I am greatly indebted to Dr M. R. Pollock for providing the facilities for this work and to him and Dr H. J. Rogers for many helpful discussions while this work was going on. Miss Ruth Coyle provided expert and invaluable technical assistance. This paper is contribution no. 583, Forest Biology Division, Research Branch, Canada Department of Agriculture, Ottawa, Ontario, Canada. This study was carried out under a work transfer from the Forest Insect Laboratory, Sault Ste. Marie, Ontario, Canada.

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Biochem. J. (1960) **75**, 395

The Biological Activity of Pure Peptides obtained by Enzymic Hydrolysis of Insulin

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(Received 27 November 1958)

Although much research has been done on the biological activity of material obtained by the action of enzymes on insulin, none of these investigations has been carried out with highly purified peptides of known structure. The determination of the structure of insulin by Sanger and his colleagues (Ryle, Sanger, Smith & Kitai, 1955) has made possible the assignment of known structures to the products of the proteolysis of insulin under con-

trolled conditions. The present investigation was undertaken to assess the biological activity, by more than one criterion, of purified peptides obtained from insulin by the action of carboxypeptidase and trypsin.

Treatment with carboxypeptidase liberates alanine from the C-terminal end of the phenylalanyl chain (Fig. 1). The residual molecule (DA insulin) was reported by Lens (1949) to have only slight hypoglycaemic activity in rabbits, whereas Harris & Li (1952) found it to be fully active by the mouse-convulsion assay. The present results confirm those of Harris & Li.

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Fig. 1. Amino acid sequence in ox insulin. ↑, Site of action of trypsin; ↗, site of action of carboxypeptidase.

The other *C*-terminal residue of insulin on the glycyl chain (asparagine) is split off much more slowly by carboxypeptidase, giving rise to a derivative (DAA insulin) with the two *C*-terminal residues removed. Although Harris & Li reported an almost quantitative removal of the asparagine residue, leading to an inactive product, we have been unable to obtain a yield of more than 27%, which made estimation of biological activity difficult since the product could not be separated from the unchanged insulin. However, Sanger & Thompson (1953) showed that a much better yield of asparagine could be obtained from *N*-acetylinsulin and it was possible to obtain a product (DAA acetylinsulin) from which the asparagine was almost quantitatively removed. (The introduction of acetyl groups, which under their conditions had reacted only with three amino groups, does not affect the activity of the insulin.)

Since the discovery of insulin, trypsin has been the enzyme most commonly used to investigate the influence of modification of the insulin molecule on its biological activity. The impurity of the enzyme preparation led to results with insulin which were not always clear. Butler, Dodds, Phillips & Stephen (1948, 1949) showed that crystalline trypsin broke only one or two peptide bonds; although only a little non-protein nitrogen was formed there was much inactivation. This, however, was not confirmed by Van Abeele & Campbell (1951), who found no loss of activity. Harris & Li (1952) found that, under the conditions used by Van Abeele & Campbell, only alanine was removed. Two bonds, in positions 22–23 (Arg. Gly) and 29–30 (Lys. Ala) in the phenylalanyl chain, are susceptible to the action of trypsin. The latter bond is split relatively rapidly giving rise to free alanine and DA insulin, the same products as those obtained with carboxypeptidase. Although the Arg. Gly bond is rapidly split in the isolated B chain from oxidized insulin, it goes relatively slowly with intact insulin, giving rise to free alanine, a heptapeptide (Gly. - Phe. Phe. Tyr. Thr. Pro. Lys) and the remainder of the molecule (DHA insulin). Various conditions of hydrolysis with trypsin were studied in an attempt to improve the yield of DHA insulin and the best yield (39%) was obtained with insulin from which the zinc had been removed, as had been found with leucine amino peptidase (E. L. Smith, personal communication). With a partition column (Andersen, 1954; Nicol & Smith, 1956) it was possible to separate the DHA insulin from unchanged insulin, and to obtain it in a pure form suitable for biological assay.

The above-mentioned derivatives were all tested for biological activity both by the rabbit-hypoglycaemic test and in the isolated rat diaphragm. The two methods were used to ensure the reli-

ability of the results and also to ascertain whether their activities differed in any qualitative way from that shown by insulin.

EXPERIMENTAL

For definition of abbreviations used for amino acids in this paper see *Biochem. J.* (1957), **66**, 6.

