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The Effects of Zinc Deficiency on Pancreatic Carboxypeptidase Activity and Protein Digestion and Absorption in the Rat

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1. Proteolytic enzyme activities were examined in the pancreas of zinc-deficient and control rats. 2. No change was detected in trypsin-plus-chymotrypsin activity. 3. Carboxypeptidase activity was appreciably lowered in zinc deficiency and returned rapidly to normal on zinc therapy. 4. In experiments in which U-^{14}C-labelled *Chlorella* protein was fed no evidence was obtained which suggested that the reduction in carboxypeptidase activity had limited the rate of protein digestion or absorption. 5. The specific activity of pancreatic protein synthesized during these experiments was appreciably lower in zinc-deficient than in control rats. 6. A higher proportion of the total activity present, in each organ examined, was in the non-protein fraction in zinc-deficient rats.

That zinc is an essential trace element in animal nutrition was first conclusively demonstrated by Todd, Elvehjem & Hart (1934) by using the rat. Numerous studies have since been carried out to determine the metabolic roles played by zinc.

The ubiquity of the element in Nature suggests that occurrences of zinc deficiency should be rare, but reports have appeared of zinc deficiency in swine (Tucker & Salmon, 1955), in cattle (Legg & Sears, 1960; Haaranen & Hyppola, 1961; Dynna & Havre, 1963) and of a combined zinc and iron deficiency in humans (Prasad, Miale, Farid, Sandstead & Schulert, 1963).

Symptoms of the deficiency, which are common to all species, include loss of appetite, retardation of growth, partial loss of hair and roughness of the skin (the last, in the pig, having been characterized as parakeratosis; Tucker & Salmon, 1955). When the deficiency is severe and prolonged the further effects include delayed sexual maturity (Prasad *et al.* 1963), lowered fertility (Haaranen & Hyppola, 1961) and retarded testicular development (Miller & Miller, 1962).

Zinc is known to be a component of the carbonic anhydrase (EC 4.2.1.1; carbonate hydro-lyase) of bovine erythrocytes (Keilin & Mann, 1940), the glutamic dehydrogenase [EC 1.4.1.3; L-glutamate–NAD(P) oxidoreductase] of bovine liver (Vallee, Adelstein & Olson, 1955), and the lactate dehydrogenase (EC 1.1.1.27; L-lactate–NAD oxidoreductase) of rabbit skeletal muscle (Vallee & Wacker, 1956). A more recent report (Harrison, 1963) suggests that a bovine malate dehydrogenase (EC 1.1.1.37; L-malate–NAD oxidoreductase) is also a zinc-dependent enzyme. Although in the rat these enzymes have not so far been proved to contain zinc, it appeared worth while to investigate the effects of zinc deficiency on their activity. Preliminary experiments showed, however, no effects of the deficiency on liver glutamate dehydrogenase and malate dehydrogenase, or on liver and skeletal-muscle lactate dehydrogenase. Neither were we able to demonstrate any substantial change in the activity of blood carbonic anhydrase.

The work of Miller & Miller (1962) and Ott, Smith, Stob & Beeson (1964), with calves and sheep respectively, indicating that the pancreas is one of the few organs to suffer an appreciable decrease in zinc concentration on giving diets low in zinc, together with the earlier report by Hove, Elvehjem & Hart (1938) that the proteolytic activity of the pancreas is reduced in zinc deficiency and the demonstration by Vallee & Neurath (1955) that zinc is an integral part of pancreatic carboxypeptidase (EC 3.4.2.1 and 3.4.2.2), prompted us to investigate the effects of zinc deficiency on the activity of this enzyme in the rat pancreas, and to determine the effects of any such change on the

utilization of dietary protein. A limited study of the effects of zinc deficiency on pancreatic trypsin-plus-chymotrypsin activity was also carried out. This work forms part of an experimental programme to determine the effects of zinc deficiency on and the requirements for zinc in farm livestock.

