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Incorporation of ^{65}Zn in the Sub-cellular Fractions of the Liver and Spontaneously Occurring Mammary Tumours of Mice after the Injection of Zinc-Glycine Containing ^{65}Zn

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(Received 20 February 1959)

Relatively little is known about the intracellular distribution of zinc in normal and tumour tissues. Heath (1949) and Heath & Liquier-Milward (1950) obtained evidence that zinc in tumour tissue is associated with nucleoprotein. Their interpretation of the results was, however, complicated by the fact that, since they adopted the method of Dounce (1943), citric acid was used in the isolation of the cell nuclei and it is certain that much or all of the zinc present would be leached out by this acid. Rosenfeld & Tobias (1951) could obtain no evidence for the association of zinc with nucleoprotein in the cell nucleus, but these workers also used a citric acid method (that described by Mirsky & Pollister, 1946) for the isolation of cell nuclei. In investigations on normal rat liver Thiers & Vallee (1957) found that the zinc was present in the nuclear, mitochondrial and 'supernatant' fractions, the lowest concentration being in the mitochondria and the highest in the supernatant and microsomal fractions.

An increased uptake of ^{65}Zn by spontaneous mammary tumours compared with normal mammary tissue was found in female mice injected with the isotope as a zinc-glycine complex (Tupper, Watts & Wormall, 1955). A similar increased

uptake of ^{65}Zn has been found in skin tumours induced in mice by 3:4-benzpyrene if the tumours are compared with normal skin (M. E. Bartholomew & R. Tupper, unpublished work). This difference in zinc metabolism between tumours and their normal tissue of origin led us to investigate more fully the binding of zinc in spontaneous mammary tumours of mice, and this paper records the results of our studies on the distribution of injected zinc in the various sub-cellular fractions separated from spontaneous mammary tumours of mice injected with zinc-glycine containing ^{65}Zn . For comparison, similar measurements have been made in the corresponding fractions of the livers of the same mice. The choice of the liver for this purpose was influenced by the fact that there is available a considerable amount of information about the intracellular particles of this organ, and furthermore the liver contains at least two zinc-containing enzymes in which we are particularly interested; at least one of these, glutamic dehydrogenase, is present in the mitochondria.

A preliminary study has also been made on the stability of the zinc compounds in these tissues, and the extent to which the zinc can be removed by prolonged dialysis at different pH values has been

determined. A brief account of some of the results presented here has been reported (Bartholomew, Tupper & Wormall, 1959).

EXPERIMENTAL

Materials

Animals. The mice used were 'pure-line' females of the C3H, ABC and Strong A strains bred in our Laboratory from animals kindly supplied to us by the Institute of Cancer Research (Royal Cancer Hospital) and the Imperial Cancer Research Fund Laboratories. The animals were fed on food pellets (Diet 41, Associated London Flour Millers), oats and water, with a supplement of lard, milk and crushed dog biscuits on 1 or 2 days in each week.

Zinc-free water. This was obtained by passing distilled water through an ion-exchange column containing a mixture, in equal proportions, of De-Acidite FF and Zeo-Karb 225 (The Permutit Co. Ltd.).

Zinc-free NaCl. A solution of NaCl was extracted with a solution of diphenylthiocarbazone (dithizone) in carbon tetrachloride until the dithizone no longer turned red. The NaCl solution was then evaporated to dryness and the 'zinc-free' salt used in the preparation of a 0.9% NaCl solution.

Zinc-free glassware. All glassware used in the dialysis experiments was soaked for at least 24 hr. in 2N-HCl and then thoroughly washed with zinc-free water.

Buffer solutions. All reagents used in the preparation of buffer solutions were of A.R. grade. Buffers at pH 4.0, 5.6, 6.4, 7.4, 8.4 and 10.0 were prepared according to the tables given by Vogel (1942). Care was taken to ensure that the buffers contained no salt which would form a precipitate with zinc. An acetic acid-sodium acetate mixture was used for buffers at pH 4.0 and 5.6, potassium hydrogen phthalate-potassium sodium phthalate for pH 6.4 and glycine-sodium glycinate for pH 8.4 and 10.0.

Sodium diethylbarbiturate buffer containing ethylenediaminetetra-acetic acid. A 0.02M-solution of disodium ethylenediaminetetra-acetate EDTA (100 ml.) was diluted to 2 l. with 0.1M-sodium diethylbarbiturate buffer, pH 7.45. The pH of the final solution was 7.4.

