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EFFECT OF COPPER ON THE COMPOSITION OF BACTERIAL CELL WALL PEPTIDES

by

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Effect of copper on the composition of bacterial cell wall peptides

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ABSTRACT

The effect of copper on the composition of bacterial cell wall peptides was investigated. When *Arthrobacter globiformis*, *Bacillus megatherium*, *Bacillus stearothermophilus*, and *Escherichia coli* were grown in 1 mM copper, the major amino acids in the peptides were repressed and some minor amino acids increased in concentration. The changes induced by Cu were reversible. Fe, Co, Mg, Mn, Ni, Pb, and Zn did not induce changes in the peptide composition. Only Mn was capable of reversing the effect of copper. Evidence is presented indicating that some or all of the original peptides were replaced by new peptides due to changes in enzyme specificity and that the changes are not genetic.

INTRODUCTION

Current ideas on sedimentary ore genesis stress the possibility of precipitation of sulfides from metalliferous brines, possibly at high pressures and elevated temperatures. The effect of such extreme conditions in modifying and limiting biological activities needs to be clarified before realistic predictions of the influence of the microorganisms on sedimentary processes can be made.

The work described here was undertaken to clarify some effects of copper on microorganisms. It is known that copper affects the growth characteristics of microorganisms and their morphology, specifically induces synthesis of cell wall components and increases osmotic fragility (4), (5). Some of the above observations indicate that copper influences the synthesis and composition of cell walls. Since peptides can form up to 60% of bacterial cell walls (23), the effect of copper on the synthesis and peptide composition in cell wall of four bacterial species was studied.

MATERIALS AND METHODS

All percentages are expressed as $^{w}/_{v}$ and all reagents were A.R. Grade unless otherwise specified. Sodium hydroxide (0.1 M) or phosphoric acid (0.1 M) was used for pH adjustment of the growth media.

Microorganisms and their growth optimum temperatures:

Arthrobacter globiformis;	32°
Bacillus megatherium;	35°
Bacillus stearothermophilus;	
Type NCA-1503-4R;	53°
Escherichia coli; B K12	37°

The microorganisms were grown in a Microferm Laboratory Fermentor (New Brunswick Scientific Co. Inc.) (6) at their appropriate growth temperature in 12 liter batches on a complex medium containing peptone, yeast extract, glucose and phosphates. One liter of an overnight culture of the organisms was used as an inoculum. The medium was stirred at 200 rev. per minute and aerated at 8 liters per minute; the pH was automatically maintained at 6.8. Growth was monitored turbidimetrically. When required, a sterile solution of metals was added in the early stages of the log phase of the culture to a given final concentration of 1 mM. Cu, Zn, Ni, Co, Mn, Mg, and Fe were added as sulfates, Pb was added as acetate. The organisms were harvested at the late log phase, washed 3 times with 1 M KCl and 3 times with distilled water. The harvesting and washing was carried out at 4°. If not used immediately the bacteria were freeze-dried and kept at -20°.

Free amino acids: 500 mg of washed cells were extracted twice with 50 ml of 5% TCA at 4°. The solid material was removed by centrifugation at 20,000 x g. The supernatant was extracted three times with ether. The aqueous layer was freed of residual ether by passing nitrogen through the sample. The amino acids in the

crude fraction were purified as described by Gehrke (10).

Cell walls: The cell walls were prepared and purified by the method of Sutton and Welker (32), dialyzed against distilled water at 4°, freeze-dried, kept over P_2O_5 for 48 hours and then stored at -20°.

X-ray diffraction: 50 mg samples of the purified cell walls were analysed by X-ray diffraction using a Phillips PW 1010 generator equipped with Cu K α tube. For the range 1° to 20° 2θ, ¼° slits with a full scale deflection of 4×10^2 counts per second (cps) were used. For the range 20° - 80° the setting was altered to 1° slits and 4×10^2 cps full scale deflection.

Cell wall hydrolysis: The purified cell walls were resuspended in 6N HCl and hydrolysed under nitrogen at 105° for 8 hours.

