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Incorporation of Sarcosine into the Actinomycins Synthesized by *Streptomyces antibioticus*

BY O. CIFERRI, A. ALBERTINI AND G. CASSANI
Department of Genetics, University of Pavia, Italy

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Sarcosine is an amino acid apparently not widely distributed in Nature. As a free amino acid, sarcosine has been demonstrated in the radial caeca of the starfish *Astropecten aurantiacus* (Kossel & Edlbacher, 1915), in the muscles of Elasmobranchii (Tarr, 1958) and in the reindeer moss, *Cladonia silvatica* (Linko, Alfthan, Miettinen & Virtanen, 1953). Sarcosine has been found bound in peptides in the antibiotics of the actinomycin (Dalgliesh, Johnson, Todd & Vining, 1950) and etamycin (Sheehan, Zachau & Lawson, 1957) groups. The reported presence of sarcosine in the hydrolysate of groundnut protein (Haworth, MacGillivray & Peacock, 1951) has recently been questioned (Greenstein & Winitz, 1961).

The absence, in cell-free extracts of actinomycin-producing Streptomycetes, of enzymes activating sarcosine by an ATP–pyrophosphate exchange (Ciferri, Di Girolamo & Bendicenti Di Girolamo, 1961) prompted the following investigation on the incorporation of sarcosine into actinomycins. As will be shown, sarcosine appears to be incorporated as such into the sarcosine portion of the antibiotic. The amino acid is, in addition, utilized as a source of glycine for the synthesis of protein. In the absence of sarcosine from the fermentation media, the data indicate that glycine provides the aminoacetic portion of the sarcosine of the antibiotic.

A preliminary note has already appeared (Ciferri, Albertini & Rossi, 1962).

While this paper was in preparation, Katz & Weissbach (1963) reported similar results for another strain of *Streptomyces antibioticus*.

EXPERIMENTAL

Materials. L-[*methyl*-^{14}C]Methionine (sp. activity 18·3 mc/m-mole), [1-^{14}C]glycine (sp. activity 7·9 mc/m-mole), [1,2-$^{14}C_2$]glycine (sp. activity 5·25 mc/m-mole) and [1-^{14}C]-sarcosine (sp. activity 8·1 mc/m-mole), were obtained from The Radiochemical Centre, Amersham, Bucks. [3-^{14}C]-Sarcosine (sp. activity 0·65 mc/m-mole) was obtained from the New England Nuclear Corp. Methyl-labelled sarcosine was also obtained by feeding the culture with methyl-labelled methionine and isolating the sarcosine of the antibiotic by the procedures reported in the text; it had sp. activity 0·031 mc/m-mole.

Micro-organism. Streptomyces antibioticus (no. 1692 of the Culture Collection of the Botanical Institute of this University) was maintained on slants of Emerson's medium (Emerson, Whiffen, Bohonas & DeBoer, 1946). A suspension of spores from such slants was used to inoculate the vegetative medium: soya-bean flour 20 g., glucose 10 g., tap water 1000 ml. (adjusted to pH 7·4 with N-NaOH). The culture was incubated at 28° for 48 hr. on an alternating shaker. The cells were recovered by centrifuging in the cold, washed twice with the fermentation medium (see below) and suspended in the same solution to give a cell concentration twice that of the vegetative-medium suspension. A portion (5 ml.) of this suspension was used to inoculate 100 ml. of the synthetic galactose–glutamic acid ('fermentation') medium (Katz & Goss, 1960). In general, the fermentations were carried out for 72 hr. under the conditions described above for the growth of the inoculum. Addition of radioactive and non-radioactive amino acids, separately sterilized, were made at the times reported in the text.

When the fermentation was completed the cells were recovered from the liquid by centrifuging, washed once with 10 vol. of 1 mM-EDTA (disodium salt) solution at pH 7·8 and stored frozen at approx. −18°.

Extraction and purification of the actinomycins. After centrifuging of the cells, the liquid medium and the EDTA washing were combined and the actinomycins extracted and purified as reported by Ciferri *et al.* (1962).

Degradation of actinomycin. Portions (10 mg.) of purified unlabelled antibiotic were degraded in three different ways (Fig. 1).

(1) Hydrolysis in 6N-HCl in a sealed tube at 110° for 24 hr. (Hanger, Howell & Johnson, 1958) resulted in the production of a melanin-like residue from the chromophoric portion of the antibiotic, which was separated by centrifuging, washed twice with water, dried and counted. The acid solution and the washings, which contained the hydrolysed peptides, were used for the separation of the amino acids (see below).

(2) Actinocinin (Brockmann & Muxfeldt, 1956) was obtained by hydrolysing the antibiotic in 20% HCl under reflux for a period (6 hr., in general) sufficient for the solution to become dark green. The solution was cooled for a few hours and the dark-green crystals were separated by filtration, washed with cold water and dissolved in a few millilitres of a saturated solution of $NaHCO_3$. Red crystals of actinocinin separated on acidification with HCl, and were recovered by centrifuging. The HCl solution was used for the separation of the amino acids of the peptide chains.

(3) Depeptidoactinomycin, a rearrangement of the chromophoric phenoxazinone, was prepared by hydrolysing

the antibiotic with 2N-$Ba(OH)_2$ and purified by the procedure outlined by Dalgliesh *et al.* (1950).

When the radioactive antibiotic was degraded, samples corresponding to 5000–10000 counts/min. (approx. 1·2–2·5 mg.) were degraded without carrier in procedure (1) and with enough unlabelled carrier to give 10 mg. in procedures (2) and (3).

The solutions from the acid hydrolysis were dried *in vacuo* over P_2O_5 and NaOH, and the solid residue was dissolved in a few millilitres of butan-1-ol–acetic acid–water (4:1:5, by vol.; top phase). The resultant solution was then applied to the top of a glass column (70 cm. × 1·2 cm.) packed with Whatman no. 1 cellulose equilibrated with butan-1-ol–acetic acid–water. The amino acids were eluted with approx. 300 ml. of the above-mentioned mixture (Johnson, Todd & Vining, 1952). The order of emergence of the amino acids, as determined by paper chromatography, was: *N*-methylvaline ≥ valine > alloisoleucine > proline > sarcosine > threonine. Hydrolysis in a sealed tube resulted in the almost total destruction of the threonine of the antibiotic (Johnson & Mauger, 1959). The fractions containing each amino acid were pooled and dried on a rotary evaporator.