Zinc-glycine solution. $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (14.068 g. = 3.2 g. of zinc) and glycine (14.684 g.) were dissolved in water. N-NaOH was added to the solution to bring the pH to 7.4 and water was then added to make a total volume of 4 l. (800 μg . of zinc/ml.). The solution was saturated with chloroform to inhibit the growth of moulds and bacteria.

Radioactive zinc. Radioactive zinc of high specific activity was separated as ZnCl_2 from cyclotron-irradiated copper by the method described by Tupper & Watts (1954).

Methods

Preparation of ^{65}Zn for injection. The ^{65}Zn was injected as a ^{65}Zn -containing zinc-glycine complex in solution at pH 7.4. The complex was prepared by dissolving glycine (10 mg.) in the stock ^{65}Zn -containing ZnCl_2 solution (1 ml.), adding N-NaOH to give a pH of about 7.4 and sufficient water to make the solution iso-osmotic. The final solution contained 6 μg . of zinc/ml. and gave a counting rate of 3×10^6 counts/min./ml. with the scintillation counter used in these investigations (background count rate was 150 counts/min.).

Injection of ^{65}Zn . Mice bearing spontaneously occurring mammary tumours (1-2 cm. diam.) were each given an intraperitoneal injection of 1 ml. of the ^{65}Zn -containing solution.

Removal of tissues. The mice were killed with coal gas 1 or 3 days after injection of the isotope. The tumours and livers were removed as rapidly as possible and placed in beakers chilled to 0-4°.

Determination of ^{65}Zn . The scintillation-counter method described by Banks, Tupper, Watts & Wormall (1955) was used to determine ^{65}Zn .

Total nitrogen determinations. These were made by the micro-Kjeldahl method, the ammonia being distilled in the Markham (1942) apparatus, into a boric acid solution containing methyl red-methylene blue indicator.

Separation of sub-cellular fractions. In some of our earlier experiments a blender (MSE Nelco) was used to prepare tumour and liver mash for the separation of sub-cellular fractions by differential centrifuging. The use of this blender was abandoned in later experiments in favour of a Potter-Elvehjem homogenizer with a Teflon (polytetrafluoroethylene) pestle (A.H.T. Co., Philadelphia, Pa., U.S.A.). Mash prepared with this homogenizer yielded sub-cellular particles showing less damage than those prepared with the blender.

The various cell suspension media investigated were 0.88 and 0.25M-sucrose and 0.25M-sucrose containing 7.3% of polyvinylpyrrolidone (Novikoff, 1955). The last-named medium gave good mitochondrial preparations but its nitrogen content was too high to enable us to make accurate allowances for it. We finally chose 0.25M-sucrose and used the method of separation described by Schneider & Hogeboom (1950).

The centrifuge used was a refrigerated machine with a superspeed attachment (MSE). The speed was not sufficiently high to enable us to separate the microsomes from the supernatant fraction.

Dialysis of mashes. Zinc-free 0.9% NaCl (50 ml.) was added to either the tumours or the livers obtained from four or five mice killed 3 days after the injection of the zinc-glycine containing ^{65}Zn , and mash was prepared in a Waring Blendor.

Samples of the mash were dialysed in sacs made from Visking transparent cellulose tubing. The ratio of the volume of mash to dialysing medium was always 1:2.5. Dialysis was carried out at 0-4° and diffusates were changed twice in each 24 hr. The mashes were dialysed against buffers at pH 4.0, 5.6, 6.4, 7.4, 8.4 and 10.0, and also against solutions at pH 7.4 containing either excess of zinc or buffered EDTA.

At the end of the dialysis period the sacs and their contents were digested with H_2SO_4 and the digests were used for ^{65}Zn determinations; similar determinations were made on samples of the undialysed mash. Recoveries of ^{65}Zn for the dialysed solution plus the corresponding diffusates were good (not less than 95%).

RESULTS

Distribution of ^{65}Zn between the sub-cellular fractions of mammary tumour and liver tissue

Microscopic observation of the nuclear fractions showed them to consist mainly of free nuclei and cell debris almost free from whole-cell contamination.

Mitochondria examined under the phase-contrast microscope were mostly spherical in form, although some filamentous granules were also present. The mitochondrial fractions were uncontaminated by cell nuclei.

