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An Examination of the Inhibitory Effects of *N*-Iodoacetylglucosamine on *Escherichia coli* and Isolation of Resistant Mutants

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1. After incubation of *Escherichia coli* with *N*-iodo[1,2-$^{14}C_2$]acetylglucosamine, 95–99% of the ^{14}C taken up by whole cells is located in a cold-trichloroacetic acid-soluble fraction. Two major components of this fraction are *S*-carboxymethylcysteine and *S*-carboxymethylglutathione. The same compounds accumulate during incubation with iodo[^{14}C]acetate but not with iodo[^{14}C]acetamide. The amount of ^{14}C associated with a cold-trichloroacetic acid-insoluble fraction are comparable for all three alkylating agents. After incubation with iodo[^{14}C]acetamide, 50% of the label bound to whole cells is recoverable in a cold-trichloroacetic acid-insoluble fraction. 2. Uptake and incorporation of ^{14}C from [U-^{14}C]glycerol is blocked at an early stage by *N*-iodoacetylglucosamine. No specific inhibition of macromolecular synthesis could be demonstrated. 3. Mutants selected for resistance to iodoacetate are partially resistant to iodoacetate and *N*-iodoacetylglucosamine, but show no resistance to iodoacetamide. 4. Mutants selected for resistance to *N*-iodoacetylglucosamine are not resistant to iodoacetate or iodoacetamide, and are defective in their ability to grow on *N*-acetylglucosamine. Resistance to *N*-iodoacetylglucosamine is not absolute, and depends on the presence of glucose or certain other sugars; there is no resistance during growth on maltose, glycerol or succinate. 5. Absolute resistance can be achieved by selecting for a second mutation conferring resistance during growth on maltose; double mutants isolated by this procedure are unable to grow on *N*-acetylglucosamine and grow poorly on glucosamine. Resistant single mutants have a slightly diminished uptake of *N*-acetyl[1-^{14}C]glucosamine, but in resistant double mutants the uptake of both [1-^{14}C]glucosamine and *N*-acetyl[1-^{14}C]glucosamine is severely diminished. 6. These observations are consistent with the presence of two permeases for *N*-acetylglucosamine, one that also permits uptake of glucosamine and another that allows entry of methyl 2-acetamido-2-deoxy-α-D-glucoside. *N*-Iodoacetylglucosamine can gain entry to the cell by both permeases.

In the preceding paper it was shown that INAG† was a potent growth inhibitor for *Escherichia coli* (Kent, Ackers & White, 1970). Non-specific effects on the uptake of carbohydrates were also demonstrated, but these are unlikely to have been solely responsible for the cessation of growth. In the present investigation we have examined possible mechanisms of toxicity by studying the fate of [1,2-$^{14}C_2$]INAG incubated with cells of *E. coli*, and by isolating mutants resistant to INAG and iodoacetate.

MATERIALS AND METHODS

Radioactive compounds. Iodo[2-^{14}C]acetic acid, iodo-[1-^{14}C]acetamide, [U-^{14}C]glycerol and [1-^{14}C]*n*-hexadecane were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The preparation of [1,2-$^{14}C_2$]-INAG is described in the preceding paper (Kent *et al.* 1970).

Assay of radioactivity. Radioactivity in whole cells, trichloroacetic acid-insoluble material and aqueous solutions was measured with a Beckman Liquid Scintillation System. The scintillation mixture used contained 7g of 2,5-bis-(5-*tert.*-butylbenzoxazol-2-yl)thiophen/l of toluene for whole cells and trichloroacetic acid-insoluble material, or 7g of 2,5-bis-(5-*tert.*-butylbenzoxazol-2-yl)thiophen and 80g of naphthalene/l of 2-methoxyethanol–toluene (2:3, v/v) for aqueous samples. [1-^{14}C]*n*-Hexadecane was used as an internal standard. Radioactivity on electrophoresis strips was determined with a Packard model 7201 Radiochromatogram Scanner.

