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The Isolation of a Toxic Substance from Agenized Wheat Flour

By P. N. CAMPBELL and T. S. WORK
National Institute for Medical Research
and E. MELLANBY
Nutrition Building, Mill Hill, London, N.W. 7

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In 1937, Melnick & Cowgill reported the occurrence of 'running fits' in dogs when gliadin was the sole source of protein in the diet. Similar symptoms were not produced when gliadin was replaced by other proteins. The authors concluded that a toxic substance accumulated in the body when gliadin was fed. Wagner & Elvehjem (1944) examined the effect of adding various supplements to a gluten-rich diet and also concluded that 'running fits' were caused by a toxic substance in wheat products rather than by any nutritional deficiency.

In 1946, Mellanby showed that wheat flour which had been 'improved' by treatment with nitrogen trichloride (agenized flour) produced symptoms in dogs similar to running fits; a flour which had not been agenized was not toxic.

Much wheat is grown to-day which, if freshly milled, produces flour which is unsuitable for use in baking. If, however, such wheat is allowed to become aged or 'oxidized' by means of long periods of storage and is then milled, the resulting flour has greatly improved baking properties. This 'oxidizing' process can be more quickly brought about by the use of various oxidizing agents or 'improvers' on the flour. Nitrogen trichloride ('agene') was introduced as an artificial ageing agent by Baker (1921), hence the term agenized flour. The use of nitrogen trichloride has gradually spread, both in this country and in the United States, until in 1946 it was estimated that over 90% of the flour milled in England was treated with nitrogen trichloride. A review, in which the use of oxidizing agents in the treatment of flour is discussed, has been published by Blish (1945).

When agenized flour forms a substantial part of the diet of dogs, the animals become progressively more restless over a period of days and eventually develop epileptiform fits. If the diet is replaced at this stage by an exactly similar diet containing untreated flour the animals recover, but if the diet of agenized flour is continued the animals become progressively worse and die. Similar fits cannot be produced by unagenized flour (Mellanby, 1946) so that it must be assumed that the gliadin used by Melnick & Cowgill (1937) had been treated with agene.

There are striking differences in the susceptibility of different species to the toxic action of agenized flour. Mellanby (1947) showed that the ferret was nearly as sensitive as the dog, but that rats and mice did not develop fits. Newell, Erickson, Gilson, Gershoff & Elvehjem (1947) showed that guinea pigs and chicks were not susceptible, and Radomski, Woodard & Lehman (1948) showed that rabbits developed typical fits.

When the present investigation was begun it was already known (Mellanby, 1947) that the toxicity of agenized flour was associated with the gluten fraction, and that other proteins could also be rendered toxic by treatment with nitrogen trichloride (Moran, 1947). In view of the widespread use of agenized flour it became important to isolate and determine the nature of the toxic substance present in wheat flour. The present communication is concerned with the method used in the isolation of the substance and with its properties. A preliminary account of this work has already been published (Campbell, Work & Mellanby, 1950). The ferret has been used as the test animal and the toxic dose has been regarded as that quantity of material which would produce a typical epileptiform fit.

While the present work was in progress Bentley, McDermott, Pace, Whitehead & Moran (1949*b*) isolated from zein treated with nitrogen trichloride a crystalline substance which was toxic to rabbits. As indicated in the appropriate section this material appears to be identical with the material isolated by us from wheat flour.

MATERIALS AND METHODS

As an example of the methods used in the isolation of the toxic substance from agenized wheat flour, the treatment of a typical batch by the method finally adopted is described. Details showing the course of the isolation, the losses involved and the toxic dose at each stage are to be seen in Table 1. It should be emphasized that the figures quoted in this table are in some cases only an approximation; for although the losses in toxicity and the variation in the toxic dose which resulted from each step were determined, it was not possible to carry out each determination during the course of the fractionation of the same sample of wheat flour owing to the large quantities of material required for each

biological test and the lack of a micro-test. The figures in the table, therefore, represent a composite picture built up from results obtained during the course of several fractionations, but they do represent the course of the fractionation in a qualitative manner. It should be further emphasized that while we have every reason for confidence in the biological test employed, i.e. the production of an epileptiform fit in a ferret, no extensive experiments have been carried out to determine the mean toxic dose of any one preparation over a series of animals, so that the figures for the toxic dose quoted are again only an approximation. The fact that it has been necessary to carry out the biological test by the addition of the test substance to the normal food of the animal rather than by injection also contributed to the difficulties.