METHODS

Animals and diet. Young Hooded Lister rats (Rowett Institute strain) of both sexes were used. Dams and young were given a semi-synthetic diet similar to that described by Mills & Murray (1960) with the exception that egg albumin was substituted for casein as the protein source. On weaning, the young were transferred to individual Perspex cages. The complete diet containing 6p.p.m. of added Zn^{2+} as $ZnSO_4,7H_2O$ was eaten readily and the animals grew well and appeared in good condition. Analysis showed an average zinc content of 0·75p.p.m. in the deficient diet. Animals receiving the deficient diet were always offered food *ad libitum,* whereas control animals were pair-fed or fed *ad libitum* as experimental conditions dictated. All animals were offered distilled water *ad libitum.*

Determination of carboxypeptidase activity. Animals were killed by a blow on the head and the duodenal and biliary portions of the pancreas were rapidly dissected out. About 50mg. was weighed and homogenized (in a glass homogenizer) in 10ml. of 0·04M-veronal–HCl buffer, pH7·5, containing 0·02M-$CaCl_2$. The homogenate was centrifuged at 100*g* for 10sec. (MSE Minor) to remove large particles. A 3ml. sample of the supernatant was transferred to a second 10ml. graduated centrifuge tube, and the zymogens were activated by adding 0·1ml. of a solution of crystalline trypsin (1mg./ml.) (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) in the veronal–HCl–$CaCl_2$ buffer. The mixture was then incubated at 37° for 3min. (Ravin & Seligman, 1951). Tryptic activity was stopped by the addition of 0·2ml. of 2% (v/v) di-isopropyl phosphorofluoridate in propan-2-ol. The mixture was left at room temperature (18–20°) for not less than 1hr.; 2·7ml. of the veronal–HCl–$CaCl_2$ buffer was then added, the mixture was stirred and then centrifuged at 2000*g* for 2–3min. Samples from this activated solution were incubated in a final volume of 1·2ml. at 37° for 20min. with 0·5ml. of the substrate solution [prepared by suspending 0·178g. of *N*-benzyloxycarbonylglycyl-L-β-phenylalanine (British Drug Houses Ltd., Poole, Dorset) in 5ml. of veronal–HCl–$CaCl_2$ buffer and adding sufficient N-NaOH solution to bring the substrate into solution and the pH to 7·5]. At the end of the incubation the samples were chilled in ice–water and 3ml. of 5% (w/v) trichloroacetic acid in ethanol was rapidly added to each. The solutions were left in the refrigerator for 1hr.; they were then centrifuged and 0·1ml. samples of the supernatant were removed for amino N determinations by the method of Moore & Stein (1954), with phenylalanine as a standard.

Protein was determined in samples of the unactivated homogenate by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine plasma albumin (Armour Laboratories, Eastbourne, Sussex) as a standard. Results are expressed as μmoles of substrate split/mg. of protein/min. in the unactivated homogenate.

Determination of trypsin-plus-chymotrypsin activity. In several early experiments the trypsin-plus-chymotrypsin activity of pancreatic tissue of zinc-deficient and control rats was determined by a method based on that of Howard & Yudkin (1963) with a pig enteropeptidase preparation as the activator of trypsinogen and chymotrypsinogen. Pancreatic homogenates were prepared in 0·04M-veronal–HCl buffer containing $CaCl_2$ to give a final concentration of 0·02M and adjusted to pH7·5. Homogenates contained between 0·5 and 0·9mg. of protein/ml. of buffer as determined by the method of Lowry *et al.* (1951). Incubations of 20min. duration were carried out by using two concentrations of homogenate in duplicate. Blank determinations in which the casein substrate was added after incubation but before the addition of the protein precipitant were carried out on each sample.

