($1.7 \times 10^{-4} \text{ M}$) the final colour attained is also increased, and above this concentration a marked colour appears in the absence of phosphate. No comparable effects are observed with Mg^{2+} , Co^{2+} , Ni^{2+} , Mn^{2+} , Fe^{2+} , Fe^{3+} or Sn^{2+} ions.

4. Cuprous ions reduce phosphomolybdate to the blue complex under the conditions for phosphate assay. It is suggested that copper ions act as electron carriers in the reduction of phosphomolybdate by ascorbic acid and that normally sufficient copper is present as impurity in the reagents. In the presence of metal-binding agents, including those occurring in tissues, phosphomolybdate reduction is inhibited as a result of metal-binding action.

5. The addition of $4.2 \times 10^{-6} \text{ M}$ copper ions to the system overcomes the effect of such concentrations of metal-binding agents as are likely to be met with in normal practice. A suitable modification to the procedure of Lowry & Lopez is described.

6. By using a somewhat higher concentration ($1.7 \times 10^{-4} \text{ M}$) of copper ions, the sensitivity of the method can be increased by 50 %, though the timing of operations becomes more critical under these conditions. Concentrations above $3.3 \times 10^{-4} \text{ M}$ interfere with the method.

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Hydrolysis of Amides by Extracts from Mycobacteria

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Amidases of bacterial origin have received less attention than amidases of animal origin. Amidase systems in micro-organisms which catalyse the hydrolysis of glutamine (Hughes & Williamson, 1952; McIlwain, 1948), asparagine (Altenbern & Housewright, 1954) and nicotinamide (Hughes & Williamson, 1953; Oka, 1954) have been described. Altenbern & Housewright (1954) were able to show the existence of two stereospecific asparaginases in extracts from *Brucella abortus*. Kirchheimer & Whittaker (1954) reported the presence of asparaginase activity in various species of *Mycobacteriaceae*.

This study deals with amidase activities of *Mycobacterium phlei* and *Mycobacterium tuberculosis* var. *bovis* B.C.G. and with the substrate specificities of the enzymes studied.

EXPERIMENTAL

Micro-organisms. The organisms used were *Mycobacterium phlei* 271 from the culture collection of this Department and *Mycobacterium tuberculosis* var. *bovis* B.C.G. 5692 (B.C.G.) obtained from the American Type Culture Collection (ATCC).

Cultivation of bacteria. The *Myco. phlei* strain was grown for 4 days at 35° in Roux bottles on nutrient agar supplemented with 0.1% Tween 80 (polyoxyethylene sorbitan mono-oleate). The bacteria were taken up in water, washed 3 times by centrifuging and then resuspended in water. The B.C.G. strain was grown in Sauton's medium (Sauton, 1912) for about a month at 35°. The pellicles were filtered off, washed three times with water and a thick suspension of washed cells was prepared.

Bacterial extracts. Cell-free extracts were prepared by disintegrating the washed bacteria in a 9 kcyc./sec. Raytheon sonic vibrator for 25 min. The cell debris was removed in the

cold by centrifuging in a Servall centrifuge at 10 000 *g* for 20 min. The cell-free extracts were then dialysed overnight against distilled water or running tap water at 6°. The protein content of the extracts was determined by Mehl's biuret method (Mehl, 1945).

Ammonia determination. Ammonia was determined either by Conway's microdiffusion method or colorimetrically by nesslerization of samples previously distilled into 0.1 *N*-HCl. Distillation was carried out for 40 min. in a water bath at 50° under a constant air current.

Amino acid determination. Amino acids were identified by paper chromatography on sheets of Whatman no. 2 filter paper with water-saturated phenol as the solvent. A 0.1% ninhydrin solution was used as the developing reagent. For quantitative determination of amino acids from paper chromatograms the method of Giri, Radhakrishnan & Vaidyanathan (1952) was employed.