Fractionations were made on mammary tumour and liver tissue from mice killed 1 or 3 days after the injection of the ^{65}Zn -glycine complex. Each fractionation was made on the pooled tissues from four or five mice. Six fractionations were carried out with the mice killed 1 day after the injection and a similar number with those killed after 3 days. The results given in Tables 1, 2 and 3 are the means for the tissues obtained from 24–30 mice.

The results showing the distribution of ^{65}Zn in the different fractions of the mash can be presented in several ways, and for comparisons of the sort under study here it is often desirable that the results should be calculated in several units or with reference to more than one standard. For example,

the ^{65}Zn content of one of the sub-cellular components of a mash can be recorded as a fraction or percentage of the amount of ^{65}Zn present in the whole mash, or all the values may be expressed in terms of $^{65}\text{Zn}/\text{mg.}$ of organic nitrogen present in that particular fraction or whole mash. In the latter case, a more accurate picture is presented of the relative zinc/protein ratios. Both methods have been used for Tables 1 and 2, where the results are given for the tissues of mice killed respectively 1 and 3 days after the injection of zinc-glycine.

Another method is to calculate all the results in terms of $^{65}\text{Zn}/\text{N}$, and then to present the results for each mash fraction as the following ratio: $^{65}\text{Zn}/\text{N}$ in the fraction, divided by the $^{65}\text{Zn}/\text{N}$ value for the original homogenate or mash from which the fraction was separated. This method, which has been adopted for the preparation of Table 3, often allows one to make a more useful assessment of the distribution of the isotope under investigation.

Table 1. *Distribution of ^{65}Zn and nitrogen between the intracellular components of mammary tumour and liver tissue after 1 day*

Tissues were obtained from mice killed 1 day after the injection of ^{65}Zn as the zinc-glycine complex.

Tables 1–3 record the mean values for each set of fractionations, the mean value being followed by the s.e. and the number of fractionations. Radioactivity results were based on net counts ranging from 4×10^4 to 1.5×10^6 counts/min.; the samples taken for assay gave net counts of 3×10^2 – 4×10^4 counts/min.

Fraction	^{65}Zn (percentage of total ^{65}Zn in whole mash)		N in the fraction (percentage of total N in whole mash)		^{65}Zn (percentage of total ^{65}Zn in whole mash)/mg. of N	
	Tumour	Liver	Tumour	Liver	Tumour	Liver
Washed nuclei and cell debris	42.07 ± 2.65 (6)	12.88 ± 1.05 (6)	40.51 ± 3.74 (6)	30.20 ± 1.64 (6)	2.37 ± 0.38 (6)	0.60 ± 0.03 (6)
Washed mitochondria	5.47 ± 0.94 (6)	4.54 ± 0.60 (6)	5.85 ± 0.86 (6)	10.64 ± 0.95 (6)	2.01 ± 0.22 (6)	0.58 ± 0.04 (6)
Supernatant (containing microsomes and soluble proteins)	41.04 ± 1.31 (6)	67.43 ± 2.06 (6)	36.74 ± 1.23 (5)	37.40 ± 0.87 (6)	2.52 ± 0.34 (5)	2.55 ± 0.20 (6)
Whole mash	—	—	—	—	2.19 ± 0.21 (6)	1.40 ± 0.09 (6)

Table 2. *Distribution of ^{65}Zn and nitrogen between the intracellular components of mammary tumour and liver tissue after 3 days*

Tissues were obtained from mice killed 3 days after the injection of ^{65}Zn as the zinc-glycine complex. See note in Table 1.

Fraction	^{65}Zn (percentage of total ^{65}Zn in whole mash)		N in the fraction (percentage of total N in whole mash)		^{65}Zn (percentage of total ^{65}Zn in whole mash)/mg. of N	
	Tumour	Liver	Tumour	Liver	Tumour	Liver
Washed nuclei and cell debris	35.20 ± 3.34 (6)	17.31 ± 1.49 (6)	40.06 ± 2.97 (6)	31.8 ± 1.28 (6)	1.86 ± 0.32 (6)	0.78 ± 0.11 (6)
Washed mitochondria	6.41 ± 0.38 (6)	9.54 ± 0.81 (6)	6.03 ± 0.31 (6)	15.92 ± 1.18 (6)	2.17 ± 0.16 (6)	0.84 ± 0.15 (6)
Supernatant (containing microsomes and soluble proteins)	45.53 ± 2.59 (6)	59.44 ± 2.34 (6)	45.11 ± 2.86 (6)	37.44 ± 1.06 (6)	2.21 ± 0.47 (6)	2.20 ± 0.24 (6)
Whole mash	—	—	—	—	2.13 ± 0.36 (6)	1.38 ± 0.14 (6)

Table 3. ^{65}Zn content of the intracellular components of mammary tumour and liver tissues from mice injected with ^{65}Zn as the zinc-glycine complex (calculated as a special ratio)

See note in Table 1.