Preparation of samples for measurement of radioactivity. Whole cells were collected by filtration on Millipore filters (type HA), washed with 5ml of cold medium and dried at 60°C for 30min. For trichloroacetic acid-insoluble material, whole cell samples were mixed with an equal

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† Abbreviation: INAG, *N*-iodoacetyl-D-glucosamine.

volume of 10% (w/v) trichloroacetic acid and kept at 4°C for 15min. Insoluble material was collected on a membrane filter, washed with 5ml of 2% (v/v) acetic acid three times and dried at 60°C for 30min.

Paper electrophoresis. High-voltage electrophoresis was carried out on Whatman no. 1 or Whatman no. 3MM paper (for preparative runs). The apparatus used was essentially that of Michl (1951) except that white spirit 100 (Esso Ltd., London S.W.1, U.K.) was used as coolant. Voltage gradients used were in the range 60–100V/cm. Three buffer systems were used: pH1.9, formic acid–acetic acid–water (1:4:45, by vol.); pH3.5, pyridine–acetic acid–water (1:10:89, by vol.); pH6.5, pyridine–acetic acid–water (25:1:225, by vol.). Amino acids and peptides were detected with collidine–ninhydrin reagent (Dawson, Elliott, Elliott & Jones, 1969). Reducing compounds were detected with the alkaline $AgNO_3$ reagent described by Trevelyan, Procter & Harrison (1950).

Reaction of iodoacetic acid and INAG with glutathione and cysteine. Alkylating agent (4μmol) was added to a solution containing 4μmol of the thiol compound and 20μmol of sodium phosphate buffer, pH8.0, to give a final volume of 0.8ml. The mixture was incubated at 37°C overnight and stored frozen.

Acid hydrolysis. Peptides were hydrolysed in sealed glass ampoules *in vacuo* with 6M-HCl for 18h at 108°C. After hydrolysis, samples were freeze-dried and taken up in minimal volumes of water (10–50μl) for electrophoresis.

Isolation of resistant mutants. Exponential-phase cells of *E. coli* ML308 were treated with *N*-methyl-*N*-nitro-*N'*-nitrosoguanidine by the method of Adelberg, Mandel & Chen (1965). After incubation with mutagen, bacteria were shaken for 5h at 37°C in 1.3% (w/v) nutrient broth to allow phenotypic expression, and were then spread on to selective agar [minimal medium containing 1.2% (w/v) agar, 0.01M-glucose and 0.1mM-iodoacetic acid or 0.05mM-INAG]. Agar plates were incubated at 37°C for 48h and then examined for resistant colonies. Twentyfive iodoacetate-resistant and the same number of INAG-resistant colonies were picked off the agar plates and purified by making single colony isolates. Conditions of growth and media are described in the preceding paper (Kent *et al.* 1970).

RESULTS

Uptake and incorporation of [1,2-$^{14}C_2$]*INAG.* Incubation of whole cells of *E. coli* ML308 with [1,2-$^{14}C_2$]INAG resulted in a rapid uptake of label, which reached a plateau at about 20min (Fig. 1). Conditions of pre-growth affected the initial rate of uptake and the plateau value. Analysis of cells at the plateau (20min) showed that 95–99% of the label inside cells was diffusible on dialysis and soluble in cold 5% trichloroacetic acid. Incubation of cells with iodo[^{14}C]acetic acid gave essentially the same result, but if iodo[^{14}C]acetamide was used a considerably smaller proportion of the label was in a trichloroacetic acid-soluble form. A comparison of the distribution of radioactivity between the acid-soluble and acid-insoluble fractions after cells had been incubated with ^{14}C-labelled alkylating agents is given in Table 1. The small amount of label associated with the acid-insoluble fraction was approximately the same in each case, but the amount in the soluble fraction is markedly less for iodoacetamide.