Insulin. Insulin (Boots Pure Drug Co. Ltd.), three times recrystallized, was used. Acetylinsulin was prepared according to the method of Fraenkel-Conrat & Fraenkel-Conrat (1950).

Technical methods. Spectrophotometric measurements were made with a Unicam SP. 500 spectrophotometer. Peptide materials were dried in a desiccator over sodium hydroxide and sulphuric acid.

Action of carboxypeptidase

DA insulin. Carboxypeptidase (The Armour Laboratories; recrystallized) was treated with diisopropyl phosphorofluoridate (DFP) to inactivate any contaminating endopeptidases as follows.

A portion (1.45 ml.) of mM-DFP was added to 0.5 ml. of the carboxypeptidase preparation containing 0.25 mg. of the enzyme. The pH was adjusted to 7.5 and the mixture was incubated at 37° for 30 min. A control of the same quantity of DFP with 0.5 ml. of water instead of enzyme was also incubated at pH 7.5. Insulin (25 mg.) was dissolved in both enzyme and control solutions. The pH of both was adjusted to 7.8 by the addition of 0.1N-acetic acid and they were incubated at 37° for 6.5 hr.

The solution was then adjusted to pH 5.4 with 0.1N-acetic acid. This precipitated the DA insulin and enzyme protein, which were washed with water, dried in a desiccator and used for biological assay. An approximate estimate of the free amino acids in the supernatant was by the spot-dilution method (Polson, Mosley & Wyckoff, 1947) with alanine and asparagine standards. The yields were 0.9 mole of alanine and 0.1 mole of asparagine/mole of insulin.

DAA insulin. Insulin (5 mg.) and 0.5 mg. of DFP-treated carboxypeptidase were incubated under the same conditions as described above for 19.5 hr. After adjustment of the pH to 5.4, the precipitate (DAA insulin) was washed with water and dried. The supernatant was found to contain 0.72 mole of alanine and 0.27 mole of asparagine. Estimation was carried out by the preparation of the dinitrophenyl (DNP) derivatives (Levy, 1954; Blackburn & Lowther, 1950). Recovery experiments had first been done with known weights of the amino acids, resulting in 95% correct recovery.

DAA acetylinsulin. Carboxypeptidase (0.1 mg.) was incubated in 2 ml. of 2 mM-DFP at pH 7.8 for 0.5 hr. and then added to 5 mg. of acetylinsulin. After incubation at 37° for 19 hr. the DAA acetylinsulin was precipitated by adjusting the solution to pH 4.2 and was washed with water and dried. The supernatant contained approx. 1 mole of alanine and 1 mole of asparagine according to the spot-dilution method (Polson *et al.* 1947).

Action of trypsin on insulin

Insulin (10 mg.) was incubated with 1 mg. of trypsin (The Armour Laboratories; three times recrystallized) at 37° and pH 7.5 for 24 hr. Individual solutions of insulin

and trypsin were kept separately and similarly incubated as controls. The enzymic digest was brought to pH 5.2 at the end of the experiment and centrifuged. The supernatant fluid contained the heptapeptide and alanine; the precipitate, consisting of a mixture of insulin, DHA insulin and a small quantity of trypsin, was dried in a desiccator.

The supernatant fluid was then subjected to ionophoresis at pH 6.5 (pyridine-acetic acid-water buffer; 10 ml.: 0.4 ml., made up to 100 ml. with water) on Whatman no. 4 paper for 3 hr. at 37.5 v/cm. The alanine and heptapeptide bands were 3 and 12 cm. respectively from the origin on the cathode side. Their positions were recognized by developing a marker strip with ninhydrin or by very rapid examination of the whole sheet under the u.v. lamp. They were cut out, eluted and their identity was satisfactorily confirmed in the first experiment by hydrolysis and paper chromatography; in subsequent experiments the eluates of the heptapeptide were used for the estimation of the extent of the trypsin breakdown of insulin. The quantity of heptapeptide released and recovered by ionophoresis was determined either by hydrolysis and determination of the amino-nitrogen content by the ninhydrin method (Moore & Stein, 1951) or by treating the eluate with α -nitroso- β -naphthol and nitric acid and measuring $E_{520\text{ m}\mu}$ due to tyrosine (Gerngross, Voss & Herfeld, 1933).