Fraction	^{65}Zn content $\left[\text{ratio } \frac{(^{65}\text{Zn}/\text{mg. of N in fraction})}{(^{65}\text{Zn}/\text{mg. of N in whole mash})} \right]$			
	1 day after injection		3 days after injection	
	Tumour	Liver	Tumour	Liver
Washed nuclei and cell debris	1.06 ± 0.07 (6)	0.43 ± 0.03 (6)	0.87 ± 0.04 (6)	0.55 ± 0.04 (6)
Washed mitochondria	0.92 ± 0.07 (6)	0.42 ± 0.02 (6)	1.08 ± 0.08 (6)	0.60 ± 0.04 (6)
Supernatant (microsomes and soluble proteins)	1.10 ± 0.04 (5)	1.82 ± 0.09 (6)	1.02 ± 0.06 (6)	1.59 ± 0.05 (6)
Whole mash	1.00	1.00	1.00	1.00

^{65}Zn was found in all three sub-cellular fractions (nuclei, mitochondria and supernatant) of both mammary tumours and liver. The distribution pattern in the tumour was, however, very different from that of the liver. From Table 3 it may be seen that the three fractions of the tumour cells have about the same ^{65}Zn concentration. In the mice killed 1 day after injection, the lowest ratio was found in the mitochondria (mean of 0.92) and the highest in the 'supernatant' fraction (1.10). The ratios for the three tumour fractions were very similar for the mice killed 3 days after the injection, though a rather low value (mean of 0.87) was obtained for the nuclear fraction.

One day after the injection most of the ^{65}Zn in the tumour was divided almost equally between the nuclei and the supernatant, with about 40 % in each. Three days after the injection, rather more ^{65}Zn (45 %) was found in the supernatant than in the nuclei (35 %).

In the liver, most of the ^{65}Zn was present in the supernatant fraction 1 and 3 days after the injection. In the liver, the mitochondrial fraction contained the lowest amount of ^{65}Zn of the three fractions studied.

Dialysis of mammary tumour and liver mash

In order to study the firmness of the binding of the zinc to the proteins or other constituents of the tissues, we have determined the extent to which the ^{65}Zn can be removed from tumour and liver mash when the mash is dialysed for a long time in solutions of different pH values. In some experiments made at pH 7.4, a considerable amount of zinc (as zinc-glycine) was added to the dialysing medium to determine whether this extra zinc would effect removal of ^{65}Zn from the tumour or liver by exchange; in other experiments the strong metal-binding or chelating agent EDTA was added to the dialysing medium.

The results showing the means for duplicate

Table 4. Effect of pH on the binding of ^{65}Zn in mouse mammary tumour and liver mash obtained from tissues labelled in vivo

pH	^{65}Zn remaining in mash after dialysis for 96 hr. (percentage of ^{65}Zn originally present in mash)	
	Liver	Mammary tumour
4.0	10.2	7.7
5.6	62.0	42.7
6.4	76.6	94.4
7.4	100.0	100.0
7.4*	40.2	31.7
7.4†	30.8	28.1
8.4	74.8	60.0
10.0	52.2	47.4

* EDTA added to buffer.

† Zinc added to buffer.

experiments are given in Table 4. At pH 7.4 no ^{65}Zn dialysed from tumour or liver mash except when EDTA or excess of zinc was present in the dialysing medium. In the pH range studied (pH 4–10) greatest removal of zinc occurred at pH 4.0, at which acidity about 90 % of the tissue-bound ^{65}Zn dialysed out from both tumour and liver mash within 96 hr. In all these dialyses, except those at pH 7.4 (where none of the ^{65}Zn was removed by dialysis) and at pH 6.4, more zinc was firmly bound in the liver mash than in the tumour tissue.