Uptake of ^{14}C *from U-*^{14}C*-labelled* Chlorella *protein.* The U-^{14}C-labelled *Chlorella* protein was obtained from The Radiochemical Centre, Amersham, Bucks. On arrival it was diluted with dried egg albumin to give a specific activity of 0·6μc/mg. and the mixture was then purified by phenol extraction and acetone precipitation according to the procedure of Dawson & Holdsworth (1962). Zinc-deficient rats weighing 63–83g. and their pair-fed controls weighing 85–93g. receiving a zinc-supplemented diet were starved overnight and at 9.00a.m. were offered sufficient U-^{14}C-labelled *Chlorella* protein plus carrier to provide a dose of 5μc/100g. live wt.; this quantity of *Chlorella* protein plus carrier was further diluted with dried egg albumin so that the total quantity of protein offered was 200mg. This was all consumed within 5min., whereupon the rats were transferred to closed chambers for 1·5hr. for collection of exhaled $^{14}CO_2$ in a series of six tubes each containing 30ml. of 2N-KOH. Rats were withdrawn at 1hr. after feeding for collection of 0·1ml. of blood from the coccygeal vein; further blood samples were taken at 2hr. and 3hr. In each case the blood was drawn directly into 1ml. of 0·3N-$HClO_4$ and the precipitate and supernatant were treated as detailed below for other tissues.

Rats were anaesthetized with diethyl ether and decapitated 3hr. after feeding and their tissues rapidly removed and homogenized in ice-cold water with an all-glass homogenizer. Ice-cold $HClO_4$ was added to the homogenate to give a final concentration of 0·5N before being rehomogenized and centrifuged at 1200*g* for 10min. (MSE Minor centrifuge). The protein precipitate was washed twice with 0·3N-$HClO_4$, twice with ethanol and once with diethyl ether, dried over silica gel to constant weight and ground to a fine powder (particle size<0·2mm.). Tissues from the digestive tract were washed free of their contents by a jet of distilled water at 0° before homogenization. Protein-free supernatants were neutralized with KOH, centrifuged to remove $KClO_4$ and evaporated slowly to dryness under infrared lamps; the residue was dissolved in 1ml. of water.

Radioisotope counting procedures. Finely powdered protein precipitates (usually 3–8mg.) were stirred into 10ml. of NE210 gel scintillator (Nuclear Enterprises Ltd., Edinburgh) in a glass vial, which was then partially immersed for 10sec. in a water bath held at 95°. Reproducibility of counting was improved by this gentle heat treatment after which the gel remains semi-fluid and regains an optically flat gel/air interface after stirring. Portions (usually 0·1ml.) of protein-free supernatant solutions were pipetted into the centre of Whatman no. 1 filter paper rectangles (3cm.×1 cm.). These papers were gently dried in a warm air stream

and laid in a glass vial containing 4ml. of a solution of 2,5-diphenyloxazole (0·5g./100ml.) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (5mg./100ml.) in xylene. The counting equipment used comprised a Panax scintillation counter (type SC–LP; Panax Equipment Ltd., Redhill, Surrey) cooled to $10 \pm 2^{\circ}$ and Panax scaler–timer (type D657/T300). A discriminator bias of 15v was used at an extra high tension of 1500v. Under these conditions the efficiency of counting protein precipitates in NE210 gel was found to be $49 \pm 2\%$; the efficiency of counting protein-free supernatants dried on filter paper in 2,5-diphenyloxazole–1,4-bis-(5-phenyloxazol-2-yl)benzene was $38 \pm 3\%$. Counts recorded in Table 3 are corrected to 100% efficiency. Counts of different samples of the protein precipitate in NE210 gel gave an S.E.M. of approx. $\pm 3\%$ at counting rates in the range 1000–10000 counts/100sec. No significant change in counting efficiency was noted when the quantity of precipitate dispersed in gel was varied in the range 3–20mg./10ml. of gel. To determine the activity of exhaled $^{14}CO_2$ an excess of 2M-$BaCl_2$ was added to the KOH traps from each respiration chamber; the combined precipitate of $Ba(OH)_2$ and $BaCO_3$ was filtered, washed with water, ethanol and diethyl ether and was dried over silica gel at room temperature. The activity of the precipitate was determined by counting in NE210 gel as described above.