With normal insulin the yield of heptapeptide was about 0.16 mole/mole of insulin. No improvement was found with calcium-stabilized trypsin (Kilby & Youatt, 1954) or when DA insulin was used as substrate. However, when insulin of a low (0.025%) zinc content (Sluyterman, 1955) was used the yield of heptapeptide was 0.39 mole.

Separation of DHA insulin. Silane-treated Celite 545 (Howard & Martin, 1950) was ground with the upper phase of a butan-2-ol-0.05M-trichloroacetic acid (1.0:2.5, v/v) equilibrated mixture. A thick slurry was made with the lower phase and a glass column (20 cm. long and 2 cm. diam.) was packed with it. The dried precipitate from the tryptic-hydrolysis experiment was dissolved in a minimum quantity of the lower phase and introduced on the column, which was developed with the lower phase at 25° for 12 hr.; 100 fractions of 1.25 ml. were collected with an automatic fraction collector. The protein concentration in each fraction was determined from $E_{275\text{ m}\mu}$ by using a Unicam spectrophotometer SP. 500. There were two obvious peaks, the first due to DHA insulin and the second to unchanged insulin. The first peak was much larger in the experiments in which zinc-free insulin was used as a substrate. The solution containing DHA insulin was concentrated *in vacuo* at room temperature to 1–2 ml. and washed twice with ether to remove trichloroacetic acid. The suspension was redissolved in 1 drop of aq. N-NH₃ solution and brought to pH 5.2. The precipitate (DHA insulin) was centrifuged off, washed with a little water, then with a large quantity of acetone and afterwards dried *in vacuo*. It was assumed that this treatment had not affected the DHA insulin, since a control sample of insulin with low zinc content, which was passed through a similar column and extracted in the same way, did not show any loss of biological activity by the two tests when compared with an untreated sample.

The composition and integrity of DHA insulin was confirmed by the DNP end-group-assay method (Sanger, 1945) and, after hydrolysis, by two-dimensional paper chromatography in a phenol-water/butanol-acetic acid system. An

amino acid analysis of DHA insulin by the Moore & Stein (1951) method carried out by Mr M. W. Rees and Miss B. Worboys of this Department gave values in agreement with those calculated for it (Table 1). A sample of insulin was analysed at the same time and gave results which agreed with its known structure (Ryle *et al.* 1955). In particular, no threonine could be detected in DHA insulin. As this amino acid occurs only in the last eight residues of the B chain, which had evidently been removed by trypsin, its absence in the analysis of DHA insulin showed that not more than 3% of the unchanged insulin could be present as a contaminant, since a higher figure would have been detected.

There was a possibility that DHA insulin might be more easily denaturable than insulin (as, for example, during the acetone extraction) and might in its pure form after tryptic digestion, be still capable of full instead of partial insulin activity. The only way in which this could be tested was to dissolve the tryptic digest of zinc-free insulin at the end of incubation, without attempting any separation, and test this directly for glucose uptake by the isolated rat diaphragm.

Zinc-free insulin (10 mg.) was dissolved in 5 ml. of 0.1N acetic acid and 2 mg. of trypsin added. Two digests were prepared. The first, which was the control, was brought to pH 3.2 and kept at 4°. This ensured that there was no trypsin action. The other was incubated under the usual conditions at 37°. Both were left for 24 hr. under these conditions, a trace of toluene being added to each. At the end of this period the second digest (at 37°) was brought to pH 2.5. To each, 3 mg. of soya-bean trypsin inhibitor (The Armour Laboratories) was added to prevent the trypsin

Table 1. *Amino acid analysis of DHA insulin*
(Moore & Stein, 1951)

DHA insulin (5.2 mg.; 0.72 mg. of N) was used. Number of residues was calculated assuming 54 N atoms/molecule (13.3 $\mu\text{g.}$ of N/residue).

Amino acid	Wt. of α -amino N ($\mu\text{g.}$)	No. of residues	No. of residues in ox insulin
Aspartic acid	38.4	2.9	3
Threonine	0	0	1
Serine	36.7	2.8	3
Proline	0	0	1
Glutamic acid	91.1	6.8	7
Glycine	39.0	2.9	4
Alanine	28.7	2.1	3
Valine	63.2	4.7	5
Half-cystine*	(40.2)	(3.0)	6
Isoleucine	10.7	0.8	1
Leucine	74.8	5.6	6
Tyrosine	38.5	2.9	4
Phenylalanine	14.6	1.1	3
Lysine	0	0	1
Histidine	24.5	1.8	2
Arginine	13.1	1.0	1
NH ₃ †	116.0	8.7	6

* Cystine analyses are unreliable with the method used.

