Table 3. *Free cupric-ion concentration in solutions each containing CuCl_2 (0.005M) and either glycine or a peptide (0.025M) at pH 7.3*

Chelating agent	Free cupric-ion concentration (M)
Glycine	$10^{-9.01}$
Glycylglycine	$10^{-8.86}$
Glycylleucine	$10^{-8.56}$
Glycyltyrosine	$10^{-9.33}$
Carnosine	$10^{-8.46}$
Diglycylglycine	$10^{-8.96}$

peptide. It is true that the copper-glycine complex of the type ML^+ readily takes up a second glycine anion. However, under the conditions envisaged here, where the copper:ligand ratio is 1:1, the extent to which this reaction can take place is limited and therefore its effect on copper binding is less marked than that of proton loss by the copper-peptide complex ML^+ .

On inspection of Table 3, which shows the free cupric-ion concentration at pH 7.3 in a series of solutions each containing cupric chloride (0.005M) and one of the chelating agents in the molar ratio of 1:5 we find that the order of metal binding under these conditions would be

glycyltyrosine > glycine > diglycylglycine > glycylglycine > glycylleucine > carnosine.

In these solutions containing excess chelating agent the order of copper binding is now markedly influenced by the value of K_2 . Since the value of K_2 for glycine ($10^{7.0}$) is very much higher than for any of the peptides, the amino acid is now second in the list, glycyltyrosine with its low pK , still remaining at the top. Furthermore, the inability of the copper-carnosine complex ML to take up a second carnosine

anion results in this peptide being displaced to the foot of the list.

It is clear that the competing powers of various ligands for copper depends to a considerable extent on the ratio of copper atoms to ligand molecules.

SUMMARY

1. Potentiometric titrations and spectrophotometric observations have been made with solutions containing cupric chloride (0.005M) and diglycylglycine in the ratio of 1:1, 1:2, 1:4 and 1:6.

2. The observations have been interpreted on the assumption of the existence in these solutions of the copper-diglycylglycine complexes ML^+ , ML , ML^- , ML_2^- , symmetrical ML_2^{2-} and asymmetrical ML_2^{2-} . Values have been calculated for the stability and dissociation constants of these complexes, and it is shown that these values are consistent with the experimental observations. A characteristic absorption spectrum has been assigned to each of these complexes.

3. The results support the view that in the process of formation of such complexes as ML , a link is established between the amide group and the copper atom, with the loss of a hydrogen ion.

4. The relative avidities of various glycine-containing peptides for cupric ions are compared with each other and with that of glycine itself, at the approximate physiological pH of 7.3.

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The Incorporation of ^{65}Zn in Mammary Tumours and Some Other Tissues of Mice after Injection of the Isotope

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The zinc content of normal tissues has been widely studied (Rost & Weitzel, 1919; Delezenne, 1919; Rost, 1921; Lutz, 1926; Dutoit & Zbinden, 1930; Simakov, 1940; Eggleton, 1940; Gettler & Bastian, 1947). Pathological material has been studied less extensively, and the zinc content of neoplastic tissue has been compared with that of normal tissues by only a few groups of workers. Some

investigators (Cristol, 1923; Labbé & Nepveux, 1927; Sugai, 1939, 1940; Addink, 1950) found that the concentration of zinc in some tumours was higher than that in normal tissues. On the other hand, Carruthers & Suntzeff (1945, 1946) and Cowdry, Carruthers & Suntzeff (1948) observed a decrease in the zinc content of epidermis associated with methylcholanthrene-induced tumours in this

tissue, and leukocytes from leukaemic subjects have been found to contain less zinc than normal leukocytes (Gibson, Vallee, Fluharty & Nelson, 1950).

In view of these conflicting observations on the zinc content of tumour tissues in relation to that of normal tissues, we decided to use ^{65}Zn to study the relative uptakes of zinc by spontaneously occurring tumours and the corresponding non-malignant tissue following a single intraperitoneal injection of the isotope. The tumour selected for the experiments described in this paper was the mammary gland tumour which develops in a high proportion of mice of certain pure-line strains. The 'control' tissues were the apparently non-malignant portions of the mammary glands of these injected tumour mice and also the mammary glands of normal non-tumour-bearing mice which had been injected with ^{65}Zn at the same time as were the tumour mice.

