THE ACTIVITY OF DIGESTION ENZYMES ON PTEROYLGLUTAMIC ACID (FOLIC ACID) AND PTEROYL-HEXA-GLUTAMYL-GLUTAMIC ACID (FOLIC ACID CONJUGATE)

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INTRODUCTION

The administration of pteroylglutamic acid (folic acid) to patients with pernicious anemia, sprue etc. has in most cases a favourable effect (SPIES[1], [2], MOORE[3]). Folic acid however does not influence all the symptoms of these syndromes, the effect of highly purified liver extracts is superior in some cases.

The activity of these liver preparations can not be attributed to the presence of small quantities of folic acid or its conjugate (in some preparations they are completely absent). In nutrition experiments on chickens the difference in effect between liver preparations and folic acid could be demonstrated by STOKSTAD AND JUKES[4].

It was demonstrated by SPIES[5] and by HEINLE AND WELCH[6] that the conjugate is not effective in pernicious anemia.

The rôle of folic acid and folic acid conjugate in nutrition is not clear. Foods contain both substances.

In vitro the free folic acid can be formed by enzymatic hydrolysis of the conjugate *e.g.*, by rat liver "conjugase", optimal activity at p_H 7 and by chicken pancreas "conjugase", optimal activity at p_H 4.5.

HEINLE AND WELCH[6] have incubated folic acid conjugate from yeast with normal gastric juice to investigate whether the conjugate might be identical with the "extrinsic factor" of CASTLE. No free folic acid was liberated from the conjugate at p_H 4.5 and 7. A small daily dose of conjugate (1 mg daily) together with 100 ml of normal gastric juice administered during a period of 11 days was ineffective in a pernicious anemia patient. In excretion experiments they found a remarkable difference between normal subjects and patients suffering from pernicious anemia. In both free folic acid administered per os was excreted in the urine, whereas after the administration of the conjugate folic acid was excreted only by normal individuals and not at all by patients with pernicious anemia.

Contrary to the inactivity of folic acid conjugate in human pernicious anemia, the conjugated form is active in folic acid deficiency in animals.

According to these experiments WELCH *et al.* came to the following summary:

"The conjugates of folic acid which occur in the diet, although utilized by normal subjects, appear to be inadequately utilized when a deficiency of the antipernicious anemia factor exists. The most reasonable hypothesis, based on the date presented, sug-

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gests that highly purified liver extracts may supply a substance that is directly or indirectly concerned with the function of at least one of the enzymes which liberates *L. Casei Factor* from naturally occurring conjugates of the vitamins."

Although WELCH *et al.*[7] were not able to demonstrate the liberation of free folic acid from the conjugate by normal gastric juice, it is probable that one of the reasons of the difference in the utilization of the conjugate by normal and by pernicious anemia patients is an abnormality in the *digestion* because:

1. It is improbable that the conjugate as such is absorbed from the intestine.

2. It is known that the digestion in the stomach of pernicious anemia patients is abnormal.

To elucidate the rôle of the digestive juices in the utilization of folic acid and folic acid conjugate we repeated and extended the experiments of WELCH *et al.*[7].

EXPERIMENTAL

The activity of the digestive enzymes on folic acid conjugate was determined by estimating the liberated folic acid. The activity of each enzyme solution was tested separately and in combination with other enzymes acting successively. The digestion experiments were carried out at p_H 2 for stomach enzymes and for pancreatic, duodenal and intestinal enzymes at p_H 7 (phosphate buffer). For the estimation of folic acid* the microbiological method with *Streptococcus lactis R* according to LUCKEY, BRIGGS AND ELVEJHEM[8] was used. The estimation of the conjugate was carried out by determining the liberated folic acid after incubation (4 hours at 37° C) with a "conjugase" preparation from rat liver as described by MIMS, TOTTER, AND DAY[9].

As conjugate substrate a yeatex solution was used containing 2 μg free folic acid and about 20 μg conjugate per g. In some experiments pure pteroyl-hexaglutamyl-glutamic acid** and pure folic acid*** were used.

The following enzyme solutions were tested:

1. Normal gastric juice before and after histamin injection.

2. Gastric juice (before and after histamin) from an untreated severe pernicious anemia patient, with characteristic macrocytic anemia, Hb value 28% and totally histamin refractair achylia gastrica.