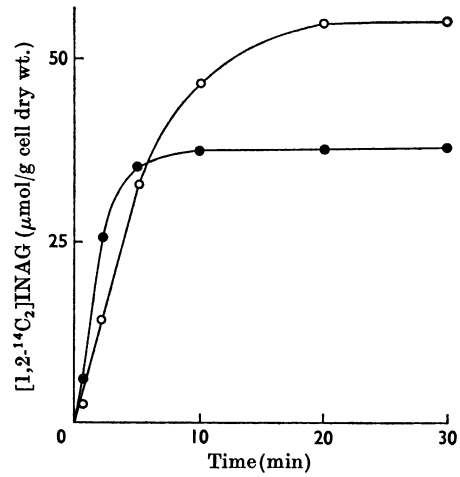

Fig. 1. Uptake of ^{14}C from [1,2-$^{14}C_2$]INAG by whole cells of *E. coli*. Cells grown in casein or minimal medium+glucose were resuspended in minimal medium containing 50μg of chloramphenicol/ml to give 600μg cell dry wt./ml. [1,2-$^{14}C_2$]INAG (0.4μCi/mg) was added to give a final concentration of 52μM. Suspensions were incubated at 37°C, and 0.2ml samples were collected on membrane filters, washed and dried and their radioactivity was measured. ●, Casein-grown; ○, glucose-grown.

Analysis of compounds accumulated during inhibition with iodoacetic acid and INAG. Gel filtration with Sephadex G-25 partially resolved the trichloroacetic acid-soluble fraction of cells labelled with [1,2-$^{14}C_2$]INAG into two components (see Fig. 2); the peak 1/peak 2 ratio varied from 2.6:1 (Fig. 2) to 6:1 in different experiments. The two peaks were separately freeze-dried and samples were subjected to paper electrophoresis. In both cases the radioactivity was associated with ninhydrin-positive material. Subsequent experiments showed that cell extracts could be subjected to electrophoresis directly, without prior gel filtration, and the same two major radioactive ninhydrin-positive spots were obtained. The method of extraction used, e.g. osmotic shock, 0.5M-perchloric acid, ultrasonic treatment at 0°C or 75% (v/v) ethanol, did not significantly alter the pattern obtained. Electrophoresis demonstrated that both components were anionic at pH6.5 and pH3.5 but cationic at pH1.9. The two components were purified by preparative paper electrophoresis at pH3.5 and hydrolysed in 6M-hydrochloric acid. One component (peak 2 in Fig. 2) was not altered by acid hydrolysis, whereas the other (peak 1) gave three new ninhydrin-

Table 1. *Location of* ^{14}C *after incubation of* E. coli *with* ^{14}C*-labelled iodoacetate, iodoacetamide and INAG*

Glucose-grown bacteria were resuspended in minimal medium containing 0.01M-glucose to give 600μg cell dry wt./ml. The culture was divided into three portions and [1,2-$^{14}C_2$]INAG (0.4μCi/mg), iodo[2-^{14}C]-acetate (83.3μCi/mg) or iodo[1-^{14}C]acetamide (37.9μCi/mg) was added to give a final concentration of 0.05mM. Cultures were shaken for 1h at 37°C, harvested, washed and extracted with 5% (w/v) trichloroacetic acid for 15min at 4°C. Results are expressed as μmol of [^{14}C]alkylating agent bound/g cell dry wt.

Fraction	Incorporation		
	Iodo[2-^{14}C]acetate	Iodo[1-^{14}C]acetamide	[1,2-$^{14}C_2$]INAG
Whole cells	24.8	0.95	17.4
Trichloroacetic acid-soluble	24.3	0.44	16.7
Trichloroacetic acid-insoluble	0.5	0.45	0.8

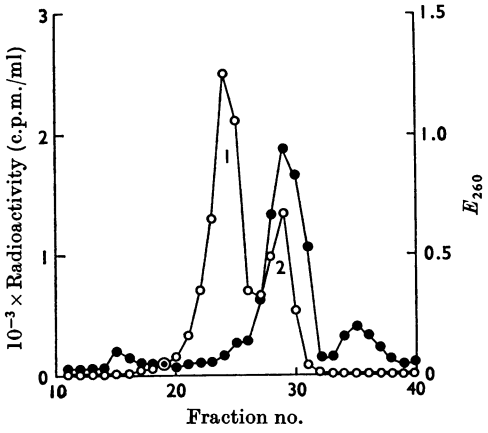

Fig. 2. Gel filtration of acid-soluble pool after incubation of *E. coli* with [1,2-$^{14}C_2$]INAG. Washed glucose-grown cells (3mg dry wt./ml) were incubated in minimal medium containing 0.05mM-[1,2-$^{14}C_2$]INAG (0.43μCi/mg) at 37°C for 75min. The labelled cells were harvested, washed and extracted in 10ml of 5% (w/v) trichloroacetic acid for 15min at 4°C. Trichloroacetic acid was removed by shaking with 10ml of ether (three times) and the neutral extract was chromatographed on a column of Sephadex G-25 (2cm diam. × 45cm; void volume 53ml) and eluted with water. Fractions (3.75ml) were collected and radioactivity (○) and E_{260} (●) determined.