The main object of these investigations was to determine whether malignant tissue differs from the corresponding normal tissue in its uptake or utilization of zinc, and these experiments form part of a study of the feasibility of obtaining specific deposition of radioactive isotopes in malignant tissue. In addition to the mammary glands and tumours a few other tissues of the injected mice were examined for ^{65}Zn in these experiments, to determine whether the tumour mice differ from normal mice in their utilization of injected zinc.

EXPERIMENTAL

Mice

Pure-line female mice of the 'C3H', 'Strong-A', and 'ABC' strains, bred in our laboratory from strains supplied to us by the Institute of Cancer Research (Royal Cancer Hospital) and the Imperial Cancer Research Fund Laboratories, were used for the experiments with tumour-bearing animals. Almost all the females of these strains ultimately develop mammary carcinomata spontaneously. Female mice of a mixed strain bred in our laboratory and showing a very low incidence of spontaneously occurring mammary carcinomata were used as controls.

All the tumour mice and most of the control animals had had one or more litters of young before being used for experimental purposes. If for any reason (e.g. cancer development in the mother) it was not possible for the young pure-line mice to be suckled by their own mothers they were foster-mothered by a lactating female of the same strain.

The animals were housed in galvanized-iron box cages and 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.

Preparation of ^{65}Zn for injection

Radioactive zinc of high specific activity was separated as ZnCl_2 from cyclotron-irradiated copper by one of the procedures previously described (Tupper & Watts, 1954;

Tupper, Watts & Wormall, 1954). As in previous animal experiments, we have found it convenient to inject the zinc as a zinc-glycine complex, for in this form the zinc is held in solution at pH 7.4 and yet it is readily available for biological utilization. This complex was prepared by dissolving 10 mg. glycine in 1 ml. of the ^{65}Zn -containing solution and adding N-NaOH to give a pH of 7.4 and water to give a final volume of 5 ml. The final solution contained $6\mu\text{g. Zn/ml.}$ and gave counting rates of 8.25×10^4 and 1.9×10^6 counts/min./ml. with the Veall-type (M. 6) and scintillation counter, respectively.

Injection of ^{65}Zn

The injections were made intraperitoneally, and each mouse received 0.025 ml. of the ^{65}Zn -containing solution/g. body weight.

The sixty tumour-bearing mice used in these experiments received their injections a few days after mammary tumours had appeared, and groups of these mice (8–10 in each group) were killed 3 hr., 1, 3, 7 and 14 days after the injection. A similar series of experiments was carried out with sixty control (non-tumour-bearing) mice. In the planning of the experiment it was intended that one group of injected mice would be killed 21 days after the injection but several of the tumour-bearing mice in this group were killed and discarded at an earlier stage since ulceration of the tumour through the skin appeared imminent. Thus results were obtained with only four tumour-bearing mice in this group (group 6), and four control mice, and in view of this small number of observations these results are not included in the Tables of this paper.

Removal of tissues

The mouse was killed with coal-gas, and a blood sample was immediately taken from the heart. No attempt was made to render the organs blood-free by perfusion.

Mammary tissue was removed by blunt-dissection, and care was taken to remove all visible lymph-nodes from the portions used for analysis. The identity of the normal mammary tissue was checked histologically in the case of the first thirty normal (non-tumour-bearing) mice used, and this precaution was only abandoned when it had been established that the technique employed ensured that mammary gland tissue containing no lymphoid tissue could be consistently obtained. A similar histological check was made on the 'normal' mammary gland tissues of the tumour-bearing mice. Between 150 and 500 mg. wet weight mammary tissue were obtained from each mouse.

The first nineteen mammary tumours investigated, and any in unusual situations or with unusual macroscopic appearances, were examined histologically and found to be typical anaplastic carcinomata.