RESULTS

In initial experiments in which activation of procarboxypeptidase was achieved by the incubation of homogenates with an enteropeptidase preparation, the mean activity of pancreatic carboxypeptidase from five deficient rats, depleted of zinc for from 28 to 82 days, was 60% of that from five rats receiving the zinc-supplemented diet. That this change is not a part of a general defect in the synthesis of proteolytic enzymes in the pancreas of zinc-deficient rats is shown by the results obtained in studies of trypsin-plus-chymotrypsin activity. In three control rats the mean activity was 0·135 unit (1unit = 1μg. of amino N liberated/mg. of pancreas protein/min.) (range: 0·114–0·182) whereas in three zinc-deficient rats the activity was 0·164 unit (range: 0·108–0·252). In a further experiment, again with enteropeptidase as activator, in which previously zinc-deficient rats (zinc-deficient diet given for 35 days) were killed at intervals, after being offered a constant daily amount of zinc-supplemented food based on the overall quantity consumed daily during the week before the experiment, the pancreatic carboxypeptidase activities rose from 34% of that of the pair-fed animals before the start of repletion to 102% of normal after 72hr. of repletion.

The enteropeptidase preparation was later found to be unstable even when stored at approx. -20° and lost most of its activity in 3 weeks. The need to standardize conditions in longer-term experiments led to the use of trypsin as activator in the experiments that are now reported.

Carboxypeptidase activity during repletion with zinc. Table 1 shows that swift recovery of carboxypeptidase activity when rats, depleted of zinc during a 35-day period from weaning, were transferred to a diet providing 6p.p.m. of zinc. Rats were killed and assays performed at daily intervals after the start of repletion. In this experiment the food intake of all animals was limited to 6g./day to obviate any effect of differing appetites on the amount of food, and hence zinc, ingested. Under these conditions the activity of pancreatic carboxypeptidase had risen to normal levels by the fourth day of repletion.

The results of these two studies on the increase of carboxypeptidase activity after zinc repletion may be compared with studies on the rate of response of the zinc-deficient rat to the oral administration of a single 100μg. dose of zinc (administered as a solution of zinc sulphate). It has been found repeatedly that the zinc-deficient rat responds rapidly to such treatment by increasing its food intake and that the maximum rate of gain in weight occurs between 24 and 48hr. after administration, i.e. before a marked increase in pancreatic carboxypeptidase is detectable.

Carboxypeptidase activity during zinc depletion. In the next experiment the time-course of change in pancreatic carboxypeptidase activity during zinc depletion was studied. Rats that had been receiving a zinc-supplemented diet to an allowance governed by the food intake of litter-mate zinc-deficient animals were given zinc-deficient diet *ad libitum* from 24 days after weaning. Animals were killed at intervals up to 21 days after the start of this treatment for assay of pancreatic carboxypeptidase

Table 1. *Effect of feeding zinc-supplemented diet to rats previously fed a zinc-deficient diet for 35 days from weaning on pancreatic carboxypeptidase activity*

Experimental details are given in the text.

Days of repletion with zinc-supplemented diet	Pancreatic carboxypeptidase activity (µmoles of substrate used/min./mg. of protein)		Mean activity of repleting rats (% of control)
	Control rats	Repleting rats (2 rats/sampling)	
0	115	58, 18	23
1	92	28, 63	49
2	92	48, 100	80
3	135	95, 60	58
4	95	115, 120	123

activity. The decline in activity after the start of zinc depletion was much more rapid than was expected (Table 2). In rats killed 4 days after the start of depletion pancreatic carboxypeptidase was only 48% of that in control rats. No further decline in activity was noted during the 21-day experimental period. These changes were not associated with any decline in the rate of growth of depleting rats until the eleventh day of depletion; the average gain in weight, initially 5·4g./day, fell to 3·4g./day by the eleventh day and to 2·1g./day between the fourteenth and twenty-first days of treatment, the final rate of growth being only 60% of that achieved by rats given the zinc-supplemented diet but otherwise treated identically.