positive constituents. Electrophoretic comparison of these with marker amino acids at pH1.9 established that the three new components behaved like glutamic acid, glycine and *S*-carboxymethylcysteine. The component that was not altered by hydrolysis (peak 2, Fig. 2) also had the same mobility as *S*-carboxymethylcysteine. An identical pattern was observed whether the cells were incubated with iodo[2-^{14}C]acetic acid or [1,2-$^{14}C_2$]-INAG. Radioactivity determinations after hydrolysis and electrophoresis showed that only *S*-carboxymethylcysteine was labelled. These results suggested that peak 1 was *S*-carboxymethylglutathione and that peak 2 was *S*-carboxymethylcysteine itself. To check this possibility, samples of these compounds were prepared by allowing the alkylating agents to react with glutathione and cysteine *in vitro*. Synthetic *S*-carboxymethylglutathione had the same electrophoretic mobility as peak 1 at pH1.9, pH3.5 and pH6.5 and gave the same pattern at pH1.9 after acid hydrolysis. The adduct formed between INAG and glutathione behaved differently, and in addition to being ninhydrin-positive could be detected with alkaline silver nitrate. Peak 1 from cells incubated with INAG is indistinguishable from *S*-carboxymethylglutathione and has no reducing power. A comparison of extracts from inhibited cells, i.e. incubated with alkylating agent, and control cells reveals that appearance of the two new components is associated with the disappearance of one ninhydrin-positive compound; subsequent analysis has shown this to be free glutathione.

Comparison of inhibition by INAG and chloramphenicol. To test whether inhibition of growth by INAG is due to any specific effect on macromolecular synthesis, its action was compared with that of chloramphenicol, an established inhibitor of protein synthesis. Inhibitor was added to cells growing in the presence of [U-^{14}C]glycerol and the subsequent fate of the label was followed (Fig. 3). It is immediately apparent that the alkylating agent, as compared with chloramphenicol, drastically diminishes the amount of label taken up by cells. Analysis of the trichloroacetic acid-soluble fraction and medium shows other major differences: chloramphenicol-treated cells accumulate and excrete large amounts of glutamic acid into the medium but this does not occur with INAG. Clearly the metabolism of glycerol is inhibited at an early stage by the alkylating agent.

Isolation of mutants resistant to alkylating agents. In the preceding paper (Kent *et al.* 1970) it was shown that *N*-acetylglucosamine could abolish the inhibitory effect of INAG. Further, the observation that the compounds accumulated during inhibition

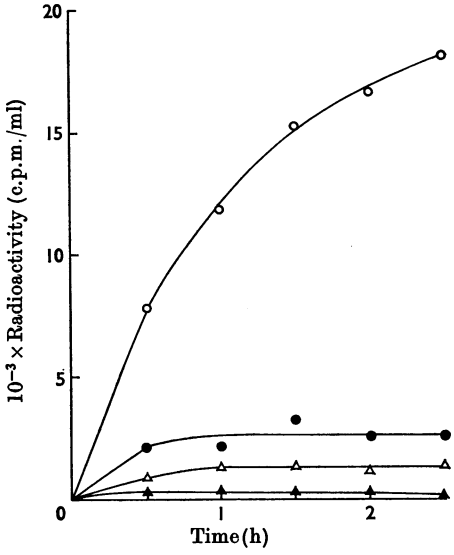

Fig. 3. Comparison of the effects of chloramphenicol and INAG on the uptake of ^{14}C from [U-^{14}C]glycerol by *E. coli*. Two cultures (2×50ml of minimal medium containing 10mM-glycerol and 100μg cell dry wt./ml)were shaken with chloramphenicol (10μg/ml) or INAG (0.1mM) for 15min at 37°C. Then 6.25μg of [U-^{14}C]glycerol (155μCi/mg) was added and the uptake of ^{14}C was followed by membrane filtration. Trichloroacetic acid-soluble ^{14}C was estimated by subtracting radioactivity for trichloroacetic acid-insoluble ^{14}C from radioactivity for whole cells. ○ and ●, Chloramphenicol; △ and ▲, INAG. ○ and △, Whole-cell ^{14}C; ● and ▲, trichloroacetic acid-soluble ^{14}C.