Enzymic solubilisation of the cell walls:

a) Myxobacter Al_1 enzyme. The technique described by Pettit was used (21).

b) Lysozyme. 100 mg of purified cell walls were resuspended in 20 ml of 0.01 M phosphate buffer pH 6.5, containing 0.1% of NaCl, 0.01 M EDTA and 80 mg of lysozyme (3.2.1.17 - Mucopeptide N-acetylmuramylhydrolyse) (Calbiochem, 3x recrystallized, > 10,000 units/mg, A grade). The suspension was incubated for 60 minutes at 30°. The undissolved material (less than 3%) was removed by centrifugation at 27,000 x g for 15 minutes. The supernatant was desalted in a Baird and Tatlock (London) chromatographic desalting apparatus at 50 mA for 15 minutes, freeze-dried and kept at -20°.

Purification of amino acids: The crude amino acids were purified on Amberlite IR-120H (10).

Separation and estimation of amino acids.

a) Gas liquid chromatography (GLC). The amino acids were esterified as described by Coulter and Hann (8). The amino acid derivatives were analysed on a Varian Aerograph Model 1520 gas chromatograph equipped with flame ionisation detectors for dual column operation using nitrogen (30 ml/min) as carrier gas. 1.5 m 6 x mm glass columns packed with 2% neopentyl glycol adipate on 80-100 mesh Chromosorb W-AW-DCMS were used. The injection port was fitted with glass liners. The stationary phase was coated on the solid support using the method of Horning, Vanden Heuvel and Creech (14), except that the packing was dried using a fluidized drier (16). During the analysis the temperature was programmed from 100° to 250° at 4° per minute and the temperature held at 250° until all material was eluted. The peaks were identified by co-injection of standards. The concentration of individual amino acids was determined from peak areas using molecular response data obtained from injection of standards.

b) Thin layer chromatography: The mixture of purified amino acids was spotted on Gelman SA Instant Thin Layer Chromatography (ITLC) plates and developed in two directions with n-butanol: acetic acid: water (60:20:20) and with $CHCl_3$: MeOH: 58% conc. NH_4OH (40:40:20) respectively. The amino acids were located with ninhydrin, $CdCl_2$ in isopropanol, and identified by comparing with standards.

Amino sugars:

a) Muramic acid:

1) Electrophoresis. The hydrolysate of purified cell walls was spotted on Whatmann 3 MM paper. Muramic acid (Sigma) was spotted on either side of the hydrolysate spot. The material was subjected to electrophoresis in 2 M pyridine-acetate buffer, pH 3.6 for 2 hours at 3KV (13). After drying the spots were located using Morgan-Elson reagent (31).

2) Thin layer chromatography. After electrophoresis the paper containing muramic acid was cut out and eluted with 0.05 M ammonia, the eluate evaporated to dryness, redissolved in distilled water and spotted on Gelman ITLC plate Type SA. The spots were developed concurrently with authentic muramic acid using butanol-pyridine-water-acetic acid (60: 40: 30: 3). After drying the materials were located by charring with sulphuric acid.

b) Glucosamine: The acid fraction of the cell wall hydrolysate was analysed for glucosamine using the procedures described for muramic acid. The glucosamine was located by comparison with a standard.

Peptides: The freeze-dried material obtained by lysis of the cell walls was redissolved in distilled water at concentrations of 10 mg per ml. 10 µl were spotted on Whatmann 3 MM paper and the electrophoresis was performed on a Miles Hivolt Electrophoretic Unit for 60 minutes at 3KV using pyridine: acetic acid: water (112.5: 31.5:900) buffer pH 5.6. The spots were located using the o-tolidine-tungstate reagent (2).

Radioactive amino acids: A mixture of radioactive amino acids containing glycine, alanine, serine, threonine, proline, valine, leucine, isoleucine, phenylalanine, tyrosine, aspartic acid, glutamic acid, lysine, arginine, histidine uniformly labelled with C^{14} and with specific activity of 1mC/mg and α-ε-diaminopimelic-1, 7-C^{14} acid with a specific activity of 13mC/mM (International Chemical and Nuclear Corporation) were incorporated into bacterial cells and the resulting radioactivity was measured as described previously (7). To check the activity of the individual amino acids, the mixture was separated by ITLC, areas corresponding to individual acids were cut out, powderized, mixed with scintillation solution (7) and their activity was measured using Bry's methods (3).