RESULTS

Amidase activities of extracts of *Myco. phlei* and *B.C.G.* Preliminary screening revealed that cell-free extracts of *Myco. phlei* catalysed the hydrolysis of L-asparagine, glycynamide, DL-leucinamide, DL-valinamide, formamide and nicotinamide. Glutaminase activity, when present, was very weak compared to that of asparaginase. Acetamide was hydrolysed to a small extent, whereas benzamide and phenylalaninamide were not.

Extracts of *B.C.G.*, like *Myco. phlei* preparations, showed considerable amidase activity towards L-asparagine, DL-leucinamide and DL-valinamide, and only weak activity towards L-glutamine; glycynamide, formamide, nicotinamide and phenylalaninamide were not attacked. Acetamide and benzamide were not tested. These results are summarized in Table 1.

Asparaginase activity of *Myco. phlei* extracts. Dialysis of the *Myco. phlei* cell-free preparation against distilled water did not appreciably affect the

activity of the asparaginase system. The presence of 0.035 *M*-KBr (Hughes & Williamson, 1952) or of 0.02 *M* pyruvate (Greenstein & Carter, 1946) did not influence the rate of hydrolysis of asparagine. These treatments did not increase the glutaminase activity.

The influence of hydrogen-ion concentration on the asparaginase activity of *Myco. phlei* was studied. As can be seen from Fig. 1, the enzyme is active

Fig. 1. Effect of pH on asparaginase activity in *Myco. phlei* extracts. The system contained: 17.6 μmoles of L-asparagine, bacterial extract (5.8 mg. of protein), phosphate buffer (0.013 *M*); total volume, 1 ml.; incubation at 37° for 4 hr.

Fig. 2. Influence of substrate concentration on asparaginase activity of *Myco. phlei* extracts. The system contained: L-asparagine in varying concentrations (0–40 μmoles), bacterial extract (2.5 mg. of protein), phosphate buffer, pH 8.2 (0.007 *M*); total volume, 1 ml.; incubation at 37° for 1½ hr.

Table 1. *Hydrolysis of amides by extracts from Myco. phlei and Myco. tuberculosis B.C.G.*

The system contained: bacterial extract (3 mg. of protein), substrate as indicated, phosphate buffer, pH 8.3 (0.02 *M*); total volume, 1 ml.; incubation at 37° for 2½ hr.

Substrates		NH ₃ formed (μmoles)	
Nature	Amount (μmoles)	With <i>Myco. phlei</i> extracts	With <i>B.C.G.</i> extracts
L-Glutamine	10	0.3	1.1
L-Asparagine	10	2.8	9.6
Glycinamide	10	5.0	0.5
DL-Leucinamide	20	9.0	8.7
DL-Valinamide	20	10.3	9.3
DL-Phenylalaninamide	20	0	0
Nicotinamide	10	6.0	0
Benzamide	20	0	—
Formamide	20	5.2	0.1
Acetamide	20	1.0	—

over a wide range of pH values (6.5–10.5) with an optimum at pH 8.0–9.0.

The rate of hydrolysis of asparagine by the *Myco. phlei* preparation was examined. At 37°, the rate of aspartic acid formation from asparagine remained constant for more than 3 hr.

Fig. 2 shows the relationship between the initial concentration of L-asparagine and the rate of formation of aspartic acid. At a concentration of 10 μ moles/ml. the reaction rate was almost maximum and increased only slightly with further addition of substrate (up to 40 μ moles/ml.).