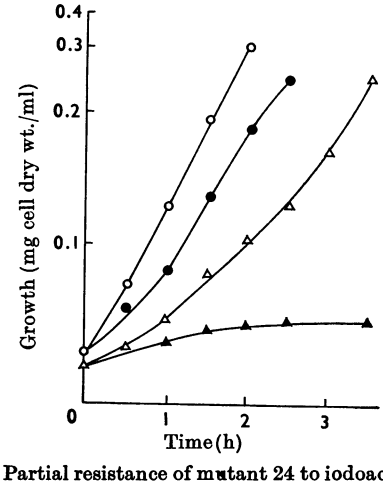

Fig. 4. Partial resistance of mutant 24 to iodoacetate and INAG. Minimal medium containing 10mM-glucose was inoculated with exponential-phase cells of mutant 24 and shaken at 37°C. Inhibitors were added at zero time to give a final concentration of 0.1mM. Growth was measured with a nephelometer. ○, No additions; ●, +iodoacetate; △, +INAG; ▲, +iodoacetamide.

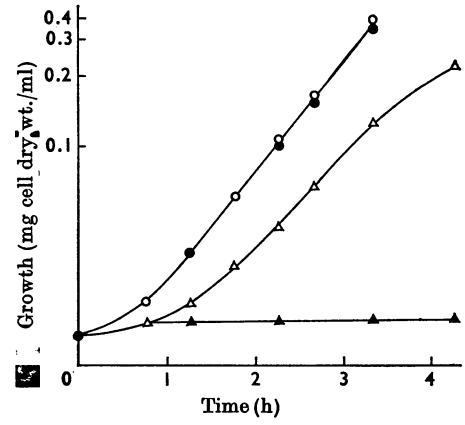

Fig. 5. Resistance of mutant 29 to INAG during growth on glucose. Minimal medium containing 10mM-glucose or 10mM-glycerol was inoculated with exponential-phase cells of mutant 29 and shaken at 37°C. Inhibitors were added at zero time to give a final concentration of 0.1mM. Growth was measured with a nephelometer. ○ and △, No additions; ● and ▲, +INAG. ○ and ●, Glucose as carbon source; △ and ▲, glycerol as carbon source.

by INAG are the same as those for iodoacetic acid implies that INAG is catabolized by cells to yield iodoacetic acid. If this is so, enzymes involved in this catabolism should play an essential role in toxicity and their loss should confer resistance. For this reason mutants resistant to INAG were isolated (see the Materials and Methods section) and characterized. Mutants resistant to iodoacetate were also isolated and checked for cross-resistance to INAG. All the INAG-resistant mutants examined showed complete resistance to INAG during growth on liquid or solid media containing 0.01M-glucose as carbon source, i.e. conditions of their selection (Fig. 4). They showed no detectable resistance to iodoacetate or iodoacetamide. All the iodoacetate-resistant mutants examined were only partially resistant to iodoacetic acid and were also partially resistant to INAG but they showed no detectable resistance to iodoacetamide (Fig. 5). Growth of wild-type *E. coli* is inhibited by iodoacetate, iodoacetamide and INAG (Kent *et al.* 1970). Mutations to INAG-resistance occurred at considerably higher frequency (one mutant/2.5×10^3 bacteria after mutagenesis and phenotypic growth) than those to iodoacetate resistance (one mutant/5.0×10^5 bacteria), which presumably explains the failure to isolate mutants that were partially resistant to iodoacetic acid and INAG by both selective procedures (only 25 INAG-resistant mutants were screened). All the mutants resistant to INAG were defective in their ability to grow on *N*-acetyl-

Table 2. *Mean generation times of* E. coli *mutants resistant to INAG*

Bacteria were shaken at 37°C in minimal medium containing 10mM carbon source, and growth was measured with a nephelometer. Results are given as time (min) taken for doubling of dry weight.