Uptake of glycine and sarcosine by growing cells. To duplicate 24 hr. old cultures, each in 100 ml. of synthetic medium, were added 10 μc of [1-^{14}C]sarcosine (1·23 μmoles) with 20 or 200 μmoles of [^{12}C]sarcosine or 10 μc of [1-^{14}C]-glycine (1·26 μmoles) with 20 or 200 μmoles of [^{12}C]glycine. At various time-intervals, 5 ml. samples were pipetted out under sterile conditions, quickly centrifuged to free them from the mycelium and frozen. Fractions (4 ml.) of each sample were extracted with 1 vol. of ethyl acetate to remove the radioactive actinomycin. The ethyl acetate layer was removed and washed with 1 vol. of water. The two aqueous phases (the mother liquor from the extraction and the wash of the ethyl acetate) were combined and portions of the solutions counted. Percentage disappearance of the amino acid from the medium was referred to the control sample taken at 0 hr. A portion of the sample taken at 36 hr. from the fermentation carried out in the presence of [1-^{14}C]sarcosine with 200 μmoles of [^{12}C]-sarcosine was examined by paper chromatography according to the procedure reported in the next section. Only one radioactive amino acid was evident and it was indistinguishable from sarcosine.

Determination of the cellular content of glycine and sarcosine. The amount of glycine and sarcosine present in the cells was determined as follows. Cells (24 hr. old) from 100 ml. of synthetic medium were recovered by centrifuging in the cold, washed twice with 1 mM-EDTA, pH 7·8, and extracted for 1 hr. at room temperature with 100 ml. of a 5% (w/v) solution of trichloroacetic acid. The suspension was filtered and the filtrate brought to a normality of 0·05 by addition of 5N-HCl. The acid solution was extracted several times with ethyl ether until all trichloroacetic acid was removed. Portions (10 ml.) of the solutions

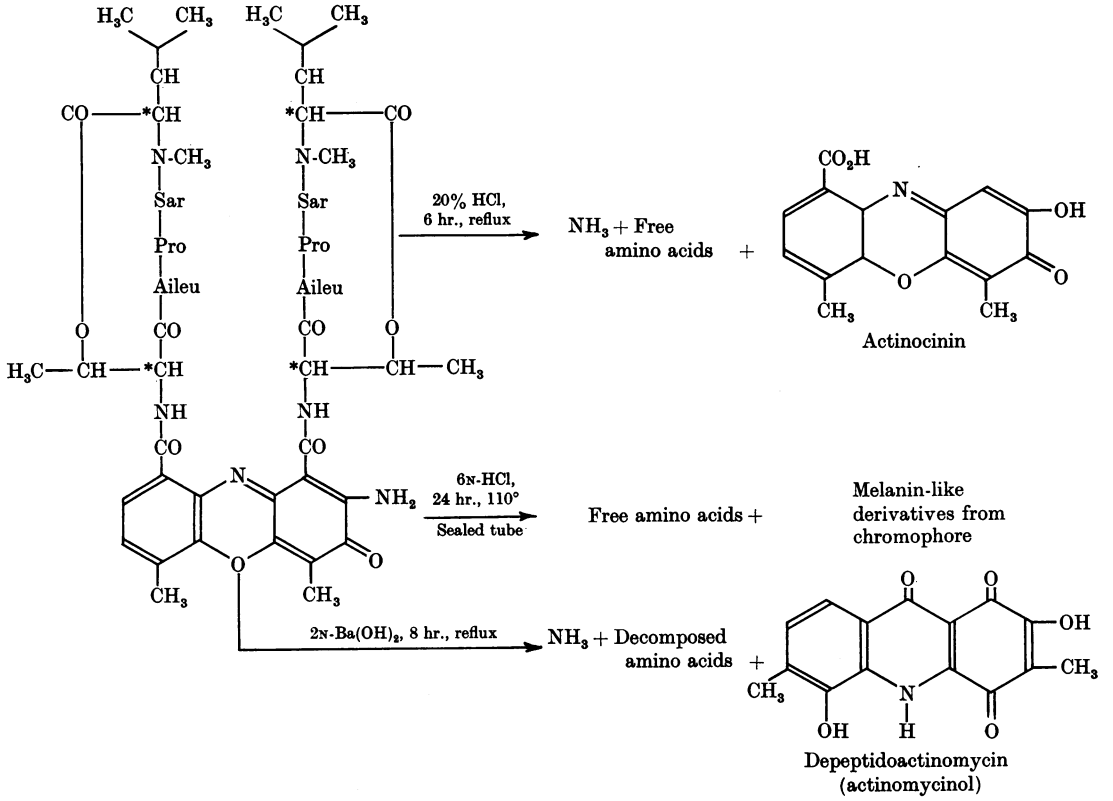

Fig. 1. Degradation of actinomycin VII (actinomycin C_3). Sar, Sarcosine; Aileu, D-alloisoleucine; Pro, L-proline. For details see the Experimental section. *, Denotes carbon atoms having the L-configuration.

were applied as bands to sheets of Whatman no. 1 filter paper together with 40000 counts/min. of [1-^{14}C]sarcosine (0·19 μg.) or of [1-^{14}C]glycine (0·17 μg.). The paper was developed in butan-1-ol–acetic acid–water (63:10:27, by vol.). The radioactive area, located by scanning with a S.E.L.O. (Società Elettronica Lombarda) Chromatoscan, was cut out, eluted and, after concentration *in vacuo*, applied to a second chromatographic sheet, which was irrigated with 80% (v/v) phenol in water. The radioactive area recovered from the second chromatogram behaved as a single component in two additional solvent systems: *tert.*-butyl alcohol–ethanol–aq. NH_3 soln. (sp.gr. 0·88)–water (4:12:1:3, by vol.); methanol–ethanol–water (9:9:2, by vol.). The content in glycine and sarcosine of the eluates was determined by ninhydrin assay (Moore & Stein, 1948) and, on the basis of the radioactivity recovered, corrected for the amount of amino acid lost during the entire procedure. Glycine content ranged, in duplicate experiments, from 3·29 to 3·5 mg./100 ml. of cell culture, whereas sarcosine was not detected (since its limit of identification was found to be 7 μg., the total sarcosine content of the cells would have been less than 0·175 mg./100 ml. of cell culture).

Purification and degradation of proteins. The proteins from approx. 2–3 g. wet wt. of cells grown for 72 hr. in 100 ml. of synthetic medium in the presence of 10 μc of [1-^{14}C]sarcosine (1·23 μmoles) or 10 μc of [1-^{14}C]glycine (1·26 μmoles) or 5 μc of [3-^{14}C]sarcosine (7·7 μmoles) were prepared by two different procedures. In one, intact cells were defatted by refluxing for 6 hr. with approx. 50 vol. of ethanol–ethyl ether (50:50, v/v) and then extracted with 5% trichloroacetic acid as follows: first with 20 vol. at room temperature for 1 hr., then with 20 vol. at 90° for 15 min. and finally with 100 vol. at room temperature for 15 min. (Arnstein & Morris, 1960). The residue was then treated in the same way except that 10% (w/v) NaCl was used instead of trichloroacetic acid. The residue was dried by washing first with ethanol–ethyl ether, then with ethyl ether ('mycelial' protein).