Copper and manganese estimation: 200 mg of dried cells were resuspended in 5 ml of concentrated HNO_3 and 2 ml of 50% perchloric acid and the suspension was evaporated on a steam bath. After three digestions the sample was made up to 5 mls with 1N HCl. The metals were then estimated with a Techtron (Model AA-5) atomic absorption spectrophotometer. The copper concentration was measured at 327.4 nm and that of manganese at 279.4 nm.

Determination of guanine-cytosine ratio (GC) in DNA. The DNA was isolated by a modification (27) of the method of Murmur (18), the buoyant density obtained by the method of Szybalski (33) and the guanine-cytosine content was estimated by the method of De Ley (9).

RESULTS

X-ray diffraction: A comparison of X-ray diffraction patterns of

purified cell walls from organisms grown in the presence or absence of copper showed considerable differences in the crystalline structure (Table 1). No significant changes were observed in the X-ray diffraction patterns of the cell walls originating from organisms grown in the absence of copper and subsequently exposed to 1 mM copper at 4° for 24 hours.

Cell wall solubilization: Attempts to solubilize the cell walls using Myxobacter Al_1 enzyme were not successful. Only 15% of cell walls originating from copper free organisms and no appreciable amount of cell walls from copper grown organisms were solubilized. When lysozyme was used for the solubilization, less than 3% of insoluble material was recovered after the lysis in both cases.

Composition of cell walls:

Peptide amino acids: The amino acids composition of the purified cell walls from *B. stearothermophilus*, *B. megatherium*, *E. coli*, and *A. globiformis* confirmed previous observations that alanine, glutamic acid and α-ε-diamino pimelic acid (DAP) or lysine are the major constituents of the cell wall peptides of gram-positive bacteria. In gram-negative organisms the quantitative division in the amino acids concentration is less distinct (24).

Effect of copper: When the organisms were grown in the presence of 1 mM copper, the cell wall peptide composition was altered considerably. The major amino acids were repressed and some minor amino acids previously present only in small quantities (or not detected) increased in concentration. An exception was *B. megatherium*, where the concentration of the major amino acid,

glutamic acid actually increased (Table 2). Although the amino acid composition of the peptides was altered the total number of moles of amino acids per unit weight of purified cell walls was not significantly changed. The changes induced by Cu were reversible: the amino acid composition of cell walls isolated from bacteria which had been grown in the presence of copper and then reinoculated into Cu-free media, were similar to those of bacteria which had not been exposed to copper.

Effect of other metals: No significant changes in the amino acid pattern were observed when any of the organisms were grown in the presence of Zn, Ni, Co, Mn, Mg, Fe, and Pb. With the exception of Mn, none of these metals was capable of reversing the effect of copper. When Mn was added at 1 mM concentration simultaneously with copper or if it was present in the medium prior to copper addition, a normal copper-free amino acid pattern was obtained.

Amino sugars: The acid cell wall hydrolysates of copper free and copper grown organisms contained material that behaved on electrophoresis and ITLC similarly to muramic acid. A component having the same chromatographic and electrophoretic property as glucosamine was identified in the hydrolysate.

Peptides: The peptides from the purified cell walls were isolated and analysed electrophoretically. Significantly different patterns were obtained for organisms grown in the presence of copper. Figure 1 shows the results obtained for *A. globiformis*. Similar results were obtained with other organisms.

Free amino acids: No particular differences in the composition of the free amino acids pools were detected in the copper grown and copper free organisms.

Incorporation of radioactive amino acids. 1 mM copper did not affect significantly the general incorporation of the amino acids into stationary cells of *B. stearothermophilus*, *B. megatherium*, and *E. coli*. In *A. globiformis* a slight enhancement of the incorporation was noticed on addition of copper. 1 mM copper completely inhibited further incorporation of C^{14}-DAP into cells of *A. globiformis*. After 10 minutes the concentration of radioactive DAP already incorporated into the cells gradually declined (Fig. 2). Similar patterns were obtained with other organisms.