Substrate specificity of asparaginase. A series of analogues and derivatives of L-asparagine were tested as potential substrates for the enzyme. As can be seen from Table 2 none of the eight compounds tested was deamidated by the asparaginase of *Myco. phlei*, with the exception of the dipeptide, glycyl-L-asparagine, which was hydrolysed to glycine, aspartic acid and ammonia. This finding indicated the presence in the *Myco. phlei* extracts of a peptidase which was catalysing the hydrolysis of the dipeptide. The question arose as to whether the asparaginase could deamidate the intact peptide or whether prior hydrolysis of the dipeptide to its monomers was essential. Since the activity of some peptidases was shown to be dependent on the presence of metal ions (Smith, 1949), ethylenediaminetetraacetic acid (EDTA) was used to elucidate this point. It was found that EDTA inhibited to the same extent the liberation of glycine and ammonia from the peptide but did not inhibit ammonia formation from L-asparagine (Table 3). Paper chromatography revealed spots of aspartic acid, glycine and the residual amount of glycyl-L-asparagine, when EDTA was added to the system containing glycyl-L-asparagine as substrate; no other spot was seen, hence glycyl-L-aspartic acid appeared to be absent. On the other hand, D-asparagine, a specific inhibitor of the *Myco. phlei* asparaginase (Grossowicz & Halpern, 1956a), inhibited the liberation of ammonia from the dipeptide, but had no effect on the formation of glycine. It appears therefore that asparaginase cannot attack the intact peptide unless the latter is first hydrolysed to glycine and asparagine.

Inhibition of asparaginase by analogues of L-asparagine. Those analogues of L-asparagine which were inactive as substrates were subsequently tested for inhibitory effects on the asparaginase system (Table 4). All the analogues of L-asparagine with an unsubstituted amide group acted as inhibitors. However, substitution of one hydrogen atom in the amide radical by a propyl or butyl group abolished the inhibitory effect of the analogue.

Differentiation between amidases of *Myco. phlei*. The remarkable specificity of the asparaginase studied suggested that the various amidase activities

Table 2. *Substrate specificity of asparaginase in extracts from Myco. phlei*

The reaction mixture contained: 10 μ moles of substrate, bacterial extract (3.5 mg. of protein), phosphate buffer, pH 8.2 (0.033M); total volume, 1 ml.; incubation at 37° for 2 hr.

Substrate	NH ₃ formed (μ moles)
L-Asparagine	8.50
D-Asparagine	0.12
N ² -Ethyl-DL-asparagine	0.02
Glycyl-L-asparagine	7.60
Glycyl-D-asparagine	0
DL-Alanyl-DL-asparagine	0.50
DL- α -Asparagine (isoasparagine)	0.40
Maleamic acid	0.22
N-n-Butyl-DL-asparagine	0.20*

* Splitting of N-n-butyl-DL-asparagine by asparaginase should yield aspartic acid. No evidence (by paper chromatography) for aspartic acid formation was found.

Table 3. *Influence of ethylenediaminetetraacetic acid (EDTA) and D-asparagine on the hydrolysis of L-asparagine and glycyl-L-asparagine by extracts from Myco. phlei*

The system contained: bacterial extract (3.6 mg. of protein), 10 μ moles of substrate as indicated, 0 or 50 μ moles of EDTA, 0 or 40 μ moles of D-asparagine, phosphate buffer, pH 8.2 (0.007M); total volume, 1 ml.; incubation at 37° for 2½ hr. All values are given after correction for controls (substrate without extract, extract without additions, extract with D-asparagine alone).

Substrate	Formation of NH ₃ and glycine in the presence of inhibitors, expressed as % of control system without inhibitor			
	EDTA present		D-Asparagine present	
	NH ₃	Glycine	NH ₃	Glycine
Glycyl-L-asparagine	57.9	61.0	17.5	100.0
L-Asparagine	98.0	—	19.0	—

Table 4. *Inhibition of asparaginase by analogues of L-asparagine*

The reaction mixture contained: 10 μ moles of L-asparagine, bacterial extract (2.3 mg. of protein), inhibitor as indicated (40 μ moles), phosphate buffer, pH 8.2 (0.007M), total volume, 1 ml.; incubation at 37° for 3 hr. All values are given after correction for controls (enzyme without substrate, substrate alone and enzyme with inhibitor only).