In the other procedure, the cells were suspended in 2 vol. of 66 mM-KH_2PO_4–Na_2HPO_4 buffer, pH 6·8, and treated in a MSE (Measuring and Scientific Equipment Ltd.) ultrasonic disintegrator for 10 min. After centrifuging (10000*g* for 10 min.), the protein from the cell-free extract ('soluble' protein) was obtained by precipitating the cell-free extract with 5 vol. of 5% (w/v) trichloroacetic acid. The precipitate was recovered by centrifuging, washed with the same solvent and suspended overnight in acetone. It was then treated as outlined for the 'mycelial' protein.

'Mycelial' and 'soluble' proteins were hydrolysed by refluxing in 6N-HCl for 20 hr. After hydrolysis, the solution was decolorized by treatment with charcoal and dried *in vacuo* over P_2O_5 and NaOH. The dry residue was taken up in a few millilitres of 1·5N-HCl and samples, corresponding to 30–50 mg. of protein, were applied to a 60 cm. × 2·5 cm. column packed with Dowex 50 (H^+ form; X4; 200–400 mesh). The amino acids were eluted with 1·5N-HCl. Radioactive amino acids were located with ninhydrin (Moore & Stein, 1948) and by radioactivity assays.

The contents of the tubes in which radioactivity was present were pooled and dried *in vacuo*. Paper chromatography of such preparations revealed the presence of one radioactive amino acid together with some unlabelled ones. Purification of the labelled compound was achieved by the same chromatographic procedure as was employed in the purification of the amino acids present in the cells, with the only exception that no labelled carrier was added. The radioactive area recovered from the second chromatogram was indistinguishable from glycine by paper chromatography in the solvent systems already described.

Isotope measurement. Samples to be counted (<0·5 mg.) were plated on to metal planchets (surface 1·88 cm.^{2}) and dried *in vacuo* or under an infrared lamp. All samples were counted with a Società Elettronica Lombarda mica-window Geiger–Müller counter tube (window thickness approx. 2 mg./cm.^{2}) under conditions in which self-absorption was negligible. The counting efficiency was 12·5%. All counts were corrected for background activity. Specific activity was expressed as counts/min./μg. of antibiotic.

Analytical methods. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystallized egg albumin (Sigma) as a standard. Actinomycin was assayed microbiologically (Goss & Katz, 1957), with crystalline preparations of the actinomycins, synthesized by the strain of *S. antibioticus* used in the investigation, as a standard.

Actinomycins were separated by circular-paper chromatography with aqueous 10% (w/v) solution of sodium *o*-cresotinate–di-*n*-butyl ether–*sym*-tetrachloroethane (4:3:1, by vol.) as solvent system (Katz & Goss, 1960). The cresotinate (an unresolved mixture of *o*-, *m*- and *p*-cresotic acid) was obtained from British Drug Houses Ltd.

More rapid separation of the actinomycins was achieved by thin-layer chromatography on alumina (Alumina G; Merck) plates with ethyl acetate–*sym*-tetrachloroethane–water (3:1:3, by vol.) as a mobile phase (Cassani, Albertini & Ciferri, 1963). Samples containing up to 0·5 mg. of actinomycin mixture, dissolved in acetone, were applied as bands to 20 cm. × 20 cm. plates (alumina thickness was approx. 0·3 mm.) and developed for 1 hr. with the solvent mixture. Three actinomycin bands, visually located by the orange colour and u.v. absorption, were always evident with R_F 0·38, 0·45 and 0·5 respectively. The bands were scraped off with a spatula and the alumina was extracted three times with 4 vol. of methanol. As determined colorimetrically (Goss & Katz, 1957), recovery ranged from 75% to 80% of the material originally applied. Samples were hydrolysed according to procedure (1) above and the acid-free hydrolysate was spotted on a sheet of Whatman no. 1 filter paper together with markers of different amounts (from 5 to 100 μg.) of D-valine and D-alloisoleucine. After development in butan-1-ol–acetic acid–water (63:10:27, by vol.) the amino acids were located by spraying with 0·2% ninhydrin solution in acetone. The actinomycin with R_F 0·38 showed spots corresponding to sarcosine, proline, *N*-methylvaline and valine and alloisoleucine appeared to be absent; the antibiotic was hence identified as actinomycin C_1 (actinomycin IV). The spot with R_F 0·45 showed, besides sarcosine, proline and *N*-methylvaline, approximately equal quantities of valine and alloisoleucine and was identified as actinomycin C_2 (actinomycin VI), whereas alloisoleucine and no valine was evident in the hydrolysate of the band with R_F 0·5 so that it was assumed to be actinomycin C_3 (actinomycin VII).

Under the same conditions, the new actinomycins produced in the presence of at least 100 μg. of sarcosine/ml. of medium had R_F 0·27 and 0·22 respectively. When the

amino acid composition of the peptide chains of these new actinomycins was examined, the actinomycin with a higher R_F showed spots corresponding only to sarcosine, *N*-methylvaline and alloisoleucine, and the actinomycin with a lower R_F showed approximately equal amounts of alloisoleucine and valine. Consequently they were identified as actinomycin F_3 and actinomycin F_1 (Brockmann, 1960).

RESULTS

Effect of the addition of sarcosine to the fermentation media. The addition of sarcosine to the synthetic ('fermentation') medium at a concentration of 1 μg./ml. had no effect on the production of actinomycin (Table 1). Higher concentrations of the amino acid appeared to have a depressing effect on the synthesis of the antibiotic. Thus 100 μg./ml. reduced the antibiotic production to 48 % of that produced in the absence of the amino acid. In another experiment, approx. 500 μg. of sarcosine/ml. decreased the production of the actinomycins to 23 % of that of the controls.

Paper or thin-layer chromatography of the actinomycins synthesized in the presence of up to 1 μg. of sarcosine/ml. indicated that these actinomycins are identical with those produced in the minimal medium. When the fermentations were done in the presence of 100–500 μg. of sarcosine/ml., besides the actinomycins of the C group other actinomycins were evident on thin-layer chromatography. Two of the actinomycins were produced in substantial amounts and, after isolation and determination of the amino acid composition of the peptide chains, identified as actinomycin F_1 and F_3 (Schmidt-Kastner, 1956; Katz & Goss, 1960).

The specific activity of the actinomycins produced in the presence of radioactive sarcosine appears to be almost identical with that of the actinomycins synthesized when the radioactive amino acid was diluted with a large excess of non-radioactive sarcosine. Possible explanations of this result will be discussed below.

Incorporation of sarcosine and glycine into actinomycins. As reported in Table 2, the radioactivity from [1-^{14}C]sarcosine is incorporated into the actinomycins synthesized by *S. antibioticus*. In all experiments, almost all the radioactivity incorporated, which ranged from 0·1 to 0·9 % of the total radioactivity supplied, was recovered in the sarcosine portion of the antibiotic. Similar results have been obtained also for another producer of actinomycin, *S. parvus* (Ciferri *et al.* 1962).