3. Gastric juice (before and after histamin) from an endemic sprue patient with steatorhoea, macrocytic anemia and emaciation, unsuccefully treated with free folic acid****.

4. Glycerol extracts from pig stomach mucosa, pig pancreas and pig intestinal mucosa.

5. Duodenal juice from normal human subjects collected before and after physostigmin injection.

The proteolytic activity of the stomach juices was demonstrated on hemoglobin and the carboxypolypeptidase activity on Cl-acetyl-tyrosine according to ANSON[10, 11].

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TABLE I

THE LIBERATION OF FOLIC ACID FROM YEAST CONJUGATE BY DIGESTIVE ENZYMES

Enzyme solution	Substrate	% free folic acid
1 Rat liver conjugase p_H 7	Yeatex	100
2 No enzyme, buffer p_H 2	folic acid	100
3 Pig stomach extract p_H 2	,, ,,	100
4 Pig stomach extract p_H 7	,, ,,	100
5 Pig stomach extract p_H 2	yeatex	0
6 Pig stomach extract p_H 7	,,	0
7 Normal gastric juice, before histamin p_H 2	,,	0
8 Normal gastric juice, after histamin p_H 2	,,	0
9 Normal human duodenal juice, before physostigmin p_H 7	,,	0
10 Normal duman duodenal juice, after physostigmin p_H 7	,,	0
11 Gastric juice, pernicious anemia patient, before physostigmin, p_H 7	,,	0
12 Gastric juice, pernicious anemia patient, after physostigmin, p_H 7	,,	0
13 Gastric juice, endemic sprue patient, before physostigmin, p_H 7	,,	0
14 Gastric juice, endemic sprue patient, after physostigmin, p_H 7	,,	0
15 Pancreas extract, pig, p_H 7	,,	90
16 Pig stomach extract p_H 2 + pig pancreas extract p_H 7	,,	0
17 Pig stomach extract p_H 2 + pig intestine extract p_H 7	,,	0
18 Pig pancreas extract p_H 7 + pig intestine extract p_H 7	,,	90
19 Pig stomach extract p_H 2 + pig pancreas extract p_H 7 + pig intestine extract p_H 7	,,	0
20 Pig intestine extract p_H 7	,,	0
21 No enzyme, buffer p_H 2	,,	0

In each experiment 2 ml substrate and 2 ml enzyme solution were incubated for four hours at 37° C. The glycerol extracts were diluted with water 1 : 10.

RESULTS

In Table I the results have been summarised. The liberated amount of free folic acid from the conjugate is given as percentage of the amount liberated by the rat liver conjugase.

From this table it is clear that no folic acid is formed from the conjugate by human and pig enzyme solutions except by the pig pancreas extract. By the pig pancreas extract an equal amount of folic acid is liberated as by the "conjugase" from rat liver. However from our experiments with combinations of enzymes it appears that folic acid is not liberated from the conjugate by pig pancreas extract if the conjugate has been first incubated with pig stomach extract.

From the difference between the activity of the duodenal juice and of the pig pancreas, which both have a carboxypeptidase-activity on Cl-acetyl-tyrosine, it may be concluded that the conjugate is not hydrolysed by the carboxypeptidase of ANSON.

We extended our experiments by estimating also the conjugate after the incubation with enzyme solutions. In Table II the results are given.

These experiments confirmed those of Table I, that if conjugate has been incubated with pig stomach extract (No. 3, Table II) or with normal (No. 10 and 11, Table II) and abnormal (No. 12–15, Table II) human gastric juice at p_H 2, no free folic acid is formed and furthermore it appears that no unaltered conjugate is left, as conjugase is not able to liberate free folic acid. That this is not brought about by a conjugase inhibitor

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TABLE II

THE ESTIMATION OF CONJUGATE OF YEATEX AFTER INCUBATION WITH DIFFERENT ENZYMES. THE CONJUGATE IS EXPRESSED AS PERCENTAGE FOLIC ACID LIBERATED AFTER TREATMENT WITH RAT LIVER CONJUGASE