When excess of zinc or the zinc-binding EDTA was added to the dialysing medium and the dialysis carried out at pH 7.4, 60–70 % of the tissue zinc, which is completely non-dialysable at this pH, can be readily removed by dialysis for 96 hr. The zinc remaining after this period dialysed extremely slowly and about 15 % of the original ^{65}Zn remained in the mash dialysed for 400 hr. against excess of zinc or for 670 hr. against buffer containing EDTA. Both tumour and liver mash contained this very firmly bound ^{65}Zn .

DISCUSSION

The results summarized in Tables 1, 2 and 3 show that there was a remarkably uniform distribution of ^{65}Zn between the three sub-cellular fractions of the tumour cells of the mice injected with zinc-glycine containing ^{65}Zn . The lowest concentration of ^{65}Zn (per mg. of nitrogen) in the tumours of the mice killed 1 day after the injection was found in the mitochondria, and the highest in the supernatant. By the third day, the ^{65}Zn contained in the mitochondria had risen slightly and that of the supernatant had fallen, but in general the differences between the ^{65}Zn concentrations of the three tumour sub-cellular fractions examined were not marked. This uniform distribution of ^{65}Zn between the three tumour fractions, and the high concentration of ^{65}Zn in the liver 'supernatant' compared with that in the liver nuclei and mitochondria, are well illustrated (as in Table 3) when the ^{65}Zn concentration in the separated fractions is assessed by the 'ratio' ($^{65}\text{Zn}/\text{mg. of nitrogen in the fraction}/^{65}\text{Zn}/\text{mg. of nitrogen in whole mash}$).

The distribution pattern of ^{65}Zn in the liver was quite different from that in the tumour. In the liver the ^{65}Zn concentrations of the nuclear and mitochondrial fractions were equal and approximately a quarter or a third that of the supernatant fraction. The ^{65}Zn content of the mitochondria and nuclei 3 days after the injection was higher than that 1 day after injection, but the incorporation of zinc into these fractions of the liver is a comparatively slow process.

Incorporation of ^{65}Zn into the tumour-mitochondrial and -nuclear fractions was considerably faster than into the corresponding liver fractions. The ^{65}Zn content of the tumour mitochondria was little greater 3 days after the injection than at the end of the 1-day period; in comparison, the concentration of $^{65}\text{Zn}/\text{mg. of nitrogen}$ in the liver mitochondria on the third day after the injection was one and a half times that at the 1-day period.

Whether the uniform distribution of ^{65}Zn between the various tumour fractions is typical of the tumour and not of the normal gland is unknown. The fractionation of normal mouse mammary-gland tissue into its sub-cellular components is very difficult because of the large amount of fat present and the small amount of tissue available, but the results of our preliminary experiments indicate that in the normal mammary gland of mice injected with zinc-glycine containing ^{65}Zn , the intracellular distribution of ^{65}Zn differs significantly from that in the mammary tumours.

From the results of the dialysis experiments, certain tentative conclusions can be reached. The ^{65}Zn in the tissues studied was mainly in a form in which it was readily exchangeable with Zn^{2+} ions,

or removable by the chelating agent EDTA. Part of the zinc in both the tumour cells and the liver cells can also be readily removed by dialysis in slightly acid or alkaline solutions. For example, at pH 5.6 there was a loss of 40–60% of the ^{65}Zn when these tissues were dialysed for 96 hr. At pH 6.4, however, about 95% of the ^{65}Zn in the tumour, and 75% in the liver mash, was not dialysable under these conditions, and it may be significant that the pK for imidazole groups in a typical protein (serum albumin) is 6.3 (Schubert, 1954). A considerable amount of evidence is available that imidazole groups have a strong affinity for zinc and, in serum albumin at least, the intrinsic affinity constant agrees well with that for simple zinc-imidazole complexes (Gurd & Goodman, 1952). The results of the dialysis experiments reported here, and other evidence we have obtained (unpublished work), are consistent with the view that much of the ^{65}Zn in these tissues and others we have studied is bound to imidazole groups of the proteins. When these tissues are dialysed at about pH 7.4 against a solution of zinc-glycine in which complex the zinc is present in a readily 'available' or exchangeable form (Tupper, Watts & Wormall, 1951), nearly all the zinc present as ^{65}Zn in both the liver and tumour tissues is readily removed. About one-sixth of the ^{65}Zn in these tissues is, however, very firmly bound and cannot be removed by long dialysis in the presence of excess of zinc-glycine or the metal-binding EDTA. In all probability this ^{65}Zn is present in zinc-containing enzymes or other zinc proteins in which the metal is an integral part of the molecule or in both.