In all cases, very little, if any, radioactivity was recovered from the chromophoric moiety of the antibiotic. Likewise, there was very little label in the other amino acids of the peptide chain of the antibiotic.

More radioactivity was incorporated if the amino acid was supplied a few hours (8 hr.) after inoculation of the fermentation medium rather than at the time of the inoculation (Table 3). If the radioactive amino acid was supplied in the vegetative medium, practically no label was evident in the antibiotic. This indicates that either sarcosine is not taken up by the micro-organism during vegetative growth, a possibility which has not been checked but which appears doubtful, or if it has been taken up it has been extensively degraded in the early part of microbial growth. In this case, very little radioactivity would be available later on for the synthesis of the antibiotic.

The fact that radioactive sarcosine is incorporated in the sarcosine portion of the antibiotic is, by itself, not a conclusive proof of the direct incorporation of the amino acid since it is possible that the amino acid was, at least in part, degraded before its incorporation into the antibiotic.

The incorporation experiments were therefore repeated with glycine, the most likely product of the metabolism of sarcosine (Table 4). These experiments demonstrated a good incorporation of glycine into the sarcosine moiety of the antibiotic (Table 5). As in the experiments in which sarco-

Table 1. *Influence of the addition of sarcosine on the biosynthesis of actinomycin*

In Expt. 1, 9·1 μc of [1-^{14}C]sarcosine (sp. activity 8·1 mc/m-mole) alone or together with 9·9 mg. of [^{12}C]sarcosine was added to each 100 ml. of synthetic medium and in Expt. 2 [^{12}C]sarcosine was added 8 hr. after inoculation. The fermentations were carried on for 72 hr. from the time of inoculation and terminated by separation of the mycelium and extraction of the antibiotic from the fermentation liquid. The percentage inhibition is referred to the production of antibiotic in the control experiments. Each result is the average of duplicate experiments.

Expt.	Addition to the synthetic medium	Actinomycin synthesized (μg./ml.)	Percentage inhibition	Sp. activity (counts/min./μg. of antibiotic)
1	None (control)	50	—	—
	[1-^{14}C]Sarcosine (1 μg./ml.)	51	0	1·52
	[1-^{14}C]Sarcosine (1 μg./ml.) and [^{12}C]sarcosine (99 μg./ml.)	24	48	1·42
2	None (control)	37	—	—
	Sarcosine (480 μg./ml.)	8·8	77	—

Table 2. *Distribution of the radioactivity incorporated from* [1-^{14}C]*sarcosine into actinomycin*

The purified radioactive actinomycin (13·1 mg.) was recovered from 600 ml. of medium to which 60 μc (7·41 μmoles) of [1-^{14}C]sarcosine was added 8 hr. after inoculation. Samples were degraded to determine the distribution of the radioactivity in the molecule of the antibiotic. The following amounts (mg.) of amino acids were recovered from Expt. 1: sarcosine 0·72; proline 0·66; alloisoleucine 0·68; valine and *N*-methylvaline 1·53. Abbreviations: MeVal, *N*-methylvaline; Val, valine; Aileu, alloisoleucine; Pro, proline; Thr, threonine; Sar, sarcosine. Values in parentheses are the percentage of radioactivity recovered in the sarcosine portion of the antibiotic.

	10^{-3} × Radioactivity (counts/min.)										
						Amino acids from the peptide chain					
Expt.	Purified antibiotic	Hydrolysed peptide	Actinocinin	Depeptido-actinomycin	Melanine residue from chromophore	MeVal + Val	Aileu	Pro	Thr	Sar
1	9·728	9·96	—	—	0·012	0·105	0·079	0·083	—	8·114 (83·3%)
2	4·0	—	—	0·06	—	—	—	—	—	—
3	7·205	6·74	0·008	—	—	0·196	0·006	0·062	0·002	5·989 (83·8%)

sine was employed, the radioactivity of the glycine molecule was not distributed in the other parts of the antibiotic. Up to 1·6% of the total radioactivity of glycine was incorporated into the antibiotic with specific activities from 2·9 to 6 counts/min./μg. of antibiotic. Such results would substantiate the view that glycine is as good a precursor of the sarcosine of the antibiotic as sarcosine.

The addition of a large excess of non-radioactive sarcosine did not decrease significantly the incorporation of labelled glycine nor did a similar excess of unlabelled glycine decrease the incorporation of labelled sarcosine. The failure to observe a significant decrease of the specific activity of the antibiotic by cross-dilution between glycine and sarcosine, as well as the lack of dilution of labelled sarcosine by unlabelled sarcosine reported in Expt. 1 (Table 1) cannot be accounted for by the presence of large intracellular pools of these two amino acids. The determination of the total amount of sarcosine and glycine present in the cells at the time of addition of the labelled amino acids revealed that the content of glycine was approx. 3·4 mg./100 ml. of cell culture whereas sarcosine was undetectable (less than 0·175 mg./100 ml. of cell culture).

An investigation into the rate of uptake of glycine and sarcosine by the cells gave rise to quite different results (Table 6). Glycine appeared to be taken up efficiently and at a fast rate. Thus when the concentrations of glycine in the medium were 0·21 and 2·01 μmoles/ml., 3 hr. after addition 89% and 55% respectively of the amino acid was taken up by the cells. In both experiments uptake of glycine was practically complete after 6 hr. from the addition of the amino acid to the medium. With sarcosine there was a much smaller rate of disappearance from the medium and a much smaller percentage of uptake by the cells during the course of the entire experiment. Indeed, when 0·21 and 2·01 μmoles of sarcosine/ml. were added to the medium, after 3 hr. 29 and 15% of the amino acid was taken up by the cells and the total amount, even after 37 hr. of incubation, was not higher than 30% of the total. Such results are similar to those that Katz & Weissbach (1963) have reported in short-term experiments with another strain of this micro-organism. In their results, too, there was a very rapid and almost total uptake of glycine and a slow and incomplete uptake of sarcosine. The authors have explained the inefficient uptake of sarcosine by the slow passage of *N*-methylamino acids across the permeability barrier.

The failure to observe dilution of the radioactivity incorporated into the antibiotic when labelled sarcosine is supplied together with unlabelled sarcosine may be explained by the fact that higher concentrations of the amino acid in the

Table 3. *Influence of the time of addition of sarcosine on the biosynthesis of actinomycin*

Growth in 100 ml. of vegetative medium was allowed to occur for 48 hr., after which the mycelium (approx. 3 g. wet wt.) was recovered by centrifuging, washed three times with the synthetic medium, suspended in the same solution and incubated for an additional 72 hr. In each experiment [1-^{14}C]sarcosine was added at a concentration of 10μc (1·23μmoles)/100 ml. of medium. Each result is the average of duplicate experiments. Controls (not shown in the Table), in which identical quantities of non-radioactive sarcosine were added 0 and 8 hr. after inoculation of the synthetic medium with mycelium grown in the presence of [1-^{14}C]sarcosine, gave respectively a production of 25 and 23μg. of antibiotic/ml. In both the purified antibiotic had a total radioactivity of 450 counts/min., corresponding to sp. activities 0·12 and 0·13.