Susbtrate	Enzyme solution	% folic acid liberated
1 Yeatex	Rat liver conjugase p_H 7	100
2 ,,	No enzyme, buffer p_H 2 + rat liver conjugase p_H 7	100
3 ,,	Pig stomach extract p_H 2 + rat liver conjugase p_H 7	0
4 ,,	Pig stomach extract p_H 7 + rat liver conjugase p_H 7	100
5 ,,	Pig pancreas extract p_H 7 + rat liver conjugase p_H 7	90
6 ,,	Pig intestine extract p_H 7 + rat liver conjugase p_H 7	100
7 ,,	Pig stomach extract p_H 2 + new yeatex p_H 7 + rat liver conjugase p_H 7	95
8 ,,	Pig stomach extract p_H 2 + pig intestine extract p_H 7 + rat liver conjugase p_H 7	0
9 ,,	Pig pancreas extract p_H 7 + pig intestine extract p_H 7 + rat liver conjugase p_H 7	100
10 ,,	Normal human gastric juice p_H2 (before histamin) + rat liver conjugase p_H7	0
11 ,,	Normal human gastric juice (after histamin) p_H2 + rat liver conjugase p_H7	0
12 ,,	Gastric juice, pernicious anemia patient, p_H2 (before histamin) + rat liver conjugase p_H7	0
13 ,,	Gastric juice, pernicious anemia patient, p_H2 (after histamin) + rat liver conjugase p_H7	0
14 ,,	Gastric juice, endemic sprue patient, p_H2 (before histamin) + rat liver conjugase p_H7	0
15 ,,	Gastric juice, endemic sprue patient, p_H2 (after histamin) + rat liver conjugase p_H7	0
16 ,,	Normal gastric juice, p_H7 + rat liver conjugase	100
17 ,,	Normal gastric juice, p_H7	0

is demonstrated in experiments 4 and 7 from Table II. In experiment No. 4, to a conjugate solution at p_H 7, pig stomach extract and conjugase at p_H7 are added successively: a normal amount of free folic acid is formed. In experiment 7 from Table II the p_H of the incubation mixture at p_H 2 is shifted to p_H 7 and new conjugate is added together with rat liver conjugase; again a corresponding amount of free folic acid is liberated. The absence of free folic acid in our experiments can never be attributed to a destruction of liberated folic acid by the acid of the buffer. This is demonstrated by experiments 2 and 3 from Table I. Free folic acid incubated with pig stomach extract under the same conditions was quantitatively recovered in the microbiological estimation.

Further investigations are in progress on the utilization of the conversion product formed from the conjugate in the normal stomach. We intend also to investigate the possible similarity of the conversion product formed from the conjugate by gastric juice and the extrinsic factor of CASTLE. It is striking that it is impossible to obtain free folic acid from both substances with rat liver conjugase. Moreover both factors are physiologically active in the same way.

SUMMARY

1. A conjugase activity (liberation of free folic acid from the conjugate) is not found in normal gastric juice at p_H2 and 7. This confirms the results of WELCH *et al.* They also found no free folic acid but they did not investigate whether the conjugate was changed by the enzymes.

2. In our experiments it is clearly shown that the vitamin B_c conjugate (pteroyl-hexaglutamyl-glutamate) from yeast is altered by incubation with normal human gastric juice and by a glycerol

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extract of pig stomach mucosa at $p_H 2$ but not at $p_H 7$. No free folic acid however is formed and rat liver conjugase is not able to liberate free folic acid from the incubation product. This is not due to conjugate inhibitors.

3. In our pernicious anemia patient, the gastric juice had a p_H of 7. The alteration of the conjugate could however not be demonstrated at $p_H 7$. Therefore a striking difference in the utilization of the conjugate exists. The folic acid deficiency of pernicious anemia patients may be explained by the inability to convert the conjugate as the p_H in the stomach is 7 and the duodenal juice can not liberate free folic acid directly.

4. The folic acid deficiency in non-tropical sprue can not be explained however in the same way, the p_H of the stomach juice being the same as of normal juices.

5. Normal duodenal juice has no conjugase activity. As the duodenal juices were always contaminated with gastric juice it was not possible to investigate whether the duodenal juice has the same conjugate converting activity at $p_H 2$ as the stomach juice.