† The value for ammonia N is considerably higher than that calculated for the theoretical amide content, indicating that the preparation was slightly contaminated with ammonia. This accounts for the slightly low values for most amino acids.

affecting the hemidiaphragms. The digests were then assayed for activity. Portions of solutions of the experimental and control digests which had contained 0.5 μ g. of insulin before incubation were used for assay.

Biological tests

Rabbit hypoglycaemia. Two or four fasting rabbits were used for the tests and were divided into two groups. Samples of blood were taken from an ear vein every hour for 3 hr. Then an injection of the test substance was made into a vein in the other ear. Duplicate blood samples were taken when possible at 5, 20, 50, 75, 90 and 120 min. The blood samples were put into 3 ml. of water containing a little fluoride, deproteinized and the glucose content was determined (Nelson, 1944; Somogyi, 1941). The other group was treated in a similar way but in this case a known weight of insulin was injected. After 2 days the groups were reversed and the treatment was repeated. The percentage fall in concentration of blood sugar was determined. Rabbits were chosen for use when their fasting blood sugar did not vary by more than 10 mg./100 ml. Not enough rabbits were used for a full-scale assay. All that was sought was a comparison under the same conditions of the action of different doses of insulin and its derivatives on the rabbit-blood sugar.

Glucose uptake of the isolated rat hemidiaphragm. This method was carried out with Gey's bicarbonate buffer (Gey & Gey, 1936) as used by Randle (1956) and others. The absolute value of the basal uptake of glucose by the hemidiaphragm varies with different experiments. Comparisons were made with experiments done simultaneously with the buffer control, and with the test substance.

RESULTS AND DISCUSSION

C-Terminal alanine

The results with DA insulin are shown in Fig. 2 and confirm the findings of Harris & Li (1952)

Fig. 2. Effect of insulin and carboxypeptidase-treated insulin (DA insulin) on the blood glucose of the fasting rabbit. The carboxypeptidase had removed 0.9 mole of alanine and less than 0.1 mole of asparagine/mole of insulin. ○, Insulin 150 μ g.; ●, DA insulin 150 μ g.

that it is fully active. The C-terminal alanine residue is thus not essential for biological activity.

C-Terminal asparagine

When 0.27 mole of asparagine had been removed the activity in the rabbit of the resulting DAA insulin was lower than that of insulin (Fig. 3) as matched in the same series of four rabbits. This again confirms the findings of Harris & Li. Clearer results were, however, obtained with the derivative of acetylinsulin (Table 2). Although acetylinsulin itself showed full activity, no activity could be detected with 150 μ g. of the DAA acetylinsulin from which both alanine and asparagine had been split completely by carboxypeptidase. With 1500 μ g. of DAA acetylinsulin there was some activity which was less than that of 150 μ g. of acetylinsulin.

The C-terminal asparagine residue of the glycyl chain is thus much more important for biological activity than is the C-terminal alanine residue of the phenylalanyl chain.

Heptapeptide sequence

The free heptapeptide (Gly.Phe.Phe.Tyr.Thr.Pro.Lys) that is split off from insulin by the action of trypsin was found to have no effect on the rabbit-blood sugar (Table 2). With the rat diaphragm 0.125, 10 and 20 μ g. gave no significant response (Table 3).

A preliminary biological test of an incompletely characterized preparation of DHA insulin revealed no hypoglycaemic activity in the rabbit (Nicol & Smith, 1956). Nevertheless, samples of the peptide obtained by evaporation *in vacuo* from the butanol

Fig. 3. Effect of insulin and carboxypeptidase-treated insulin (DAA insulin) on the blood glucose of the fasting rabbit. The carboxypeptidase had removed 0.72 mole of alanine and 0.27 mole of asparagine/mole of insulin. ○, Insulin 150 μ g.; ●, DAA insulin 150 μ g.