Determinations

Radioactive zinc (^{65}Zn). For most of these determinations the tissue sample was digested with H_2SO_4 and the radioactivity of the digest measured with a scintillation counter (as described by Banks, Tupper, Watts & Wormall, 1954*a, b*), but a few of the earlier determinations were made by the older procedure of Banks, Tupper & Wormall (1950) using a Veall (1948)-type liquid counter. Measurements were made for sufficient time to give a total count with a probable error of $\pm 1\%$ when the scintillation-counter was used and $\pm 2.5\%$ when Geiger-Müller counters were employed.

Total nitrogen. Total N was determined by the micro-Kjeldahl method, the ammonia being distilled in the apparatus described by Markham (1942), into a boric acid solution containing methyl red-methylene blue indicator.

RESULTS

It was not possible to separate the ducts and alveoli, which are the mammary gland components from which carcinomata arise, from the relatively large amounts of fat in which they are embedded. Because of this variable fat content of the samples it was considered that ^{65}Zn contents based on a unit weight of tissue would have little significance. We have, therefore, related the amount of ^{65}Zn in each tissue to the nitrogen content, and the results are expressed as the percentage of the injected $^{65}\text{Zn}/\text{mg.}$ tissue nitrogen.

As pointed out by Thomson, Heagy, Hutchison & Davidson (1953), the relating of the results of tissue analysis to dry or wet weight of the tissue has serious disadvantages, and they advocate the expression of analytical results in relation to deoxyribonucleic acid content. Unfortunately this method could not be adopted in our present investigations, since the amount of tissue available was often too small for the necessary determinations.

Comparison of mammary gland carcinoma with 'normal' (apparently non-malignant) mammary gland tissue

The results presented in Table 1 show that, except with the mice killed 3 hr. after the injection of ^{65}Zn (i.e. group 1) the carcinoma tissue had a higher mean $^{65}\text{Zn}/\text{total N}$ ratio than had the 'normal' mammary gland tissue from either

Table 1. *Comparison of the ^{65}Zn contents of mammary gland carcinoma and normal mammary gland tissue*

The mice were given a single intraperitoneal injection of ^{65}Zn -containing zinc-glycine. The ^{65}Zn contents of the tissues are given as mean values $\pm$ standard error. The number of tumours or mammary glands examined (the whole mammary gland tissue obtained from each mouse being counted as one gland) are given in brackets.

Group	Interval between injection and killing (days)	^{65}Zn contents (as % injected $^{65}\text{Zn}/\text{mg.}$ tissue N)		
		Mammary gland		Mammary gland carcinoma
		Non-tumour-bearing mice	Tumour-bearing mice*	
1	0.125	0.164 ± 0.046 (8)	0.143 ± 0.031 (8)	0.134 ± 0.038 (8)
2	1	0.162 ± 0.019 (6)	0.128 ± 0.017 (7)	0.248 ± 0.046 (9)
3	3	0.153 ± 0.034 (7)	0.102 ± 0.006 (5)	0.235 ± 0.015 (12)
4	7	0.096 ± 0.019 (7)	0.075 ± 0.013 (7)	0.148 ± 0.011 (9)
5	14	0.052 ± 0.008 (9)	0.043 ± 0.006 (7)	0.065 ± 0.007 (10)

* The 'normal' mammary gland tissues from tumour-bearing mice were obtained from the same mice as the corresponding carcinomata, but in a few cases, particularly with mice, with large tumours, it was not possible to obtain sufficient of the 'normal' tissue for analysis.