6. In the pig, free folic acid can be formed from the conjugate by pancreas enzymes.

RÉSUMÉ

1. On ne trouve pas d'activité conjugase (libération d'acide folique libre à partir du conjugat) dans le suc gastrique normal, à $p_H 2$ ni à $p_H 7$. Ceci confirme les résultats de WELCH *et al.* Ceux-ci constatèrent l'absence d'acide folique libre mais ne recherchèrent pas si le conjugat était modifié par l'enzyme.

2. Nous montrons ici de façon nette que le conjugat de la vitamine B_c (pteroyl-hexaglutamyl-glutamate) de la levure est modifié après incubation avec du suc gastrique humain normal, ou avec un extrait glycérinique de muqueuse stomacale de porc, à $p_H 2$ mais non à $p_H 7$. Il ne se forme toutefois pas d'acide folique, libre, et la conjugase du foie de rat est incapable de libérer de l'acide folique libre du produit résultant de l'incubation. Ce fait n'est pas dü à la présence d'inhibiteurs de la conjugase.

3. Chez le malade atteint d'anémie pernicieuse, étudié ici, le suc gastrique avait un p_H de 7. Mais aucune modification du conjugat n'a pu cependant être montrée à $p_H 7$. Il existe donc une différence frappante dans l'utilisation du conjugat. La carence en acide folique des malades atteints d'anémie pernicieuse peut s'expliquer par leur incapacité à transformer le conjugat, puisque le p_H du liquide stomacal est de 7 et que le suc duodénal ne peut libérer directement l'acide folique.

4. La carence en acide folique dans la sprue non tropicale ne peut cependant s'expliquer de la même façon, le p_H du suc gastrique est le même que celui du suc gastrique normal.

5. Le suc duodénal normal n'a pas d'activité conjugase.

Le suc duodénal utilisé étant toujours touillé de suc gastrique, il n'a pas été possible de rechercher si le suc duodénal possède la même activité de transformation du conjugat à $p_H 2$ que le suc gastrique.

6. Chez le porc, l'acide folique peut être libéré du conjugat par des enzymes pancréatiques.

ZUSAMMENFASSUNG

1. Konjugase-Aktivität (Freisetzung freier Folsäure aus dem Konjugat) wird in normalem Magensaft zwischen $p_H 2$ und $p_H 7$ nicht gefunden. Dies bestätigt die Resultate von WELCH *et al.* Sie fanden ebenfalls keine freie Folsäure, untersuchten aber nicht, ob das Konjugat durch die Enzyme verändert wurde.

2. Bei unseren Experimenten wird deutlich gezeigt, dass Vitamin B_c-Konjugat (Pteroyl-hexaglutamyl-glutamat) aus Hefe durch Inkubieren mit normalem menschlichen Magensaft und mit einem Glyzerolextrakt aus Schweinemagenmucose bei $p_H 2$, aber nicht bei $p_H 7$, verändert wird. Dabei entsteht jedoch keine freie Folsäure und Rattenleberkonjugase kann aus dem Inkubationsprodukt keine freie Folsäure freisetzen. Das beruht nicht auf Konjugase-Inhibitoren.

3. Bei unseren, an perniziöser Anämie leidenden Patienten, hatte der Magensaft einen $p_H 7$. Die Veränderung des Konjugats tritt aber, wie gezeigt wurde, bei $p_H 7$ nicht auf. Dadurch besteht ein scharfer Unterschied in der Benutzung des Konjugats. Der Folsäuremangel von Patienten mit perniziöser Anämie könnte durch die Unfähigkeit, das Konjugat umzusetzen erklärt werden, da der p_H des Magensafts 7 beträgt und der Duodenalsaft freie Folsäure nicht direkt freisetzen kann.

4. Der Folsäuremangel bei nicht-tropischer Sprue kann jedoch nicht in gleicher Weise erklärt werden, da der p_H des Magensafts derselbe wie bei normalem Saft ist.

5. Normaler Duodenalsaft hat keine Konjugaseaktivität. Da der Duodenalsaft immer mit Magensaft verunreinigt war, war es nicht möglich um zu untersuchen, ob Duodenalsaft bei $p_H 2$ dieselbe, Konjugat umsetzende Aktivität hat wie der Magensaft.

6. Beim Schwein kann aus dem Konjugat durch Pankreasenzyme freie Folsäure gebildet werden.

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