Bacteria	Glucose	Glucosamine	*N*-Acetylglucosamine
Wild-type	49	87	64
Mutant 26	52	87	318
Mutant 29	48	90	110
Mutant 34	48	85	84
Mutant 41	48	>3000	>3000
Double mutant 26L	51	378	>3000
Double mutant 34M	50	750	>3000

Table 3. *Activity of* N*-acetylglucosamine-catabolizing enzymes in* E. coli *mutants resistant to INAG*

Bacteria were grown in minimal medium containing 10mM-glucose, harvested, washed and disrupted in a French pressure cell. The disrupted cell suspension was centrifuged at 30000*g* for 30min and enzyme activities in the supernatant were determined. Enzyme activities are expressed as μmol of substrate metabolized/h per mg of protein at 37°C. n.d., Not determined.

Bacteria	Glucosamine 6-phosphate deaminase	*N*-Acetylglucosamine 6-phosphate deacetylase	*N*-Acetylglucosamine kinase*
Wild-type	0.44	1.95	0.47
Mutant 26	3.34	0.76	0.37
Mutant 29	0.37	1.60	0.45
Mutant 41	<0.01	1.20	0.45
Double mutant 26L	3.05	0.45	n.d.
Double mutant 34M	0.18	1.88	n.d.

* EC 2.7.1.9; this is an ATP-dependent kinase.

glucosamine as sole source of carbon (Table 2). Most mutants exhibited a slightly diminished growth rate, i.e. they resembled mutant 34, but mutant 41 did not grow at all on *N*-acetylglucosamine or glucosamine. The mutants were further characterized by assaying enzymes involved in *N*-acetylglucosamine catabolism (White, 1968) and the results are presented in Table 3. Mutant 41 has no detectable deaminase activity, and mutant 26 has a diminished deacetylase activity, but mutant 34 has normal activities of all three enzymes. Failure to locate any defect in mutant 34 (the most common type) suggested that permeation of *N*-acetylglucosamine might be affected. Loss of deaminase alone is not sufficient to confer resistance, as a mutant lacking deaminase isolated by a different selective procedure (White, 1968) is sensitive to INAG. It therefore seems that mutant 41 must have an additional defect. A mutant lacking the deacetylase (White, 1968) is also sensitive. Although all INAG-resistant mutants were resistant to INAG during growth on glucose, they were sensitive when grown on glycerol, succinate and maltose (conditionally resistant). Conditions under which the mutants are resistant are given in Table 4; the pattern of resistance obtained in liquid medium occasionally differed from that on agar. Mutant 26 is sensitive on lactose, but a secondary mutant was isolated that is resistant on this disaccharide (26L). Similarly, mutant 34 is sensitive on maltose and a resistant secondary mutant was isolated (34M). Both double mutants (26L and 34M) resistant to INAG under all conditions of growth (unconditionally resistant) are unable to grow on *N*-acetylglucosamine and grow poorly on glucosamine (see Tables 2, 3 and 4). If the ability of mutants 34 and 34M to take up [1-^{14}C]glucosamine and *N*-acetyl-[1-^{14}C]glucosamine is compared with that of wild-type, marked differences are observed (Table 5). Although mutant 34 takes up a normal amount of [1-^{14}C]glucosamine, mutant 34M takes up only 10% of the value for the wild-type. Further, mutant 34 has only a slightly decreased uptake of *N*-acetyl-[1-^{14}C]glucosamine, whereas mutant 34M takes up

Table 4. *Pattern of sensitivity and resistance of* E. coli *mutants during growth on different carbon sources*

Resistance was tested by streaking cultures on to minimal agar containing 10mM-carbon source and 0.05mM-INAG. Growth was checked after 16h at 37°C. n.g., No growth in absence of inhibitor; +, resistant; −, sensitive.