SUMMARY

1. The distribution of ^{65}Zn in the nuclear, mitochondrial and 'supernatant' (soluble proteins plus microsomes) fractions of mammary tumours and livers of mice, 1 and 3 days after the mice have been injected with the isotope in the form of a ^{65}Zn -containing zinc-glycine complex, has been studied. The results are expressed as $^{65}\text{Zn}/\text{mg. of nitrogen}$ in any particular fraction, and also as a special ratio: ($^{65}\text{Zn}/\text{nitrogen}$ in the sub-cellular fraction)/($^{65}\text{Zn}/\text{nitrogen}$ in the whole mash).

2. ^{65}Zn was found in nuclear, mitochondrial and supernatant fractions of mammary tumours and livers.

3. The tumour cells showed a remarkably uniform distribution of ^{65}Zn between the three fractions, but the liver supernatant fractions contained three or four times as much ^{65}Zn (per mg. of nitrogen) as did the nuclei and mitochondria.

4. Part of the ^{65}Zn incorporated into these tissues can be removed by dialysis in slightly acid or alkaline solutions but not normally at pH 7.4.

The results of these dialyses are consistent with the view that the zinc is mainly bound to imidazole groups of the proteins.

5. About four-fifths of the ^{65}Zn present in both the liver and the tumour is freely exchangeable with Zn^{2+} ions at pH 7.4 and can be removed by dialysing the mash against buffers containing excess of exchangeable zinc or the zinc-binding ethylenediaminetetra-acetic acid.

6. About one-sixth of the ^{65}Zn in these tissues could not be removed by exchange or by combination with ethylenediaminetetra-acetic acid, and this fraction probably represents ^{65}Zn in zinc bound to protein.

We are indebted to the British Empire Cancer Campaign for grants which have partly covered the cost of these investigations, and to Dr T. E. Banks for valuable help and advice in connexion with the apparatus for measuring the ^{65}Zn .

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Incorporation of Canavanine by *Staphylococcus aureus* 524 SC

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(Received 16 March 1959)

Previous experiments (Richmond, 1959) have shown that *Staphylococcus aureus* 524, which is normally exacting for arginine, can, when incubated in a fully synthetic amino acid medium containing canavanine in place of arginine, increase in turbidity and synthesize cell protein. Under these conditions, however, the cultures do not synthesize active forms of β -galactosidase, hyaluronidase, phosphatase or the lytic enzyme. It seemed possible therefore that canavanine was incorporated into cell protein in place of arginine, resulting in the formation of inactive enzymes.

A number of workers have reported that certain amino acid analogues can be incorporated into protein of micro-organisms in place of normal amino acids and that this process is associated with variable effects on the enzyme-forming ability of the organisms (Munier & Cohen, 1956, 1959; Cowie & Cohen, 1957; Pardee & Prestidge, 1958; Cohen & Munier, 1959).

This paper describes the detection of canavanine in the protein of *S. aureus* which has been grown in its presence.

MATERIALS AND METHODS

Organism. *Staphylococcus aureus* 524 SC (Rogers, 1953) has been used throughout these studies. The organism was maintained as described by the author (Richmond, 1959).

Media. Hedley-Wright broth (Rogers, 1954) and the fully synthetic amino acid media 18AA and 17AA(-Arg) have been described previously (Richmond, 1959). Experiments involving the use of radioactive lysine were carried out in 18AA medium from which the lysine had been omitted (17AA-Lys medium).

Amino acids and derivatives. The compounds used in these studies and their sources were as follows. L-Canavanine (Dr J. Mandelstam and Dr R. Davies); dinitrophenyl (DNP)-arginine (Dr F. Sanger); DNP- ϵ -lysine (Dr R. R. Porter). The [^{14}C]lysine was obtained from The Radiochemical Centre, Amersham, Bucks. It was used at a specific activity of 0.1 $\mu\text{C}/\text{m-mole}$.

Preparation of organisms for experimental purposes. Unless otherwise stated, organisms were prepared as described previously (Richmond, 1959) and inoculated into 17AA(-Arg) medium containing 50 μg . of canavanine/ml. The culture was incubated at 35° and shaken at the maximum rate (87 cye./min., 1 ft. throw) of a graded shaker (Kantorowicz, 1951); growth was followed turbidimetrically