Utilization of U-^{14}C-labelled Chlorella *protein.* Table 3 presents results of determinations of the specific activity of the lipid-free perchloric acid-insoluble fraction (hereafter referred to as the 'protein' fraction) of tissues from zinc-deficient and pair-fed control rats killed 3hr. after feeding on U-^{14}C-labelled *Chlorella* protein at a level providing 5μc of ^{14}C radioactivity/100g. body wt. Outstanding among these results is the lower specific activity of pancreatic protein from zinc-deficient rats than from their pair-fed control litter mates. The mean specific activity of this fraction from zinc-deficient rats was only 72% of that from control animals. A similar but smaller trend is seen on comparing the specific activity of this fraction with those from

Table 2. *Effect of feeding zinc-deficient diet to control rats on pancreatic carboxypeptidase activity*

Experimental details are given in the text.

Days of depletion	Pancreatic carboxypeptidase activity (μmoles of substrate used/min./mg. of protein)	Activity (% of mean activity on day 0)
0	138	—
	145	—
5	63	44
	73	52
7	85	60
	127	91
11	75	53
	70	49
14	90	64
	82	59
21	63	44
	70	49

Table 4. *^{14}C radioactivity of the 0·5N-perchloric acid-soluble fraction of tissues from zinc-deficient and pair-fed control rats expressed as a percentage of the total ^{14}C radioactivity of each tissue*

Experimental details are given in the text. Results are given as means ± S.E.M. for four animals/treatment.

	^{14}C radioactivity of 0·5N-$HClO_4$-soluble fraction (% of total)	
Tissue	Pair-fed control rats	Zinc-deficient rats
Blood (3hr.)	30·6±3·9	34·2±4·3
Liver	16·3±1·4	28·8±9·5 (19·5±3·9*)
Pancreas	6·3±0·6	9·2±1·9
Kidney	36·0±4·2	43·6±3·1
Muscle	41·2±3·2	49·2±6·7

* Value when inexplicably high result for one animal excluded.

Table 3. *Specific activity of the protein fraction of tissues from zinc-deficient and pair-fed control rats 3hr. after feeding U-^{14}C-labelled* Chlorella *protein*

Experimental conditions are given in the text.

		Specific activity (counts/100sec./mg. of protein)						
						Blood		
Pair no.	Rats	Pancreas	Kidney	Muscle	Liver	1hr.*	2hr.*	3hr.
1	Control	1872	190	24	223	37	57	56
	Zinc-deficient	1174	144	17	218	25	66	63
2	Control	1611	158	23	209	—	—	79
	Zinc-deficient	1275	150	23	219	—	—	67
3	Control	1318	147	25	207	—	—	49
	Zinc-deficient	1077	125	16	264	—	—	43

* Sampled from coccygeal artery and vein 1hr. and 2hr. after giving ^{14}C-labelled protein.

Table 5. ^{14}C *radioactivity of the non-protein fraction of whole blood at intervals after the ingestion of U-*^{14}C*-labelled* Chlorella *protein (5µc/100g. body wt.) by zinc-deficient and pair-fed control rats after 18hr. starvation*

Experimental details are given in the text. The radioactivity of protein-free supernatants of whole blood is given as counts/100sec./ml.

	Pair-fed control rats				Zinc-deficient rats			
Expt. no.	I	II	III	IV	I	II	III	IV
Rat no.	478	483	482	474	476	481	480	472
Interval between feeding and blood sampling								
1hr.	4079	—	—	4917	8977	—	—	4800
2hr.	2470	—	—	5282	5314	—	—	4638
3hr.	4172	3669	3962	3147	4524	4499	6263	5174

Table 6. *Percentage of radioactivity of U-*^{14}C*-labelled* Chlorella *protein exhaled as* $^{14}CO_2$ *during a period of 3hr. after oral administration*

	Pair-fed control rats			Zinc-deficient rats		
Expt. no.	I	II	III	I	II	III
Rat no.	478	483	482	476	481	480
% of administered radioactivity recovered as $^{14}CO_2$	14·9	15·8	13·2	13·6	14·1	16·0
Sp. activity of exhaled CO_2 (counts/100sec./mg. of $BaCO_3$)	348	249	250	202	234	301

Table 7. *Recovery of radioactivity administered as U-*^{14}C*-labelled* Chlorella *protein estimated from total radioactivity found in blood, liver, kidney, pancreas, muscle and exhaled* $^{14}CO_2$

The radioactivity in blood was estimated by assuming that blood accounts for 8% of the total live weight. The radioactivity in muscle was estimated by assuming that skeletal muscle accounts for 40% of the total live weight.