Inhibitor added	Inhibition (%)
D-Asparagine	81
N ² -Ethyl-DL-asparagine	84
Glycyl-D-asparagine	34
DL-Alanyl-DL-asparagine	66
DL- α -Asparagine (isoasparagine)	25
N-n-Butyl-DL- α -asparagine	0
Maleamic acid	31
N-n-Propyl-maleamic acid	0
N-n-Butyl-DL-asparagine	0

Table 5. *Effect of heating in the presence of substrates on the amidase activities of Myco. phlei extracts*

The system contained: 30 μ moles of L-asparagine or nicotinamide or 10 μ moles of glycineamide, bacterial extract (5.3 mg. of protein) after heating at 65° for 15 min. in the presence of 50 μ moles of the appropriate substrate and subsequent dialysis to remove the substrate, phosphate buffer, pH 8.2 (0.017M); total volume, 1 ml.; incubation at 37°: asparaginase system for 1 hr., nicotinamidase for 30 min. and glycineamidase for 10 min.

Treatment of extract	NH ₃ formed (μ moles) from		
	L-Asparagine	Glycinamide	Nicotinamide
Untreated (control)	4.4	4.1	5.5
Heated in the absence of substrate	0	3.3	3.3
Heated in the presence of L-asparagine	1.9	3.2	2.1
Heated in the presence of glycineamide	0.3	3.0	2.3
Heated in the presence of nicotinamide	0.3	3.0	4.6

of *Myco. phlei* might be due to different enzymes. Evidence for this hypothesis was provided by the selective inactivation of the asparaginase upon storage. The extracts were normally used within 2-3 days after preparation. Storage for 2-4 weeks at 0° caused a considerable decrease (50 % or more) in asparaginase activity without affecting appreciably the amidases of nicotinamide, glycineamide and leucinamide. Further evidence was provided by three different approaches: (a) selective protection of enzymes against heat inactivation by the addition of the corresponding substrates; (b) selective inhibition of enzyme activity by substrate analogues; (c) the effect of addition of a second amide (as measured by ammonia production) to an enzyme system already saturated with one amide.

(a) Three samples of the enzyme preparation were incubated at 65° for 15 min. in the presence of asparagine, glycineamide and nicotinamide respectively. After dialysis for 18 hr. the activity of each sample towards the three substrates was determined. For comparison, the activities of an untreated extract and of extract heated in the absence of substrate were determined simultaneously. Results of a typical experiment are shown in Table 5. It is quite evident that the enzymes are selectively protected from heat inactivation by the presence of the corresponding amides. Thus, for example, the presence of asparagine during the heating of the extract protected the asparaginase but not the nicotinamidase. It can also be seen that in the absence of the substrate, the asparaginase is much more susceptible to heat (65° for 15 min.) than are the nicotinamidase and glycineamidase activities. In other experiments, under slightly different conditions (temperature and time of exposure, concentration of protecting substrate, etc.), essentially similar results were obtained. For example, asparaginase activity was completely destroyed when heated at a slightly lower temperature (59° for 15 min.) in the absence of asparagine, regardless of the presence of glycineamide or nicotinamide. The presence of asparagine during heating resulted in the preservation of 90 % of the original

Table 6. *Effect of D-asparagine on the hydrolysis of other amides by extracts from Myco. phlei*

The system contained: substrate as indicated, bacterial extract (3.8 mg. of protein), phosphate buffer, pH 8.2 (0.007M); total volume, 1 ml.; incubation at 37° for 2½ hr. All values are given after correction for controls (enzyme without substrate, enzyme with D-asparagine, substrate without enzyme).

Substrate		NH ₃ formed (μ moles)	
		No inhibitor added	30 μ moles of D-asparagine added
L-Asparagine	10	8.9	4.0
Glycinamide	10	4.0	3.8
Formamide	10	8.4	8.5
DL-Leucinamide	20	8.9	9.1
DL-Valinamide	20	8.3	7.9
Nicotinamide	10	9.2	9.4

activity. On the other hand, under the same conditions the glycineamidase activity was only slightly affected (20 %); this small inactivation was not prevented by the presence of glycineamide or of the other substrates.