Medium	Time of addition (hr.)	Actinomycin synthesized (μg./ml.)	10^{-3} × Radioactivity in purified antibiotic (counts/min.)	Sp. activity (counts/min./μg. of antibiotic)
Vegetative	0	21	0·622	0·18
Fermentation	0	25	10·4	2·55
Fermentation	8	22	20·28	5·72

Table 4. *Incorporation of glycine and sarcosine into actinomycin*

In Expt. 1, 10·5μc (2μmoles) of [1,2-$^{14}C_2$]glycine was added, alone or together with 200μmoles of sarcosine, to each 100 ml. of medium. In Expt. 2, to each 100 ml. of medium were added 10μc of [1-^{14}C]glycine or of [1-^{14}C]sarcosine (1·26 and 1·23μmoles respectively) alone or together with 126μmoles of sarcosine or 123μmoles of glycine. In both experiments addition of the amino acids took place 8 hr. after inoculation of the fermentation medium and all fermentations were carried on for 72 hr. Each result is the average of two experiments.

Expt.	Addition to synthetic medium	Antibiotic recovered (mg.)	10^{-3} × Radioactivity in purified antibiotic (counts/min.)	Sp. activity (counts/min./μg. of antibiotic)	Percentage of total radioactivity incorporated into antibiotic
1	[1,2-$^{14}C_2$]Glycine	2·784	9·9	3·55	1·65
	[1,2-$^{14}C_2$]Glycine and [^{12}C]sarcosine	1·843	5·52	2·99	0·94
2	[1-^{14}C]Glycine	1·781	9·384	5·26	1·69
	[1-^{14}C]Glycine and [^{12}C]sarcosine	1·408	8·541	6·06	1·53
	[1-^{14}C]Sarcosine	2·915	5·246	1·79	0·94
	[1-^{14}C]Sarcosine and [^{12}C]glycine	2·336	5·056	2·16	0·91

Table 5. *Distribution of the radioactivity incorporated from radioactive glycine and sarcosine into actinomycin*

The purified antibiotic from the experiments reported in Table 4 was degraded as follows: actinomycin from Expt. 1 with 20% HCl under reflux, and actinomycin from Expt. 2 in a sealed tube (see Experimental section). The hydrolysed peptides were streaked on a sheet of Whatman paper no. 1 and run overnight in butan-1-ol–acetic acid–water. The paper was then dipped in a dilute solution of ninhydrin (0·01%, w/v, in acetone) and left overnight at room temperature. The ninhydrin-positive bands corresponding to the amino acids were cut, eluted and counted. Abbreviations are as given in Table 2. Values in parentheses are the percentages of radioactivity recovered in the sarcosine portion of the antibiotic.

	10^{-3} × Radioactivity (counts/min.)							
				Amino acids of peptide chain				
Expt. 1	Purified antibiotic	Actinocinin	Melanine residue from the chromophore	MeVal + Val	Aileu	Pro	Thr	Sar
[1,2-$^{14}C_2$]Glycine; [1,2-$^{14}C_2$]glycine and [^{12}C]sarcosine	15·283	0·103	—	1·010	0·169	0·215	0·202	10·2 (66·7%)
2								
[1-^{14}C]Glycine	9·384	—	0·145	0·014	0·008	0·031	—	8·881 (94·6%)
[1-^{14}C]Glycine and [^{12}C]sarcosine	8·541	—	0·068	0·009	0·014	0·012	—	6·425 (75·1%)
[1-^{14}C]Sarcosine	5·246	—	0·081	0·038	0·012	0·008	—	5·1 (97·2%)
[1-^{14}C]Sarcosine and [^{12}C]glycine	5·056	—	0·05	0·028	0·006	0·015	—	5·216 (103·1%)

medium result in higher uptake of the amino acid by the cells (Table 6) and higher incorporation of the amino acid into the antibiotic (Table 7).

Thus in Expts. 1 and 2 of Table 6, when 10 μc of [1-^{14}C]sarcosine was supplied together with 1·78 and 17·8 mg. of [^{12}C]sarcosine/100 ml. of medium, approx. 0·007 and 0·05 mg. of the exogenous sarcosine was incorporated into the antibiotic. These amounts represent 1·36 and 1·40% of the total sarcosine taken up by the cells in each experiment. Since approximately ten times as much sarcosine is taken up by the cells when they are grown in the presence of the highest concentration of sarcosine (Tables 6 and 7) the same amount of radioactivity is incorporated into the antibiotic and hence there is no dilution of the specific activity of the antibiotic.

The lack of cross-dilution between glycine and sarcosine has not been satisfactorily explained. It may well be that the two amino acids are incorporated into the antibiotic by different mechanisms and therefore the incorporation of one into the antibiotic is not interfered with by the presence of the other, which is incorporated by a different mechanism. Indeed, sarcosine may be incorporated into the sarcosine of the antibiotic without transformation into glycine (see below). On the other hand, glycine may be transformed into sarcosine by a reaction that does not give rise to free sarcosine, e.g. on a precursor of the antibiotic molecule. One can envisage, for instance, methylation of a pentapeptide containing glycine and valine, which would give rise to peptides containing sarcosine and *N*-methylvaline.

When the actinomycin produced in the presence of [1,2-$^{14}C_2$]glycine was degraded (Table 5, Expt. 1) approx. 6·6% of the radioactivity present in the antibiotic was recovered in the *N*-methylvaline fraction. Katz & Weissbach (1963) reported similar results, which they attributed to an in-

Table 6. *Disappearance from the medium of sarcosine and glycine during growth of* Streptomyces antibioticus

[1-^{14}C]Sarcosine (10μc; 1·23μmoles) together with 20 and 200μmoles of [^{12}C]sarcosine, as well as 10μc of [1-^{14}C]glycine (1·26μmoles) together with 20 and 200μmoles of [^{12}C]glycine, were added 24 hr. after inoculation to duplicate flasks containing 100 ml. of medium each. Samples (5 ml.) were quickly removed at intervals, centrifuged to remove the mycelium and extracted with 1 vol. of ethyl acetate. The ethyl acetate was removed and washed with 1 vol. of water. The two aqueous portions were combined and samples plated for counting. Percentage of the labelled amino acids remaining was referred to the control samples taken at 0 hr. Each result is the average of two experiments. At the end of the experiment, antibiotic production was 23·4, 22·6, 24·1 and 31·1 μg./ml. in Expts. 1–4 respectively.