	Pair-fed control rats			Zinc-deficient rats		
Expt. no.	I	II	III	I	II	III
Rat no.	478	483	482	476	481	480
% of administered radioactivity recovered	23·9	26·5	22·9	21·4	24·1	26·2

kidney and muscle tissue. This was not found in liver or blood.

In Table 4 the activity of the fraction soluble in 0·5N-perchloric acid is expressed as a percentage of the total ^{14}C activity in each organ. Despite the large standard errors of some of these values it is apparent that a higher proportion of the total activity remained in the soluble fraction in zinc-deficient than in control animals. All organs showed this difference; the magnitude of the difference between organs did not appear to be related to tissue vascularity and thus probably did not arise solely from the presence of occluded blood.

Changes in the 0·5N-perchloric acid-soluble activity of whole blood at intervals after feeding U-^{14}C-labelled *Chlorella* protein are given in Table 5. These changes in activity with time follow a similar pattern to those reported by Dawson & Holdsworth (1962) for plasma amino acid activity in normal rats fed on U-^{14}C-labelled *Chlorella* protein. A comparison of the levels of activity, in control and zinc-deficient rats, 1hr. after feeding does not suggest that there exists any limitation to proteolysis and absorption of labelled amino acids into the bloodstream of the zinc-deficient animals that could be a direct consequence of the lower pancreatic carboxypeptidase earlier demonstrated in these animals. After 3hr. the 0·5N-perchloric acid-soluble radioactivity in the blood of zinc-deficient rats remained appreciably higher than that of their control litter mates, presumably as a consequence of a decreased rate of incorporation

of ^{14}C-labelled amino acids into tissue proteins or a decreased rate of oxidation of their deaminated residues. This second possibility appears unlikely as measurements of $^{14}CO_2$ output during the 3hr. interval between isotope administration and slaughter of experimental animals (Table 6) indicate that no difference exists between deficient and control animals, between 13 and 16% of the ingested activity appearing in this form in individual animals of both these groups. No significant difference in the specific activity of exhaled carbon dioxide was apparent. Table 7 presents an estimate of the total recovery of administered *Chlorella* protein activity in the various tissues examined plus that present in exhaled $^{14}CO_2$. The absence of any difference between the animals of the two groups again suggests that the low carboxypeptidase activity found in the zinc-deficient animals was not limiting proteolysis and absorption of activity from the gastrointestinal tract.

DISCUSSION

The present work shows that the activity of carboxypeptidase in the pancreas of the rat falls rapidly when a zinc-deficient diet is given and recovers with equal rapidity when a zinc-supplemented diet is given. We have not determined whether these changes are merely a consequence of an inadequate supply of zinc for activation of the apoenzyme or a defect in the production of the apoenzyme itself; this point requires further investigation in view of the fact that the studies on the incorporation of activity from ingested U-^{14}C-labelled *Chlorella* protein suggest that protein synthesis in the pancreas is impaired. However, it is noteworthy that, despite this last finding, the synthesis of trypsin by the pancreas is apparently proceeding normally in the zinc-deficient animal.

Coleman & Vallee (1960) and Vallee (1961) have shown in studies *in vitro* that the zinc-free apoenzyme of carboxypeptidase is reactivated with respect to its action both as a peptidase and an esterase when Co^{2+}, Ni^{2+}, Mn^{2+} or Fe^{2+} ions are added to the assay system. In the present experiment no attempt was made to deplete the rats of either iron or manganese reserves, so that, provided that the concentration of the apoenzyme is not diminished in the zinc-deficient animal, our results suggest that the substitution of either Fe^{2+} or Mn^{2+} for Zn^{2+} does not proceed *in vivo* at a rate sufficient to maintain the activity of this peptidase.