(b) The second method was based on the assumption that if asparagine, nicotinamide and other amides were hydrolysed by the same enzyme, a substance inhibitory to one of these reactions (e.g. splitting of asparagine) would inhibit the hydrolysis of the other substrates (e.g. nicotinamide). Previous findings have shown (Grossowicz & Halpern, 1956a) that D-asparagine inhibits competitively the hydrolysis of L-asparagine in extracts of *Myco. phlei*. The effect of D-asparagine on the hydrolysis of other amides was therefore tested. The data presented in Table 6 show that D-asparagine had no effect on the deamidation of substrates other than L-asparagine. Similarly, 3-acetylpyridine inhibited competitively the deamidation of nicotinamide, but had no effect upon the hydrolysis of other amides tested (Grossowicz & Halpern, 1956b).

(c) Further addition of glycineamide to an enzyme system already saturated with this substrate did not increase the rate of ammonia production. On

Table 7. *Effect of addition of one amide to an enzyme system saturated with another amide*

The system contained: varying amounts of one substrate (20–60 μ moles), or a mixture of nicotinamide and glycineamide (30–60 μ moles of each), *Myco. phlei* extract (2.6 mg. of protein), phosphate buffer, pH 8.2 (0.007M); total volume, 1 ml.; incubation at 37° for 2½ hr.

Substrate added		NH ₃ formed (μ moles)
Nature	Amount (μ moles)	
Nicotinamide	20	4.2
Nicotinamide	30	4.7
Nicotinamide	40	4.8
Nicotinamide	60	5.4
Glycinamide	20	2.7
Glycinamide	30	3.4
Glycinamide	40	4.0
Glycinamide	60	4.4
Nicotinamide + glycineamide	30	8.8
Nicotinamide + glycineamide	60	10.1

the other hand, addition of a different substrate (e.g. nicotinamide) to the same system doubled the amount of ammonia formed (Table 7).

DISCUSSION

The specificity of the asparagine deamidase of *Myco. phlei* is very remarkable. The experiments reported here seem to indicate that the free α -amino and amide groups, as well as the proper optical configuration of the substrate, are essential for asparaginase activity. Substitution of one hydrogen in the α -amino group of asparagine abolishes the activity of the compound as a substrate and transforms it into an inhibitor, provided that the amide amino group remains unsubstituted. Maleamic acid, which has no α -nitrogen, also inhibits the hydrolysis of L-asparagine, but *N*-*n*-propylmaleamic acid (with a substituted amide group) is without any effect. D-Asparagine (the unnatural enantiomorph of L-asparagine) and isoasparagine (with the amino group adjacent to the amide group) proved to be specific inhibitors of the enzyme. L-Asparagine and isoasparagine substituted in the amide-amino group are inactive, both as substrates and as inhibitors.

The selective inhibition of asparaginase and nicotinamidase by substrate analogues, the selective protection against heat inactivation in the presence of the corresponding substrate and the 'addition effect' caused by the simultaneous presence of two different substrates, indicate quite clearly the presence of several distinct amidases. On the basis of the data presented it is suggested that *Myco. phlei* possesses at least three different amidases:

L-asparaginase, nicotinamidase and a third enzyme which catalyses the hydrolysis of glycineamide. Whether the hydrolysis of glycineamide, leucineamide, valineamide and formamide is catalysed by the same or different enzymes has yet to be investigated.

SUMMARY

1. The amidase activity of extracts from *Mycobacterium phlei* and *Mycobacterium tuberculosis* (B.C.G.) were studied. Extracts from either of the two species catalysed the hydrolysis of L-asparagine, leucineamide and valineamide; a slight activity was found towards L-glutamine and no activity at all towards phenylalanineamide. Extracts from *Myco. phlei* but not from B.C.G. were also active towards glycineamide, formamide and nicotinamide, but did not hydrolyse benzamide.

2. The kinetics and substrate specificity of the *Myco. phlei* asparaginase were investigated. The enzyme attacked L-asparagine only and was inactive towards D-asparagine, isoasparagine, maleamic acid or L-asparagine substituted in the α -amino or amide-amino groups.

3. D-Asparagine and other analogues of L-asparagine (isoasparagine, maleamic acid and *N*²-ethyl-DL-asparagine) inhibited the asparaginase activity. A free amide-amino group is essential for rendering the substance inhibitory.

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