When copper was added at the growth inhibitory concentration of 1.5 mM to the stationary cells, the incorporation of the amino acids into *A. globiformis* continued for about 30 minutes at a gradually decreasing rate. Both the incorporation into the proteins and the incorporation into the free amino acids pool including the RNA complexes were affected (Fig. 3). Similar results were obtained with other organisms.

When the mixture of amino acids from the cell walls of 1 mM Cu-grown *A. globiformis* was analysed by ITLC, no spot corresponding to DAP was observed. The radioactivity originally present in DAP was located in lysine.

Determination of guanine-cytosine ratio in DNA: DNA was isolated from *B. stearothermophilus* grown in the absence or presence of copper and/or from the organisms grown in the absence of copper but exposed to it for 24 hours at 4°. The values for the buoyant density of the DNA of 1.7124 ± (GC 52.8%) were obtained in all cases.

Copper and manganese estimation: The Cu and Mn contents of whole cells of *A. globiformis* are presented in Table 3.

DISCUSSION

The copper induced variation in the composition of amino acids in the cell wall peptides could result from at least three changes in the cellular composition of the micro-organisms:

a) alteration in the composition of the cellular amino acid pool,

b) changes in the relative amounts of cell wall peptides,

c) replacement of some or all of the original peptides by peptides of different composition.

We have shown that the first possibility was unlikely since copper did not alter the amino acid composition of the pool.

To establish if the variation of the amino acids was due to the changes in peptides, the cell walls had to be solubilized with a minimum of damage to the peptides. As the product of the lysis by lysozyme is O-β-N-acetylglucosaminyl -(1 → 6)-O-β-N-acetylmuramic acid peptide (26), (12), it was necessary to establish that the changes in the electrophoretic pattern of the solubilized cell walls was due to peptides and not due to some changes in the amino sugars. The results of the amino sugar analysis supported the suggestion that the changes occurred in the peptide moieties.

As the electrophoretic pattern obtained with the copper grown cell walls indicates presence of material absent in the copper free cell walls, it is suggested that the amino acid variation was caused by synthesis of new peptides.

The peptides in the bacterial cell walls are synthesized by individual enzymes, each being specific for a certain amino acid (1), (30). The formation of the peptide bonds requires Mn (29). It has been shown that the specificity of at least one of these enzymes, the glutamyl adding enzyme, is dependent on the presence of Mn (19). The effect of additions of Mg, Co, Cd, Al, Zn, Ni, Hg, and Ca on the enzyme specificity was investigated and it was found that only Co and Mg partially restored the enzyme's specificity. Furthermore, it has been shown that with peptidases that replacement of Zn by Mn, Fe, Co, Ni, Cd, or Hg leads to changes in the specificity of the enzymes (20). It may be significant that the changes in specificity due to metals, involved enzymes which *in vivo* recognize individual amino acids. It is suggested that in our work, the change of the amino acid composition was possibly due to changes of enzyme specificity by Cu-Mn competition. The replacement of one amino acid by a different one in the bacterial cell wall peptides has been reported previously (28).

DAP is present only in bacterial cell walls (22). Therefore, the results obtained with the incorporation of radioactive DAP indicate directly the changes occurring in the cell wall peptides. From the results obtained (Table 2) it is evident that the incorporation into the cell wall peptides was affected

significantly with 1 mM Cu. In *A. globiformis* the GLC analysis showed the absence of DAP from the peptides (Table 2). The ITLC analysis of the cell wall amino acids showed that the residual radioactivity after 50 minutes was due to lysine. This was probably due to the fact that in bacteria, DAP and lysine are part of a common synthetic pathway and lysine can be formed by decarboxylation of DAP (25). The time required to remove DAP from the cell wall peptides was correlated with the observation where turnover of cell wall peptides of 0.6 generation times was reported (17). The observation that only the cell wall peptide synthesis and not the overall protein synthesis was significantly affected by copper was consistent with the observation that in 1 mM copper the growth pattern of the organisms was not significantly changed (4).