There is a dearth of information on the quantitative importance of carboxypeptidase in the overall process of amino acid release from proteins before their absorption by the animal. With this in mind the study of the utilization of U-^{14}C-labelled *Chlorella* protein was undertaken to determine whether or not the loss of more than 50% of the activity of this enzyme limited the uptake of amino acids from ingested protein. Estimates of the activity recovered from liver, kidney, pancreas, muscle and blood 3hr. after giving the labelled protein source, and that recovered from exhaled carbon dioxide, lend no support to the suggestion that the decline in carboxypeptidase activity has limited peptide hydrolysis in the digestive tract before amino acid absorption (Table 7). Further, the activity of the non-protein fraction of blood taken at 3hr. (or at 1hr. and 2hr. in one pair of animals) does not suggest that there was any delay in the entry of labelled amino acids into the bloodstream in the zinc-deficient animal. An attempt was made during this work to determine the extent of peptide breakdown by direct examination of the activity of fractions of the digestive tract contents, but sampling difficulties, leading to extreme variability of results, led to the abandonment of this approach.

From these findings we conclude that although pancreatic carboxypeptidase activity falls in zinc deficiency this metabolic defect is not directly responsible for the growth arrest that follows within 2 weeks of the introduction of weanling rats to a diet providing less than 1 μg. of zinc/g.

This conclusion is also supported by a further observation. If the low carboxypeptidase activity were the primary defect it would be expected that a rise in activity would precede all other responses on feeding zinc to a deficient animal. The rise in activity on feeding zinc was consistently noted after 3 days and activity was normal after 5 days of repletion. This time-scale must, however, be compared with that obtained from measurement of changes in food intake after zinc repletion. An important effect of zinc deficiency is a fall in food consumption, and in a recent study (J. Quarterman, unpublished work) it has been found that within 4hr. of offering a zinc-supplemented diet to depleted animals the rate of food intake greatly increases, suggesting that within this period a major biochemical defect has been rectified by traces of the zinc supplement. The mechanism underlying this rapid response is at present being investigated.

The finding that a higher proportion of the ^{14}C activity in the 0·5N-perchloric acid-soluble fraction of tissues from zinc-deficient rats than pair-fed controls suggests that a defect exists in the incorporation of absorbed amino acids into protein in zinc-deficient animals. Since it was not possible to determine the specific activity of amino acid pools within each tissue, this cannot, however, be affirmed with certainty although the finding was that less ^{14}C activity is incorporated into pancreas, kidney and muscle protein in the zinc-deficient rat (Table 3); also, work showing that the total protein

contents of liver and pancreas are lower in zinc deficiency (R. B. Williams, unpublished work) lend support to this suggestion. In this connexion we have already reported the existence of a major defect in nucleic acid metabolism in the liver accompanied by elevated plasma uric acid concentrations in the zinc-deficient rat (Williams, Mills, Quarterman & Dalgarno, 1965).

Since we have failed to show a decrease in the activity of several of the zinc-containing dehydrogenases in the zinc-deficient animal and since the present study shows that although pancreatic carboxypeptidase activity is low in these animals this is not imposing a significant constraint to amino acid absorption, it remains most probable that the dramatic effects of zinc deficiency are related to the changes we have observed in nucleic acid metabolism and protein biosynthesis. The findings of Theuer (1965) that zinc deficiency in the rat delays the incorporation of L-[U-^{14}C]leucine into plasma protein and possibly into liver proteins support this suggestion but it is noteworthy that in these studies Theuer found a marked increase in the proportion of the total activity of intraperitoneally administered L-[U-^{14}C]leucine or L-[U-^{14}C]lysine exhaled as $^{14}CO_2$ during a 3hr. period by zinc-deficient animals as compared with control rats given zinc-supplemented diet *ad libitum*. In our experiments no such difference in $^{14}CO_2$ output was apparent on comparing results for deficient and pair-fed control animals. The reason for this discrepancy is not apparent, but it may perhaps be relevant that Theuer (1965) also found no significant differences in $^{14}CO_2$ output when L-[U-^{14}C]glutamate was parentally administered to deficient and control rats. If this effect of zinc deficiency on carbon dioxide output results from the catabolism of certain amino acids only it would probably be masked in our experiments where a U-^{14}C-labelled protein was fed.

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