Table 2. Maximum fall of blood glucose of fasting rabbits after intravenous injection of insulin derivatives

Substance	No. of rabbits	Dose (μg.)	Maximum fall of blood glucose (percentage of original blood glucose)	Remarks
Insulin	8	150	55	—
Acetylin insulin	6	150	54	Full activity
DAA acetylin insulin	2	150	4	No activity
	2	1500	34	Less than 10 % of original activity
DHA insulin	4	150	9	No activity
	4	1000	48	Approx. equivalent to 150 μg. of insulin
DHA insulin	2	1000	63	Insulin activity not impaired
Insulin		200		
Heptapeptide	2	1000	8	No activity
Heptapeptide	2	1000	56	Insulin activity not impaired
Insulin		150		

Table 3. Effect of the heptapeptide on the glucose uptake of the isolated rat diaphragm

Medium contained 2.5 mg. of glucose/ml. One hemidiaphragm in 1 ml. of medium was incubated for 2–3 hr. at 37° under O₂ + CO₂ (95:5) (Randle, 1956). Figures in parentheses show the number of hemidiaphragms used.

Amount of heptapeptide (μg.)	Uptake of glucose (mean ± S.D.; mg. of glucose/g. of tissue/hr.)		P (%)
	Control	Experimental	
0.125	1.57 ± 0.26 (5)	1.54 ± 0.26 (5)	> 5
10	1.81 ± 0.20 (4)	2.21 ± 0.16 (6)	> 5
20	3.23 ± 0.51 (5)	3.61 ± 0.51 (5)	> 5

Fig. 4. Log dose-response curve of glucose uptake in the rat diaphragm caused by insulin and DHA insulin. Experimental details were as given in Table 3 (Randle, 1956). ○, Insulin; ●, DHA insulin.

eluate, but this time without heat, showed some biological activity in the rabbit and with the isolated rat diaphragm. These biologically active samples were more completely characterized by DNP *N*-terminal end-group assays and by analysis, and it was shown that not more than 3 % of unchanged insulin could be present and that no new free amino groups were produced. The results of the biological assays are shown in Table 2. The DHA insulin showed little activity in the rabbit at a dose of 150 μg. but at 1000 μg. showed activity in different rabbits, which in any individual animal could be matched by a control of 150 μg. of a sample of zinc-free insulin which had also been passed through the silane-treated Celite column and extracted in exactly the same way as was DHA insulin.

The results of the experiments with glucose uptake of the isolated rat diaphragm gave a dose-response curve with DHA insulin that was parallel to that of untreated insulin, showing that the nature and mechanism of their actions were similar (see Fig. 4). It was calculated that 1 μg. of DHA insulin had the same activity as 0.174 μg. of insulin (the 95 % confidence range was 0.108–0.279). Since not more than 3 % of unchanged insulin could be present in the DHA insulin used in these experiments, the biological activity of 10–28 % found above could not be attributed to contamination with unchanged insulin. The interpretation of

such a result would appear to be that the part of insulin corresponding to the heptapeptide is not essential for full biological activity and should not be regarded as the 'active centre' but that it clearly has some influence on the activity.

There was the possibility that a mixture of DHA insulin, the heptapeptide and alanine might give the biological activity of insulin. Table 4 shows that this is not the case, the mixture having no more activity than in the glucose uptake of the rat diaphragm with DHA insulin alone.

Again the possibility that DHA insulin might be more susceptible to denaturation demanded a check that the column procedure had not thus affected its activity. This was done in the experiment in which a known weight of the mixture of trypsin and insulin which had been incubated at 37° was tested without any fractionation. It was found to have lower biological activity than a control mixture which had been kept at 4° (Table 5). This showed that DHA insulin could not possess the same activity as insulin, since if this was so the incubated mixture containing it and unchanged insulin would have had the same activity as the control. This experiment also confirmed that a mixture of DHA insulin, heptapeptide and alanine (which were all in the incubated mixture) did not combine in solution to produce the biological activity of insulin.

Table 2 also shows the results of experiments in which insulin was assayed in the presence of the heptapeptide and of DHA insulin respectively. The insulin activity was unchanged, showing that neither has any inhibiting action on insulin.

Mirsky (1957) showed that the peptides in insulin hydrolysates caused competitive inhibition of insulinase in the liver of the intact rabbit and thus prolongation of insulin action. DHA insulin and the heptapeptide in our experiments, however, did not individually prolong insulin action when injected simultaneously with it into the whole animal. One would have expected prolongation of action if either peptide had depressed insulinase action.