Table 2. *The ^{65}Zn contents of some tissues of tumour-bearing and non-tumour-bearing mice following an intraperitoneal injection of ^{65}Zn -containing zinc-glycine*

Group	Interval between injection and killing (days)	^{65}Zn content (as % injected $^{65}\text{Zn}/\text{mg.}$ tissue N)									
		Liver		Lung		Kidney		Skin		Blood	
		T*	NT*	T	NT	T	NT	T	NT	T	NT
1	0.125	0.527 ± 0.107 (8)	0.427 ± 0.084 (7)	0.137 ± 0.011 (8)	0.153 ± 0.020 (8)	0.348 ± 0.038 (8)	0.289 ± 0.019 (7)	0.055 ± 0.015 (8)	0.065 ± 0.015 (8)	0.095 ± 0.025 (8)	0.154 ± 0.056 (7)
2	1	0.348 ± 0.032 (9)	0.401 ± 0.030 (8)	0.154 ± 0.013 (9)	0.181 ± 0.023 (6)	0.217 ± 0.016 (9)	0.238 ± 0.016 (8)	0.043 ± 0.005 (3)	0.045 ± 0.004 (3)	0.031 ± 0.003 (8)	0.031 ± 0.003 (5)
3	3	0.276 ± 0.024 (7)	0.269 ± 0.023 (10)	0.157 ± 0.012 (8)	0.138 ± 0.008 (9)	0.160 ± 0.013 (8)	0.142 ± 0.008 (9)	0.049 ± 0.006 (5)	0.045 ± 0.009 (4)	0.042 ± 0.008 (8)	0.029 ± 0.003 (7)
4	7	0.166 ± 0.011 (8)	0.129 ± 0.015 (8)	0.101 ± 0.013 (9)	0.096 ± 0.010 (10)	0.127 ± 0.027 (8)	0.080 ± 0.008 (9)	0.039 ± 0.005 (6)	0.035 ± 0.003 (6)	0.047 ± 0.016 (8)	0.039 ± 0.014 (7)
5	14	0.105 ± 0.017 (9)	0.069 ± 0.009 (9)	0.058 ± 0.007 (8)	0.054 ± 0.004 (9)	0.059 ± 0.008 (8)	0.044 ± 0.003 (10)	0.028 ± 0.004 (7)	0.028 ± 0.004 (6)	0.017 ± 0.002 (8)	0.015 ± 0.001 (10)

* T and NT denote, respectively, tumour-bearing and non-tumour-bearing mice. For details about the presentation of the results see Table 1.

tumour-bearing or non-tumour-bearing mice. The difference between the ^{65}Zn contents of the tumours and the 'normal' mammary gland tissues removed from the same animal is statistically significant, the value of P being less than 0.01 and very little greater than 0.001. If the results for group 1 (containing the mice killed 3 hr. after the injection of ^{65}Zn) are excluded, the difference is still greater, the value for P then being a little less than 0.001.

With all the groups studied, except group 6, the mean values for the ^{65}Zn contents of the 'normal' mammary gland tissue of the injected tumour mice were less than those for the corresponding non-tumour-bearing mice, but the differences were not statistically significant, the value for P being between 0.1 and 0.2.

For reasons given earlier in this paper there were few tumour mice available for examination 21 days after the injection, and the results for this small group (group 6) are not included in Table 1. It is of interest, however, that they were in general agreement with those recorded for groups 2-5, the mean values, corresponding to those given in columns 3-5 of Table 1, being 0.032, 0.039 and 0.058 for three, two and four mice, respectively.

*^{65}Zn content of a few other tissues
of the injected mice*

The results of the determination of the amount of ^{65}Zn in the blood, liver, lung, kidney and skin of the tumour-bearing and the 'normal' mice, at various intervals after the intraperitoneal injection of ^{65}Zn are given in Table 2. Statistical analysis of these results showed that there was no significant difference (the values of P being 0.2 or more in every case), as far as the blood and the few organs examined are concerned, between the values for the tumour mice and those for the normal mice. There was a similar agreement between the corresponding values for the animals in group 6, the mean values for the ^{65}Zn content (as % injected $^{65}\text{Zn}/\text{mg. tissue N}$) ranging from 0.049 and 0.052 for the livers to 0.012 and 0.012 for the bloods of the tumour and non-tumour mice, respectively.