	N-Acetylglucosamine	Glucose	Lactose	Maltose	Glycerol	Succinate
Wild-type	+	−	−	−	−	−
Mutant 26	+	+	−	−	−	−
Mutant 29	+	+	+	−	−	−
Mutant 34	+	+	+	−	−	−
Mutant 41	n.g.	+	+	−	−	−
Double mutant 26L	n.g.	+	+	+	+	+
Double mutant 34M	n.g.	+	+	+	+	+

Table 5. *Uptake of* [1-^{14}C]*glucosamine and* N-*acetyl*[1-^{14}C]*glucosamine by* E. coli *mutants* 34 *and* 34*M*

Cells grown in minimal medium containing 10mM-glucose+10mM-*N*-acetylglucosamine were washed and resuspended in fresh minimal medium containing 50μg of chloramphenicol/ml to give 500μg cell dry wt./ml. [1-^{14}C]Glucosamine (18.9μCi/mg) or *N*-acetyl[1-^{14}C]glucosamine (14.0μCi/mg) was added to give a final concentration of 0.1mM. Glucose and fructose were added to give 10mM solutions where indicated. A 0.2ml sample of whole cells was collected by membrane filtration after incubation for 1min at 37°C. Results are expressed as μmol of ^{14}C-labelled substrate taken up/min per g dry wt.

	[1-^{14}C]-Glucosamine	Glucose + [1-^{14}C]-glucosamine	Fructose + [1-^{14}C]-glucosamine	*N*-Acetyl-[1-^{14}C]-glucosamine	Glucose + *N*-acetyl-[1-^{14}C]-glucosamine	Fructose + *N*-acetyl-[1-^{14}C]-glucosamine
Wild-type	21.28	0.23	12.45	37.53	10.56	27.58
Mutant 34	28.12	0.38	15.80	28.52	0.29	22.88
Double mutant 34M	2.02	0.21	0.77	2.68	1.15	4.06

only 7% of the value for the wild-type; the diminished uptake in mutant 34 is more evident in the presence of glucose when it is virtually zero. Iodoacetate-resistant mutants show the same partial resistance to iodoacetate and INAG under all conditions of growth. Analysis of the trichloroacetic acid-soluble pool of INAG-resistant mutants after incubation with INAG and glucose reveals a normal glutathione pool and no *S*-carboxymethylglutathione or *S*-carboxymethylcysteine. Iodoacetate-resistant mutants form the carboxymethyl derivatives of glutathione and cysteine in the presence of iodoacetate and INAG.

DISCUSSION

Earlier work had shown INAG to be a potent inhibitor of growth for *E. coli* (Kent *et al.* 1970). It is not possible to say whether the accumulation of *S*-carboxymethylglutathione and *S*-carboxymethylcysteine is a primary event in the inhibition of growth by iodoacetate and INAG or merely a secondary effect. As yet no specific function has been assigned to glutathione (Boyland & Chasseaud, 1969), and Roberts, Cowie, Abelson, Bolton & Britten (1957) were unable to demonstrate that it has any essential role in the metabolism of *E. coli*. Nevertheless, in mammalian systems, glutathione–protein mixed disulphides have been found and it would be relevant in view of the present findings to determine whether similar structures play any biochemical part, especially in membrane phenomena, in bacteria (Mize, Thompson & Langdon, 1962; Huisman & Dozy, 1962; Revesz & Modig, 1965; Huisman, Dozy, Horton & Nechtman, 1966; Modig, 1968; Jackson, Harrap & Smith, 1968; Harding, 1969). Previous work on the cysteine pool in *E. coli* (Wheldrake, 1967) has shown it to be 400-fold lower than that of glutathione. In the present experiments the intracellular concentration of *S*-carboxymethylcysteine was three- to six-fold lower than that of glutathione. This large difference could be accounted for by a cellular degradation of *S*-carboxymethylglutathione to yield *S*-carboxymethylcysteine; enzymes of this type are certainly present, as *E. coli* can utilize glutathione and some of its derivatives, e.g. *S*-acetylglutathione, as sole source of sulphur for growth (Roberts *et al.* 1957). If cells are inhibited by preincubation with INAG, addition of glutathione or cysteine causes no enhancement of recovery. Although the reaction *in vitro* of iodoacetate with glutathione and cysteine is well documented (Dickens, 1933), little work has been published on alkylation *in vivo*. Schroeder, Woodward & Platt (1933) suggested that the thioether of glutathione is formed when yeast is inhibited with iodoacetate.