X-ray diffraction analysis of bacterial cell walls has been used to evaluate the sub-microscopic character of the cell walls, in particular the lipids (11), (15). Our work shows that cell walls freed from lipids, give a characteristic pattern and that such a pattern is altered if the organisms were grown in the presence of copper. It is not known if such changes are due to the alteration of the peptide composition or to a different arrangement of large cell wall subunits.

Since the guanine-cytosine contents of the DNA from cells grown in the presence and absence of copper were identical and since copper treated cells resumed a normal peptide composition when grown in a Cu-free medium, we assume the observed changes were not genetic.

The results of our work support the following suggestions:

Copper changes the chemical composition of the cell wall peptides by induction of synthesis of new peptides. The mechanism responsible for such changes could be due to an alteration of the specificity of the peptide synthesizing enzymes caused by Cu-Mn competition. The consistency of the amino acids/dry weight cell walls ratio indicates that the terminating mechanism for the peptide synthesis is not affected by copper.

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X-ray diffraction analysis of bacterial cell walls grown in the presence or absence of copper

Organisms	Position of major peaks in nm	
	-Cu	+Cu
E. coli	236.3	86.7
	157.2	73.2
	74.6	42.0
	45.6	
	43.6	
B. stearothermophilus	490.8	95.3
	245.4	90.7
	162.2	79.4
	40.3	70.8
B. megatherium	447.3	107.3
	251.5	100.5
	230.4	90.6
	209.5	70.3
	193.0	
	190.5	
A. globiformis	111.5	
	97.5	
	90.0	

Table 1. Characteristic peaks obtained by X-ray crystallographic analysis with purified bacterial cell wall from organisms grown in the presence or absence of copper.

The percentage of individual amino acids in bacterial cell walls grown in the presence or absence of copper

	Organisms							
	E. coli		*B. stearothermophilus*		*B. megaterium*		*A. globiformis*	
Amino acids	-Cu	+Cu	-Cu	+Cu	-Cu	+Cu	-Cu	+Cu
Ala	33	22	36	12	60	19	53	42
Asp	2	9	2	9	-	12	1	3
Cys	-	-	1	5	-	-	-	-
DAP	16	7	28	5	27	6	20	-
Glu	23	10	22	12	13	17	19	8
Gly	1	1	1	10	1	10	-	-
Leu	2	9	5	13	-	9	1	2
Lys	7	18	-	-	-	3	1	6
Phe	1	1	2	8	-	3	1	1
Pro	1	1	1	12	-	3	-	-
Ser	1	7	1	-	-	9	1	10
Thr	1	3	1	7	-	5	1	3
Tyr	11	9	-	-	-	-	-	-
Val	1	3	1	7	-	4	2	25
	m moles of amino acids/100g cell-walls							
	277	213	240	230	316	339	216	232

Table 2. Composition of cell wall peptides of organisms grown in the presence or absence of copper. The concentration of individual amino acids are expressed as a percentage of the total amino acids forming the peptides.

Cu and Mn content of the whole cells of *A. globiformis*

Metal added	Cu µg/g	Mn µg/g
None	37	30
Cu	3862	15
Mn	35	4560
Mn + Cu	3375	1870
Cu reinoculated*	40	31

Table 3. The metal indicated was added to 1 mM final concentration in the early log phase. The cells were harvested at the end of the log phase, freeze-dried, stored over P_2O_5 for 48 hours and Cu, and Mn were estimated by the atomic absorbtion technique.

* The inoculum was grown in the presence of Cu, harvested and washed and then reinoculated into copper free medium; resultant growth was harvested and treated as described above.

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Fig. 1. Electrophoretic pattern of cell wall peptides isolated from *Arthrobacter globiformis* grown in the presence or absence of 1 mM copper.