Time after addition of amino acid to medium (hr.)	[1-^{14}C]Sarcosine and [^{12}C]sarcosine		[1-^{14}C]Glycine and [^{12}C]glycine	
	Expt. 1 (20μmoles)	Expt. 2 (200μmoles)	Expt. 3 (20μmoles)	Expt. 4 (200μmoles)
0	100	100	100	100
0·5	82	86	26	68
1	80	88	22	76
2	77	87	13	71
3	71	85	11	45
6	71	83	10	9
13	70	81	8	6
25	70	80	5	5
37	71	80	5	4

Table 7. *Incorporation of sarcosine into the cells of* Streptomyces antibioticus *and into actinomycin*

Calculations refer to the data reported in Table 6 for the 37 hr. values of Expts. 1 and 2.

Sarcosine added to 100 ml. of medium at 0 hr.		Sarcosine taken up by cells at 37 hr.		Actinomycin produced (mg.)	10^{-3} × Radioactivity incorporated into antibiotic (counts/min.)	Exogenous sarcosine	
[^{12}C]Sarcosine (mg.)	[1-^{14}C]Sarcosine (counts/min.)	(%)	(mg.)			Incorporated into antibiotic (mg.)	In the cells, which has been incorporated into antibiotic (%)
0·178	$2033·3 \times 10^3$	30	0·534	2·34	7·9	0·0073	1·36
1·78	$2000·0 \times 10^3$	20	3·56	2·26	5·6	0·0501	1·40

corporation of C-2 of glycine into the methyl group of methionine, which then is used as a precursor of the methyl groups of sarcosine and *N*-methylvaline.

Incorporation of the methyl group of methionine into actinomycins. It has been reported that the methyl group of methionine is the precursor of the two methyl groups of the chromophore, and that it may be used also as a source of the methyl groups of the sarcosine and the *N*-methylvaline of the peptide chain (Birch, Cameron, Holloway & Richards, 1960; Sivak, Meloni, Nobili & Katz, 1962). These experiments were repeated with our culture with the additional goal of producing methyl-labelled sarcosine.

The results (Table 8) show that the methyl group of methionine is effectively incorporated into the antibiotic with a percentage incorporation (up to 11 %) that is much higher than that obtained with either radioactive sarcosine or radioactive glycine. As before, the addition of a large amount of sarcosine to the fermentation medium considerably reduced the amount of antibiotic produced. The addition of sarcosine, in the fermentations carried out in the presence of methionine, resulted in an inhibition of the production of the antibiotic (approx. 60 %). In all cases, however, the radioactivity from the methyl group of methionine appeared to be distributed equally in the methyl groups of sarcosine, *N*-methylvaline and (presumably) the chromophore. After acid hydrolysis of the purified actinomycin, 59 and 63 % of the radioactivity remained associated with the amino acids of the antibiotic (since the acid hydrolysis leads to the destruction of the chromophoric moiety, theoretically 66 % of the radioactivity should have been recovered in the peptide portion). When the amino acids of the peptide chains were separated the sarcosine from both experiments accounted for 32 % of the total radioactivity of the antibiotic and the *N*-methylvaline for 31 and 27 % (theoretically each amino acid should account for 33 % of the radioactivity).

These results prove that in a synthetic medium the methyl group of methionine is utilized as a source of the methyl groups of the sarcosine and of the *N*-methylvaline. In addition, it is possible that it is used for the methyl groups of the chromophore.

In the presence of a very large excess of non-radioactive sarcosine, the percentage incorporation of methionine was decreased by approximately one-third with respect to that observed in the absence of sarcosine. Similarly the specific activity of the antibiotic was decreased by one-third. These findings suggest that sarcosine is incorporated into the sarcosine of the antibiotic.

Incorporation of methyl-labelled sarcosine. Proof of a direct incorporation of sarcosine into the sarcosine of the antibiotic was obtained in the experiments reported in Tables 9 and 10. Addition to the synthetic medium of methyl-labelled sarcosine resulted in incorporation of the label solely into the sarcosine of the antibiotic. This indicates that sarcosine is incorporated into the sarcosine portion of the antibiotic without preliminary demethylation. If this was not so, either the sarcosine of the antibiotic should not have been labelled or the label should be equally distributed in the methyl group of the antibiotic (sarcosine, *N*-methylvaline and chromophore) as found when methyl-labelled methionine is employed.

Incorporation of sarcosine into protein. The protein from the mycelium grown in the presence of radioactive sarcosine was extracted and fractionated, as described in the Experimental section, to determine the distribution of the radioactivity in the amino acids. The protein hydrolysates from cells grown in the presence of [1-^{14}C]sarcosine gave a single radioactive peak in a position which corresponded to that of glycine. Up to 86 % of the radioactivity of the protein was recovered in this peak. Separation by paper chromatography (see

Table 8. *Incorporation of methionine into actinomycin*

[*methyl*-^{14}C]Methionine (10μc; 0·54μmole) alone or together with 540μmoles of sarcosine was added at the time of inoculation to each 100 ml. of synthetic medium. The fermentations were carried on for 72 hr. The purified antibiotic was hydrolysed and the amino acids were separated as in Table 5. Values in parentheses are the percentages of radioactivity recovered in the sarcosine and *N*-methylvaline portions of the antibiotic.

Addition	Antibiotic recovered (mg.)	10^{-3} × Radioactivity in antibiotic (counts/min.)	Sp. activity (counts/min./μg. of antibiotic)	Percentage of total radioactivity incorporated	10^{-3} × Radioactivity in sarcosine and *N*-methylvaline portions of antibiotic (counts/min.)	
					Sarcosine	*N*-Methylvaline
[*methyl*-^{14}C]Methionine	2·171	307·97	141·8	11	101·04 (32·8%)	96 (31·1%)
[*methyl*-^{14}C]Methionine and [^{12}C]sarcosine (480μg./ml.)	1·389	136·17	98	4·9	43·875 (32·3%)	37·73 (27·7%)

Table 9. *Incorporation of* [3-^{14}C]*sarcosine into actinomycin*

In Expt. 1 0·45μc (15μmoles) of biologically produced [3-^{14}C]sarcosine and an equal amount of synthetic [1-^{14}C]sarcosine were added 24 hr. after inoculation and the fermentations carried on for an additional 48 hr. In Expt. 2 5μc (7·7μmoles) of synthetic [3-^{14}C]sarcosine was added to each 100 ml. of synthetic medium and the fermentation carried on for a total of 72 hr. Each result of Expt. 2 is the average of two experiments; Expt. 1 was performed on only one sample.

Expt.	Addition to the synthetic medium	Time of addition (hr.)	Actinomycin synthesized (mg.)	10^{-3} × Radioactivity in purified antibiotic (counts/min.)	Sp. activity (counts/min./μg. of antibiotic)	Percentage of total radioactivity
1	[1-^{14}C]Sarcosine	24	4·14	0·486	0·11	0·43
	[3-^{14}C]Sarcosine	24	3·84	0·931	0·24	0·83
2	[3-^{14}C]Sarcosine	0	5·76	11·65	2·01	0·22
	[3-^{14}C]Sarcosine	20	4·576	15·265	3·31	0·27

Table 10. *Distribution of the radioactivity incorporated from* [3-^{14}C]*sarcosine into actinomycin*

Actinomycin from Expt. 2 of Table 9 was degraded and the components were isolated as reported in Table 5. Values in parentheses are the percentages of radioactivity recovered in the sarcosine portion of the antibiotic.

