Extract A	2.5
	Extract A + heated extract A	6.7
	Extract A + heated extract A + versene	1.1
2	Extract B	6.7
	Extract B + $\alpha\alpha'$dipyridyl	1.1

Expt. 1: Organism grown in presence of glucose
Expt. 2: Organism grown in absence of glucose
Extract A (in 1% sodium thioglycollate) prepared as in Table IX (1 ml/3.2 ml)
Extract B (in 1% sodium thioglycollate) prepared after 2 h anaerobic incubation of *Cl. sporogenes* in presence of a mixture of alanine and proline (1 ml/3.2 ml)
Buffer = 0.028 *M* $NaHCO_3$. Gas = 93% N_2 and 7% CO_2
Triglycine = 0.0066 *M*; Versene = 10 mg/3.2 ml; $\alpha\alpha'$dipyridyl hydrochloride = 2 mg/3.2 ml

as a result of energy-yielding reactions, it is difficult to assume that this is the mechanism involved in the maintenance and enhancement of peptidase activity. It seems more reasonable to assume that the enzyme-metallic complex needed for optimal peptidase activity is altered in some way, during incubation, with resulting inactivation, and that energy is required to restore and to maintain the complex in an active form.

Experiments with 10^{-2} *M* potassium cyanide and sodium azide show that the peptidase is insensitive to these substances, indicating the likelihood that the ferrous ion required for optimal activity must be present in a bound form.

Effects of mapharsen

Tests with $1.8 \cdot 10^{-4}$ *M* mapharsen showed that this arsenoxide brings about 25% inhibition of peptidase activity. Although this is an appreciable effect for such a low concentration of arsenoxide, the enzyme is obviously not as labile a thiol compound as other enzymes occurring in *Cl. sporogenes*[4].

DISCUSSION

The simplest explanation for the described phenomena, in which the presence of energy-yielding substrates enhances the peptidase activity of *Cl. sporogenes*, is that the energy supplied by the alanine-proline or glucose-proline reactions maintain ferrous iron as an enzyme complex within the cell. It is known that energy is required for the maintenance of the sodium/potassium extracellular-intracellular ratio in mammalian tissues, particularly brain[10,11,12] and erythrocytes, and there seems to be no reason why similar considerations should not apply to the maintenance of intracellular ferrous ions in the form of an enzyme complex.

Our results give no evidence of differential leakage of ferrous ions from *Cl. sporogenes* under different conditions of incubation. Owing to the close agreement

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between the degree of peptidase activation by a heated extract of *Cl. sporogenes* and its ferrous iron content, it seems certain that the ferrous ion is implicated. The fact that the peptidase system is not inhibited by azide or cyanide (10^{-2} *M*) argues against the possibility that free or loosely bound ferrous iron is involved. Doubtless, an iron chelate is formed, possibly of the nature described by SMITH *et al.*[13] for manganese in the case of prolidase and leucine aminopeptidase. The inhibition caused by versene and $\alpha\alpha'$-dipyridyl is probably indicative of the fact that these compounds form more stable chelates with iron than that formed by the peptidase. Thus, in their presence, new chelates are formed, resulting in loss of peptidase activity.

Our evidence has also indicated that the arsenoxide, mapharsen, will inhibit the tripeptidase activity. Thus, the enzyme probably contains a labile thiol group. Whether the binding of ferrous iron with the enzyme involves the –SH group is unknown.

It has long been recognised that ferrous iron is an activator of peptidase activity in *Cl. sporogenes*. MASCHMANN[14] noted that ferrous iron, or preferably a mixture of ascorbic acid and ferrous iron or of cysteine and ferrous iron, activated peptidases in *Cl. sporogenes*. Also GROS *et al.*[15] reported a stimulation by penicillin of diglycine hydrolysis in the presence of a ferrous iron-cysteine mixture, thus implicating ferrous iron and thiol groups in peptidase activity.

The possibility has been considered that the amino acid, or glucose-amino acid, interactions maintain the peptidase-iron system in the reduced form necessary for optimal activity. Several facts, however, are not in support of this view, *e.g.*:

(1) Ferricyanide has little inhibitory effect on peptidase *per se*; yet its presence results in loss of peptidase activity when it is used as a hydrogen acceptor in the amino acid reaction.

(2) The presence of thioglycollate is insufficient to maintain optimal peptidase activity. In earlier experiments with easily inactivated thiol enzymes of *Cl. sporogenes*, particularly hydrogenase[4,6], it was found that the presence of thioglycollate is sufficient to prevent loss of activity, the thioglycollate presumably maintaining these enzymes in a reduced form.

(3) The presence of 2:4 dinitrophenol which does not affect the rate of the amino acids interactions, nor peptide hydrolysis *per se*, results in diminished peptidase activity when present during incubation of *Cl. sporogenes* with the amino acids.

(4) Although pyruvate is rapidly metabolised anaerobically, it cannot maintain the peptidase activity in *Cl. sporogenes* unless an acceptor amino acid is also present.

It is concluded, therefore, that the interactions of the amino acids, or of glucose or pyruvate with amino acids, provides *Cl. sporogenes* with a factor necessary for restoring and maintaining peptidase activity. It seems most likely that these interactions provide the energy necessary to produce and maintain an active enzyme-ferrous-thiol complex, required for optimal peptidase activity, at the low ferrous ion concentrations present in the cells. In the absence of energy-yielding substrates, the complex deteriorates, but may be restored by the addition of excess ferrous ions and thiol compounds. Whether the deterioration consists of a dissociation of the intracellular ferrous ion from the peptidase complex, with subsequent oxidation or removal of the iron, or whether it consists of a more profound change in the nature of the peptidase-iron compound is a matter for further investigation.

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SUMMARY

1. Peptidase activity of resting cells of *Cl. sporogenes* is maintained optimally in presence of energy-yielding substrates. Under conditions where there is active breakdown of amino acids or of glucose in the presence of *Cl. sporogenes*, a relatively high rate of triglycine hydrolysis is observed.

2. Interference with reactions of energy-producing substrates by ferricyanide or 2:4 dinitrophenol results in a decreased peptidase activity.

3. Ferrous, manganous and molybdate ions increase triglycine hydrolysis. The stimulations by ferrous and manganous ions are not additive.

4. Peptidase activity of *Cl. sporogenes* is inhibited by versene and $\alpha\alpha'$dipyridyl, but not by cyanide or azide (10^{-2} *M*).

5. Peptidase activity probably depends on thiol groups for activity, since it is inhibited by low concentrations of the arsenoxide, mapharsen.

6. The addition of a heated extract of *Cl. sporogenes* to extracts of this organism increases the rate of triglycine hydrolysis. The stimulation is proportional to the ferrous ion concentration of the heated extract.

7. It is suggested that the peptidase-iron complex necessary for optimal peptidase activity of *Cl. sporogenes* is of such a nature that it can be maintained under conditions where energy is supplied. In the absence of energy-yielding substrates, the complex deteriorates only to be restored by the addition of excess ferrous ions and thiol compounds.

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