Smith, Hill & Borman (1958) have shown that a considerable number of amino acids can be removed by leucine aminopeptidase from the *N*-terminal end of both chains of insulin without complete inactivation. It appears from our experiments, however, that this was not the case with the *C*-terminal end. The removal of asparagine from this end of the glycyl chain results in considerable inactivation, as does the removal of eight amino acids from the phenylalanyl chain. It is possible that this end of the insulin molecule, especially in the region of the second interchain disulphide linkage, may have a particular importance in the mechanism of the biological activity of the hormone.

Table 4. *Effect of products of trypsin-treated insulin on the glucose uptake of the isolated rat diaphragm*

Experimental details are as given in Table 3. Figures in parentheses show the number of hemidiaphragms used.

Expt. no.	Substrate	Amount (μg.)	Glucose uptake (mean ± s.d.; mg. of glucose/g. of tissue/hr.)	P (%)
1	Control	—	1.81 ± 0.30 (4)	—
2	Insulin	0.5	3.41 ± 0.25 (6)	<1 (1 v. 2)
3	DHA insulin	5	3.83 ± 0.25 (6)	<1 (1 v. 3)
4	Heptapeptide	10	2.21 ± 0.25 (6)	>5 (1 v. 4)
5	Alanine	10	1.78 ± 0.30 (4)	>5 (1 v. 5)
6	DHA insulin + heptapeptide + alanine	5 } 10 } 10 }	4.00 ± 0.25 (6)	>5 (3 v. 6)

Table 5. *Effect of trypsin-treated insulin and unincubated control on the glucose uptake of the isolated rat diaphragm*

Experimental details were as given in Table 3 and as described in text. Figures in parentheses show the number of hemidiaphragms used.

Expt. no.	Substrate	Amount (μg.)	Glucose uptake (mean ± s.d.; mg. of glucose/g. of tissue/hr.)	P (%)
1	Buffer control	—	1.35 ± 0.34 (6)	—
2	Control digest (unincubated)	0.5	5.23 ± 0.34 (6)	<1 (1 v. 2)
3	Incubated digest	0.5	3.91 ± 0.34 (6)	<1 (1 v. 3); <5 (2 v. 3)

SUMMARY

1. The action of carboxypeptidase on insulin and acetylinsulin has been investigated. The results of previous workers that the removal of C-terminal alanine does not affect the biological activity of the insulin molecule, but that removal of C-terminal asparagine does so, have been confirmed.

2. The action of trypsin on insulin has also been investigated. The peptides resulting have been purified, characterized and tested for biological activity. The peptide DHA insulin, which is insulin without the eight amino acid residues of the carboxyl end of the phenylalanyl chain, still retains about 15 % of the biological activity of the intact insulin molecule.

I wish to thank Professor F. G. Young, F.R.S., and Dr F. Sanger, F.R.S., for much valuable advice and encouragement; Dr L. F. Smith for the gift of acetylinsulin and assistance in the column procedures; Mr T. Hollingsworth and Mr R. G. Carpenter, of the Department of Human Ecology, Cambridge, for advice on statistical analysis and computation.

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 Eggleston (1958) studied decarboxylases from animal and plant sources and stated that pyridoxal phosphate in some cases acted in conjunction with a metal whereas in others the action was independent of a metal.
 Happold & Turner (1957) have shown activation of glutamic acid-aspartic acid transaminase (from heart muscle) by Mg^{2+} ions, but they thought that the effect was due mainly to the acceleration of non-enzymic decarboxylation of oxaloacetic acid, one of the reaction products, thus giving an apparent increase in activity. A preliminary communication from this Institute (Patwardhan, 1958) has

Biochem. J. (1960) **75**, 401

Glutamic-Aspartic Transaminase of *Dolichos lablab*: Participation by Iron as a Cofactor

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(Received 14 August 1959)

The possibility of a metal participating in enzymic transformations of amino acids that are pyridoxal phosphate-dependent was suggested by the studies of Metzler, Ikawa & Snell (1954). Experimental evidence for metal participation in these types of enzymic reactions was given by Yanofsky (1952), who showed that inhibition of serine dehydrase by metal-binding agents was partially reversed by Mg^{2+} ions. Similar results have been obtained with kynureninase of *Neurospora* (Jakoby & Bonner, 1953), cystathionase of *Escherichia coli* (Wijesundera & Woods, 1953) and tryptophanase (Happold & Struyvenberg, 1954).