	10^{-3} × Total radioactivity (counts/min.)							
				Amino acids from peptide chain				
Time of addition of [3-^{14}C]-sarcosine (hr.)	Purified antibiotic	Melanine residue from the chromophore	Hydrolysed peptide	*N*-Methyl-valine and valine	Alloisoleucine	Proline	Threonine	Sarcosine
0	11·65	0·016	11·3	0·068	0·048	0·464	—	10·71 (91·9%)
20	15·625	0·036	14·925	0·095	0·044	0·204	—	14·25 (91·2%)

Table 11. *Incorporation of radioactive glycine and sarcosine into proteins*

All cells were recovered from 100 ml. of synthetic medium fermented for 72 hr. in the presence of 10μc of radioactive amino acid (1·23μmoles of [1-^{14}C]sarcosine, 1·26μmoles of [1-^{14}C]glycine and 7·7μmoles of [3-^{14}C]-sarcosine).

Cells grown in the presence of	Type of protein	Protein recovered (mg.)	Percentage of labelled amino acid incorporated into protein	Amount of protein hydrolysed (mg.)	10^{-3} × Radioactivity in hydrolysed protein (counts/min.)	10^{-3} × Radioactivity recovered in glycine fraction
[1-^{14}C]Sarcosine	'Soluble'	120	0·47	50	10·9	9·48 (86·9%)
[1-^{14}C]Sarcosine	'Mycelial'	221	1·0	40	9·75	7·7 (78·9%)
[1-^{14}C]Glycine	'Mycelial'	282	2·52	30	15·35	8·3 (54·07%)
[3-^{14}C]Sarcosine	'Mycelial'	155	0·03	155	1·925	—
		158	0·02	158	1·025	—

the Experimental section) of the amino acids of the radioactive peak showed that all the radioactivity was associated with a single compound and that the compound was indistinguishable from glycine. Such results were always obtained with both 'mycelial' and 'soluble' proteins if the cells were grown in the presence of either [1-^{14}C]glycine or of [1-^{14}C]sarcosine (Table 11). The proteins from the cells grown in the presence of [3-^{14}C]sarcosine had very little radioactivity, so were not fractionated. The latter results are to be expected if, for the synthesis of proteins, demethylation of sarcosine to glycine is taking place. With methyl-labelled sarcosine, the reaction must lead to unlabelled glycine and hence to very little labelling of all proteins. The fate of the methyl group of sarcosine has not been ascertained; probably it does not follow to a great extent the metabolic fate of the methyl group of methionine and other methyl donors.

It may be added that attempts to find non-radioactive sarcosine in the protein derived from mycelia grown in the presence of large amounts of this amino acid (up to 500μg./ml.) were always unsuccessful.

DISCUSSION

The results presented suggest that in this micro-organism sarcosine may either be incorporated directly into actinomycin or demethylated to glycine,

which then appears in the proteins. When sarcosine is not supplied to the fermentation medium, the results obtained with isotopic glycine and methionine suggest that glycine acts as a precursor of sarcosine receiving a methyl group from methionine.

Enzymic proof of the conversion of sarcosine into glycine is, at present, lacking since it has been impossible to demonstrate, by manometric assay, the presence in cell-free extracts of *S. antibioticus* of a sarcosine dehydrogenase (Ratner, Nocito & Green, 1944) or a sarcosine demethylase (Bloch & Schoenheimer, 1940). However, resting cells of *S. antibioticus* appear to convert sarcosine into glycine, though to a very small extent.

In addition, the manner in which sarcosine is incorporated into the antibiotic remains to be elucidated. It is possible that it reacts with other amino acids of the antibiotic to give a free peptide or an anthranoylpeptide (Brockmann, 1960) or is enzymically added, for instance, by a stepwise addition (Ito & Strominger, 1960*a*, *b*) to the phenoxazinone chromophore of the antibiotic independently synthesized (Katz & Weissbach, 1962*a*). Further work must be performed on the overall reactions leading to the synthesis of the antibiotic. The information so far available indicates that: (1) methionine contributes the methyl groups of the sarcosine, the *N*-methylvaline and the chromophore; (2) in the absence of sarcosine, glycine is the precursor of the aminoacetic portion of sarcosine; (3) tryptophan, or compounds related to tryptophan, are precursors of the chromophore (Sivak *et al.* 1962); (4) the D-valine and *N*-methylvaline of the peptide portion are derived from L-valine (Katz & Weissbach, 1962*b*). The other two amino acids of the peptide, proline and threonine, which are present in the L-form, probably derive from the common biogenetic pathways of protein amino acids. No evidence is at present available on the biogenesis of D-alloisoleucine but possibly it is derived from L-isoleucine. A search for mutants, specifically blocked in the biosynthetic routes of the antibiotic, is now being made.

In general, the reactions associated with the biosynthesis of proteins do not appear to be involved in the biosynthesis of the peptide portion of the antibiotic since it has been impossible to demonstrate in cell-free extracts of the micro-organism activation by an ATP–pyrophosphate exchange of the amino acids present only in the antibiotic (*N*-methyl-L-valine, sarcosine, D-alloisoleucine and D-valine). Likewise, tests for the enzymic transfer of radioactive sarcosine to soluble RNA have been always negative. However, since the tests are not positive for all the amino acids present in proteins, it is impossible to conclude that these reactions are not present in the amino acids that occur only in the antibiotic.

SUMMARY

1. The addition of sarcosine to cultures of *Streptomyces antibioticus*, growing on a chemically defined medium, results in the incorporation of the amino acid in the sarcosine portion of the actinomycins synthesized by the micro-organism. In addition, sarcosine is utilized as a source of glycine for the synthesis of protein.

2. In the absence of sarcosine from the fermentation medium, glycine provides the aminoacetic moiety and methionine the methyl group of the sarcosine of the antibiotic.

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REFERENCES

Arnstein, H. R. V. & Morris, D. (1960). *Biochem. J.* **76**, 323.

Birch, A. J., Cameron, D. W., Holloway, P. V. & Richards, R. W. (1960). *Tetrahedron Lett.* no. 25, 26.

Bloch, K. & Schoenheimer, R. (1940). *J. biol. Chem.* **135**, 99.

Brockmann, H. (1960). *Ann. N.Y. Acad. Sci.* **89**, 323.

Brockmann, H. & Muxfeldt, H. (1956). *Angew. Chem.* **68**, 67.