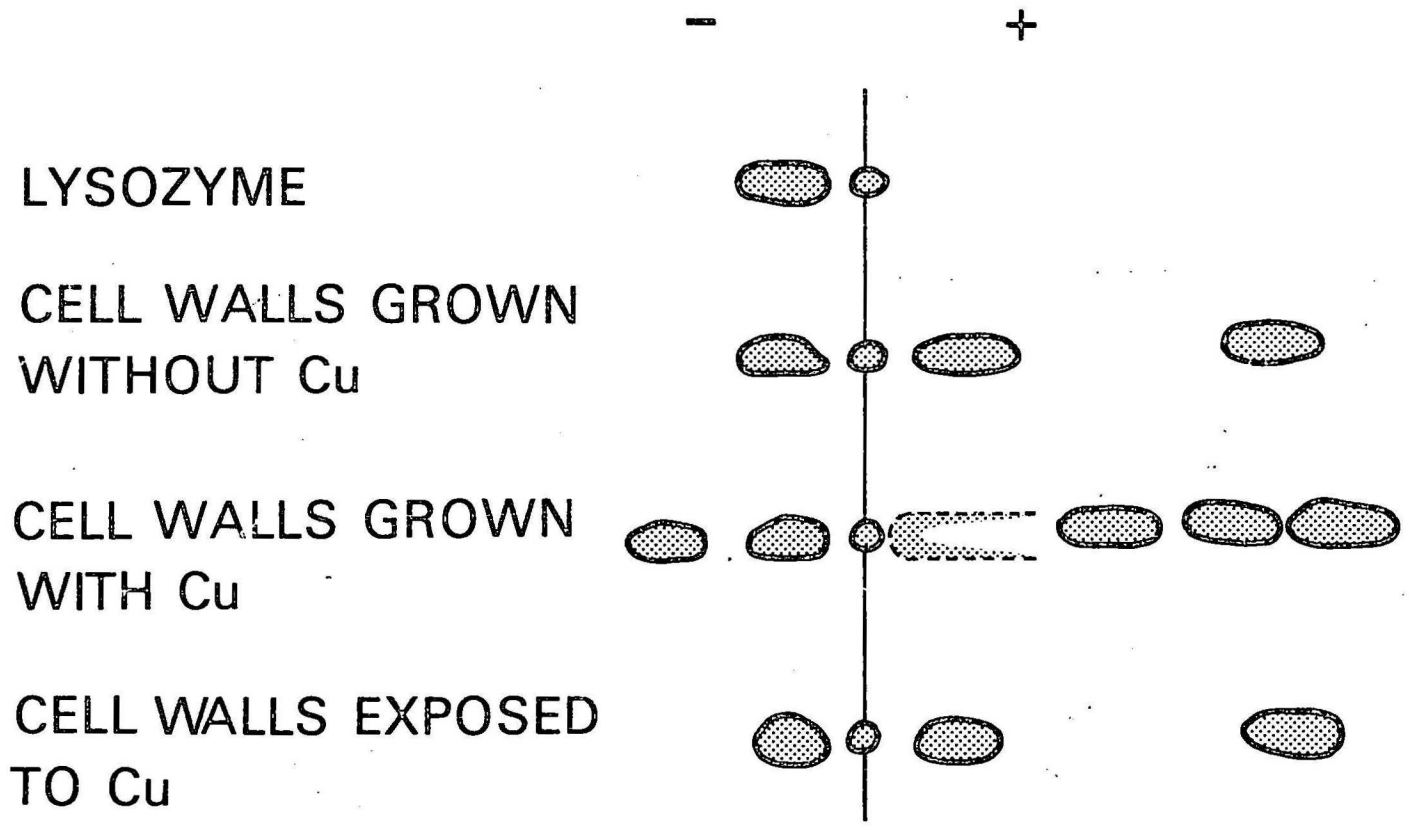

−
+
LYSOZYME
CELL WALLS GROWN WITHOUT Cu
CELL WALLS GROWN WITH Cu
CELL WALLS EXPOSED TO Cu

Fig. 2. Incorporation of radioactive amino acids into stationary cells of *Arthrobacter globiformis*.

– . – . – Incorporation of a mixture of amino acids into cellular proteins.

– O – O – O – Incorporation of DAP into cell wall peptides.