DISCUSSION

Heath (1949) and Heath & Liquier-Milward (1950) observed that transplanted mammary gland carcinomata and transplanted fibrosarcoma contained more ^{65}Zn (on a weight basis) than did normal mammary gland and striped muscle respectively, 18 hr. after the mice had been given a subcutaneous injection of the isotope. In order to try to obtain a stricter comparison between tumour tissue and 'corresponding normal tissue' in the experiments described in this paper, we have studied a spontaneously occurring tumour and a series of two

'control' tissues, and have also calculated the ^{65}Zn contents in relation to the tissue total nitrogen. We believe that in this way it has been possible to obtain a reasonably accurate assessment of the relative zinc requirements, or capacities for utilizing zinc, of tumour and non-tumour tissues.

Thus the observation that mammary gland tumour tissue takes up and retains considerably more of the injected ^{65}Zn than does normal mammary gland tissue is significant and will be investigated further. The difference is too small to be of value for therapeutic radiological purposes, but it is of interest in relation to the metabolic utilization of zinc. Apart from the presence of zinc in carbonic anhydrase (Keilin & Mann, 1939, 1940) little is known about the tissue constituents with which this metal is associated. Lipovitellin contains fairly firmly bound zinc (Tupper *et al.* 1952, 1954) but this lipoprotein is apparently found only in egg-yolk. Heath (1949) and Heath & Liquier-Milward (1950) obtained evidence that at least some of the zinc in tumour tissue is associated with nucleoprotein in the cell nuclei, but Rosenfeld & Tobias (1951) were unable to find appreciable amounts of ^{65}Zn in the nuclear fractions of liver, kidney, lung and transplanted mammary tumour tissues of mice after the intravenous injection of ^{65}Zn as zinc chloride. Hoch & Vallee (1952) and Vallee, Hoch & Hughes (1954) have obtained evidence for the presence of zinc-containing proteins in human leukocytes.

Our limited study of the distribution of ^{65}Zn in some of the organs of the injected mice did not reveal any significant difference between the tumour-bearing and the non-tumour-bearing animals with respect to the uptake of injected zinc by liver, lung, kidney and skin.

SUMMARY

1. The distribution of ^{65}Zn in mice with spontaneously occurring mammary tumours and in non-tumour-bearing mice has been studied at various intervals after a single intraperitoneal injection of a ^{65}Zn -containing zinc-glycine complex.

2. The tumour tissue had a small but statistically significant higher ^{65}Zn content/(mg. tissue nitrogen) than had 'normal' (apparently non-malignant) mammary gland tissue of the same mouse at 1, 3, 7 and 14 days, but not at 3 hr., after the injection of ^{65}Zn . The mean tumour values 1, 3 and 7 days after the injection were about twice those for normal mammary gland tissue.

3. 'Normal' mammary gland tissue from the injected tumour-bearing mice generally contained a little less ^{65}Zn than did the corresponding tissue from the non-tumour mice, but the difference was not statistically significant.

4. The ^{65}Zn contents of the blood, liver, kidney, lung and skin of the tumour-bearing mice were not

statistically different from the corresponding values obtained with normal mice.

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Actinomycete Metabolism

INCORPORATION OF ¹⁴C-LABELLED COMPOUNDS INTO STREPTOMYCIN

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Recent work (Numerof, Gordon, Virgona & O'Brien, 1954) has indicated that streptomycin can be elaborated by *Streptomyces griseus* with D-glucose as sole carbon source. The radioactive labels of added [¹⁴C]glycine and acetate were significantly incorporated only in the guanidine side chains. As these carbon atoms arise largely, if not entirely, from carbon dioxide (Hunter, Herbert & Hockenhull, 1954), atoms from the substrate are presumably incorporated only after these have been completely metabolized by the organism. Karow, Peck,

Rosenblum & Woodbury (1952) have demonstrated the incorporation of the labelled carbon of [¹⁴C]-glucose into streptomycin. They did not, however, submit their product to chemical degradation to determine the distribution of the radioactivity within the molecule. Their results have been considerably extended in our work, and we have shown that glucose is incorporated equally into all three monosaccharide residues in streptomycin. In an endeavour to discover some of the intermediate pathways involved in this process, a study has also been made of the incorporation of [¹⁴C]streptamine and N-methyl-L-glucosamine into the antibiotic by *Strep. griseus*.

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