Cassani, G., Albertini, A. & Ciferri, O. (1963). *J. Chromat.* (in the Press).

Ciferri, O., Albertini, A. & Rossi, P. (1962). *Biochem. biophys. Res. Commun.* **7**, 264.

Ciferri, O., Di Girolamo, M. & Bendicenti Di Girolamo, A. (1961). *Nature, Lond.*, **191**, 411.

Dalgliesh, C. E., Johnson, A. W., Todd, A. R. & Vining, L. C. (1950). *J. chem. Soc.* p. 2946.

Emerson, R. L., Whiffen, A. J., Bohonas, N. & DeBoer, C. (1946). *J. Bact.* **52**, 357.

Goss, W. A. & Katz, E. (1957). *Appl. Microbiol.* **5**, 95.

Greenstein, J. P. & Winitz, M. (1961). *The Chemistry of Amino Acids*, vol. 1, p. 22. New York: Academic Press Inc.

Hanger, W. G., Howell, W. C. & Johnson, A. W. (1958). *J. chem. Soc.* p. 496.

Haworth, R. D., MacGillivray, R. & Peacock, D. H. (1951). *Nature, Lond.*, **167**, 1068.

Ito, E. & Strominger, J. L. (1960*a*). *J. biol. Chem.* **235**, PC5.

Ito, E. & Strominger, J. L. (1960*b*). *J. biol. Chem.* **235**, PC7.

Johnson, A. W. & Mauger, A. B. (1959). *Biochem. J.* **73**, 535.

Johnson, A. W., Todd, A. R. & Vining, L. C. (1952). *J. chem. Soc.* p. 2672.

Katz, E. & Goss, W. A. (1960). *Antibiot. & Chemother.* **10**, 221.

Katz, E. & Weissbach, H. (1962*a*). *J. biol. Chem.* **237**, 882.

Katz, E. & Weissbach, H. (1962*b*). *Biochem. biophys. Res. Commun.* **8**, 186.

Katz, E. & Weissbach, H. (1963). *J. biol. Chem.* **238**, 666.
Kossel, A. & Edlbacher, S. (1915). *Hoppe-Seyl. Z.* **94**, 264.
Linko, P., Alfthan, M., Miettinen, J. K. & Virtanen, A. I. (1953). *Acta chem. scand.* **7**, 1310.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Moore, S. & Stein, W. H. (1948). *J. biol. Chem.* **176**, 367.
Ratner, S., Nocito, V. & Green, D. E. (1944). *J. biol. Chem.* **152**, 119.
Schmidt-Kastner, G. (1956). *Naturwissenschaften*, **43**, 131.
Sheehan, J. C., Zachau, H. G. & Lawson, W. B. (1957). *J. Amer. chem. Soc.* **79**, 3933.
Sivak, A., Meloni, M. L., Nobili, F. & Katz, E. (1962). *Biochim. biophys. Acta*, **57**, 283.
Tarr, H. L. A. (1958). *Annu. Rev. Biochem.* **44**, 602.

Biochem. J. (1964) **90**, 92

The Iodination of Chymotrypsinogen

BY A. N. GLAZER AND F. SANGER
Medical Research Council, Laboratory of Molecular Biology, Cambridge

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The use of isotopic-labelling techniques in the determination of amino acid sequences has proved particularly valuable in connexion with the study of certain enzymes whose active centres can be specifically labelled (Cohen, Oosterbaan, Jansz & Berends, 1959; Sanger, 1963). In this way a sequence around a single amino acid residue may be isolated and determined. Isotopic-labelling methods have the advantages that they can be applied on a very small scale, and, if appropriate techniques are used, they avoid the necessity of complete purification of a peptide provided that it can be separated from all other radioactive products. In attempts to apply these methods in a more general way with isotopic iodine we have studied the iodination of tyrosine and histidine residues in peptides and have applied the labelling technique to chymotrypsinogen.

In general, iodine should react with all tyrosine and histidine residues in a protein and it would not be expected that there would be any specific reaction, for instance at an active centre. The reactivities of different individual residues do, however, frequently vary and it may be possible to devise special ways of labelling or protecting special sites. With chymotrypsinogen it was found that an almost specific reaction was in fact obtained with one of the tyrosine residues. The present paper describes these results together with some preliminary studies on the iodination of small model peptides.

MATERIALS AND METHODS

Materials

Chymotrypsinogen (crystallized; salt-free), lot 541, and chymotrypsin (three-times crystallized), lot 6004–5, were obtained from Worthington Biochemical Corp. Monoiodohistidine and di-iodohistidine were prepared by Dr D. C. Shaw. Chromatographically pure L-histidyl-L-alanine was given by Dr A. Patchornik. Acetyl-L-histidine was given by Dr A. Neuberger. 3-Iodo-L-tyrosine and 3,5-di-iodo-L-tyrosine were obtained from British Drug Houses Ltd. Glycyl-L-tyrosine was obtained from Roche Products Ltd. Acetyl-L-tyrosine ethyl ester was obtained from the California Corp. for Biochemical Research. Mercaptoethanol was obtained from L. Light and Co. Ltd. and was re-distilled. All other reagents used were of analytical grade. The ^{131}I and ^{125}I were obtained from The Radiochemical Centre, Amersham, Bucks., as carrier-free solutions of iodide in dilute sodium hydroxide, pH 8–10, with a specific activity of 1 mC/ml. Sephadex G-25 (medium grade; 100–270 mesh) was obtained from Pharmacia, Uppsala, Sweden. *Streptomyces griseus* protease was given by Dr M. Nomoto.

Methods

Iodination with ethanolic iodine solution. The iodine solution (either 0·1 or 0·4%) was prepared by dissolving iodine in ethanol. A sufficient amount of carrier-free radioactive iodide was added to give a final specific activity of 1 μC/10 μl. A solution containing 0·01–0·1 μmole of the compound(s) to be iodinated was evaporated to dryness and redissolved in 50 μl. of aq. ammonia (sp.gr. 0·880). The appropriate amount of iodine solution was then added and the reaction allowed to proceed in a closed tube for several hours at room temperature. The reaction mixture was then evaporated to dryness either in a desiccator over NaOH or by evaporation in the fume-cupboard. In some cases, specified in the text, the reaction was carried out in an ice bath and stopped 30 sec. after mixing by the addition of 1 μl. of mercaptoethanol.

Iodination with iodine monochloride. A portion (0·01–0·1 μmole) of a solution of the compound(s) to be iodinated was evaporated to dryness and redissolved in a suitable volume of 0·2M-Na_2HPO_4. The solution of radioactive iodine monochloride prepared as described below was then added. The ratio of the volume of 0·2M-Na_2HPO_4 solution to that of the stock iodine monochloride solution added was always kept at 3·2:1; this proportion gave a final pH of 8–8·2 in the iodination mixture. Iodination was carried out at room temperature or in an ice bath and, whenever required, the reaction was stopped by the addition of mer-