Although label associated with the acid-insoluble fraction after incubation with iodo[2-^{14}C]acetate or [1,2-$^{14}C_2$]INAG (Table 1) only represents a small part of total cellular radioactivity, this is not true for the cells treated with iodo[1-^{14}C]acetamide, where the acid-soluble and acid-insoluble fractions are equivalent. Lawley & Brookes (1968) have reported that acid-insoluble radioactive material formed during incubation of *E. coli* with iodo[1-^{14}C]-acetamide is principally due to alkylated cysteine residues in proteins; nucleic acids are not alkylated. Thus it is possible that alkylation of enzymes essential to growth (represented by the trichloroacetic acid-insoluble radioactivity) is responsible for the inhibitory effects of all three alkylating agents. On the other hand, mutations conferring partial resistance to iodoacetate and INAG give no resistance to iodoacetamide, suggesting that their mode of action is different. The mechanism of this partial resistance is not clear, but does not appear to involve glutathione, as *S*-carboxymethylglutathione and *S*-carboxymethylcysteine are still found after exposure to the inhibitor.

A comparison with inhibition by chloramphenicol (Figs. 3 and 4) indicates the lack of any specific effect

on protein and nucleic acid synthesis by INAG. Although the results demonstrate the inhibition of glycerol utilization at an early stage, this does not mean that subsequent stages are not affected in addition.

Results obtained with INAG-resistant mutants show that INAG is taken up and catabolized by the same pathway as *N*-acetylglucosamine. Although deacetylation occurs during inhibition of wild-type *E. coli*, this step is not essential for the development of toxicity, as mutants lacking *N*-acetylglucosamine 6-phosphate deacetylase, isolated by a different selective procedure (White, 1968), can still be inhibited. It is not known whether deacetylation of inhibitor in wild-type *E. coli* occurs before or after adduct formation. Experiments on the uptake of labelled glucosamine and *N*-acetylglucosamine (Table 5) show that mutant 34 has a permeability defect specific for *N*-acetylglucosamine. A second mutation in the double mutant (34M) affects the uptake of glucosamine and *N*-acetylglucosamine. Thus there appear to be two (at least) distinct uptake mechanisms for *N*-acetylglucosamine (see also White, 1968); one, specific for *N*-acetylglucosamine, is poorly blocked by glucose; the other, which also transports glucosamine, is powerfully blocked by glucose. Because of this it is not possible to obtain unconditional resistance to INAG by a single mutation. Conditionally resistant mutants, such as mutant 34, have lost the specific transport system and depend on glucose to prevent uptake via the non-specific system. Unconditional mutants, such as mutant 34M, have also lost the non-specific system and no longer depend on glucose. The method used to isolate mutants would have eliminated any that had lost only the non-specific system, as glucose would be unable to prevent INAG entering via the specific system. The role of the non-specific system in glucose transport is uncertain, but those mutants lacking it (mutants 34M and 26L) grow at normal rates on glucose. Although other sugars are not as effective in inhibiting the non-specific system, it appears that catabolism of glucose is essential for inhibition, as methyl α-glucoside (a non-metabolizable analogue of glucose previously used for transport studies; Cohen & Monod, 1957) does not protect mutant 34 from INAG. Romano & Kornberg (1968) have suggested that acetyl-CoA regulates the entry of some sugars in *Aspergillus nidulans*. If uptake via the non-specific permease were controlled by the intracellular concentration of a metabolite such as acetyl-CoA, the effects obtained with different sugars (Table 4) could be due to variations in the pool size of the metabolite inhibitor. Catabolite repression does not appear to be involved, as cells of mutant 34 pregrown on maltose grow immediately on a mixture of glucose and INAG. Although loss of the specific permease only results in a 24% decrease in the rate of *N*-acetylglucosamine uptake, there is a corresponding increase in the mean generation time (Table 2), which implies that growth on *N*-acetylglucosamine is limited by transport; this has also been shown for the lactose system (Rickenberg, Cohen, Buttin & Monod, 1956) and the galactose system (Horecker, Thomas & Monod, 1960). The use of metabolizable substrates for transport assays (as in Table 5) prevents any precise conclusions being made at this stage about the nature of the two permeases for *N*-acetylglucosamine. In the following paper a non-metabolizable transport analogue is described, and resistant mutants are shown to have a defective phosphoenolpyruvate-dependent phosphorylation system (White, 1970).

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