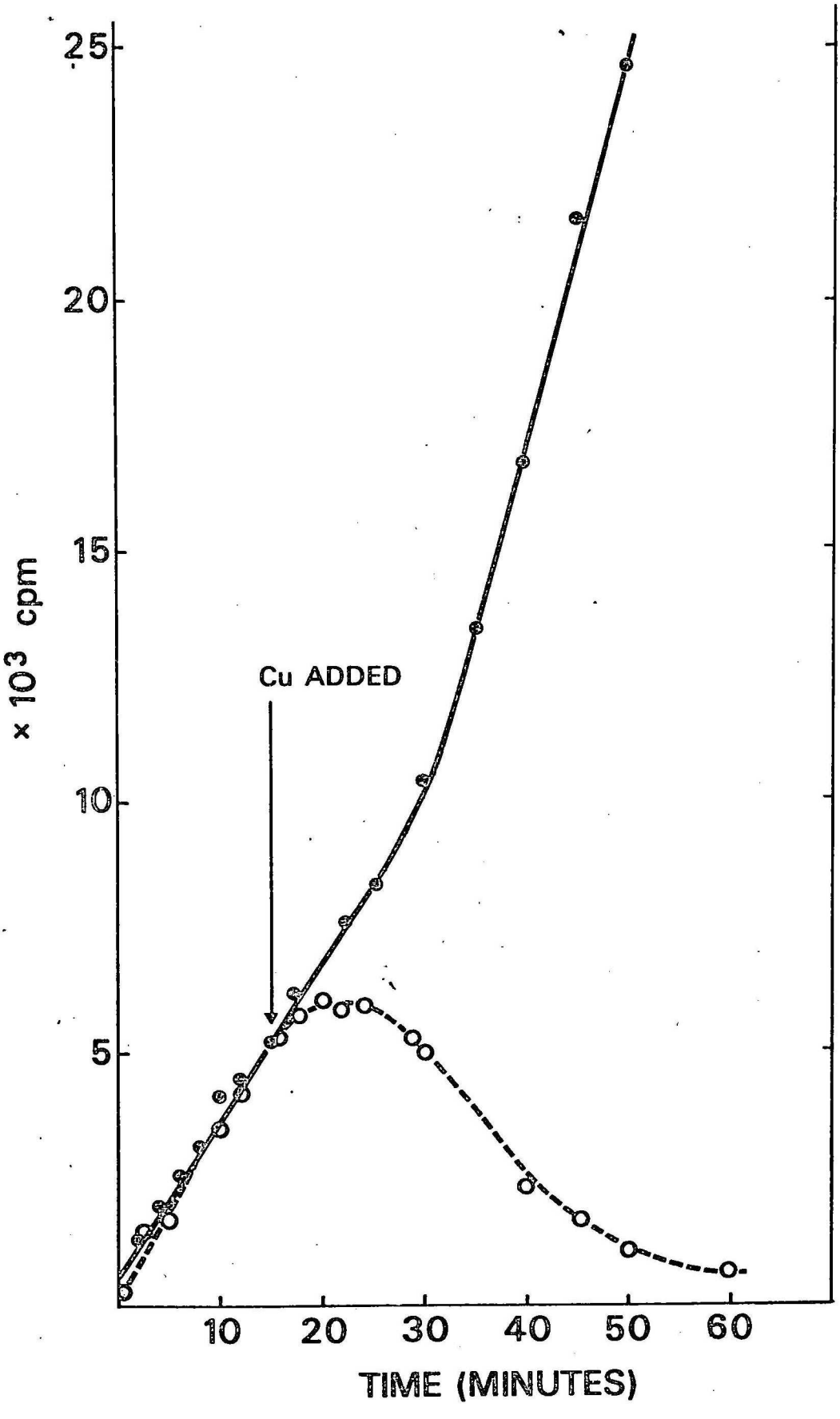
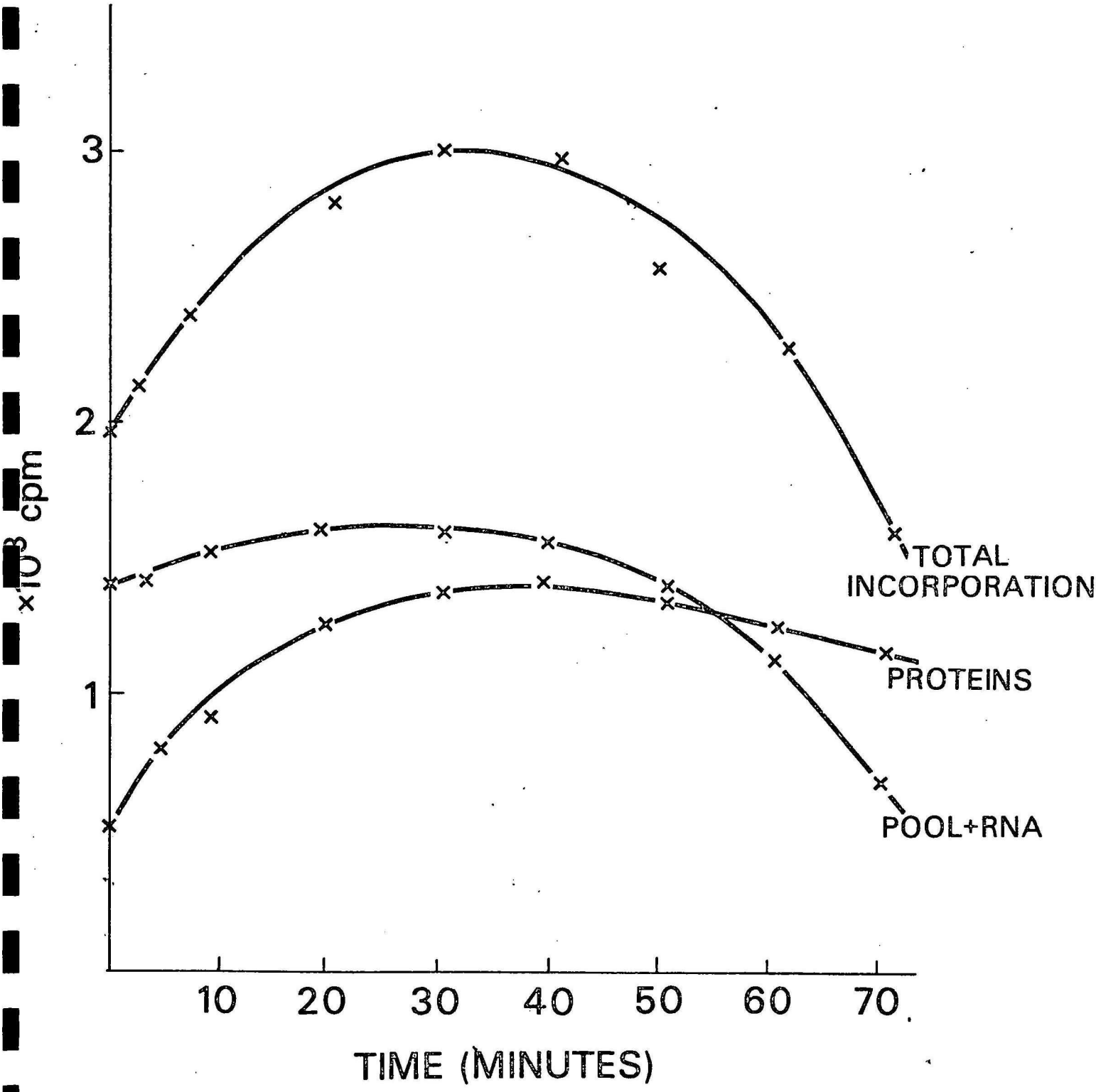

25
20
15
10
5
× 10³ cpm
Cu ADDED
10
20
30
40
50
60
TIME (MINUTES)

Fig. 3. Incorporation of radioactive amino acids into stationary cells of *Arthrobacter globiformis* in the presence of 1.5 mM copper.


3
2
1
×10³ cpm
TOTAL INCORPORATION
PROTEINS
POOL+RNA
10
20
30
40
50
60
70
TIME (MINUTES)