Separation of gluten from flour and treatment with pepsin and trypsin

Flour (17·7 kg.) which had been treated with 11 g. NCl_3 by Wallace and Tiernan Co. Ltd., Chiswick, London, was placed in a muslin bag in small batches and kneaded with running water until all the water-soluble material had been removed. The insoluble gluten was then mixed with 13 l. of distilled water containing 30 ml. of conc. H_2SO_4. Pepsin (40 g.) (pepsin powder, B.P., supplied by Chas. Zimmerman and Co. Ltd.) was added and the whole incubated at 37–38° with constant stirring for 24 hr. The pH of the suspension was then brought to 8·1–8·4 by the addition of 5 N-NaOH and trypsin (40 g., supplied by British Drug Houses Ltd.) was added. A little toluene was added to the suspension which was incubated as before for 48 hr. The pH of the suspension, which tended to fall during the early part of the digestion, was checked every few hours and adjusted to 8·1–8·4 by the addition of 2 N-NaOH. If the pH at any time fell below 7·0 it was assumed that the trypsin had been destroyed and a further 40 g. of trypsin were added. The suspension was then filtered and the residue washed with distilled water. The total volume of the digest and washings was about 14 l. which contained approximately 150 g. N and was equivalent to 101 toxic doses (fraction *A*, Table 1).

Preliminary experiments on enzymic digest

Before attempting to develop a method of fractionation, some general knowledge of the properties of the toxic substance had to be obtained.

Heat stability. A sample of digest was boiled for 1 hr. at pH 5·0 and the precipitated protein was removed; activity was not decreased.

Acid stability. Samples of digest were boiled with 6 N-H_2SO_4 for 1, 4 and 18 hr. The SO_4^{--} was removed as $BaSO_4$ and each sample was biologically tested. Up to 4 hr. there was less than 20% loss in activity (a 20% loss was considered to be the minimum detectable without recourse to treatment of a group of animals and statistical analysis of the result). The sample heated for 18 hr. retained less than half of the activity of the digest.

Alkali stability. A sample of digest was heated for 1 hr. with excess 2 N-$Ba(OH)_2$ and Ba^{++} removed as $BaSO_4$. No loss in activity could be detected.

Dialysis. A sample of digest was dialysed against successive portions of distilled water until no more material dialysed. The dialysate and residue possessed the same toxicity relative to their N content, but only 25% of the activity was retained in the dialysis sac.

Liberation of NH_2 groups by acid. The Van Slyke HNO_2 method was used to estimate the apparent NH_2-N:total N ratio. When the digest was boiled with 6 N-H_2SO_4 for 4·5 hr. the proportion was increased from 27 to 54%, while boiling with conc. HCl for 3·5 hr. increased the ratio to 61%. Thus a greater degree of hydrolysis in a shorter time was effected with HCl than with H_2SO_4. The loss in activity after boiling for 3·5 hr. with conc. HCl was slightly greater than after boiling for 4·5 hr. with 6 N-H_2SO_4, but the average molecular weight as indicated by the ratio NH_2:total N was smaller after HCl hydrolysis and this was accordingly adopted as a standard hydrolytic procedure.

Dialysis of enzymic digest

The enzymic digest (*A*, 14 l., 150 g. N) was concentrated under reduced pressure on a water bath (40–60°) to approximately 6 l. and was dialysed in cellophan sacs against 15 l. of distilled water at approximately 5°. Half the dialysate was removed after the first 2 days and replaced with an equal volume of distilled water. This process was repeated three times, after which the whole of the dialysate was replaced by distilled water three times. At the end of this time the amount of dialysable material which could be collected in 2 days did not represent more than 1% of the total N originally contained in the sacs. The combined dialysates were concentrated to 5·5 l. under reduced pressure at 40–60°. The total N in the combined concentrated dialysates was 125 g. (fraction *B*).

Acid hydrolysis of the dialysate

The dialysate (*B*, Table 1) was hydrolysed in four batches with conc. HCl by boiling 1·5 l. with 6 l. of 10 N-HCl for 3·5 hr. under a reflux condenser. Most of the acid was subsequently removed by evaporation of the hydrolysate under reduced pressure on a water bath at 40°. The residual gum was diluted with a little water and reconcentrated to remove a further quantity of HCl. The combined filtered hydrolysates were diluted to 27 l. with distilled water and treated with the anion-exchange resin 'Deacidite E' (obtained from The Permutit Co.). The resin (approx. 2 kg.), after being well washed with distilled water, was activated by allowing it to stand overnight in 5 l. of 2 N-NaOH, washed with 10 l. of distilled water by decantation, loaded into a porcelain pipe (11·5 × 75 cm.), and washed with distilled water until 100 ml. of effluent required less than 3 ml. of 0·02 N-HNO_3 to neutralize it to methyl orange. The acid hydrolysate solution was added to the column and the effluent collected. When the pH of the effluent fell below 5·5 the column was washed clear of hydrolysate with distilled water and was regenerated with 0·5 N-NaOH (30 l.) and washed as before with distilled water. The remaining acid hydrolysate was then added together with the acid effluent obtained from the first column. The combined effluents were concentrated under reduced pressure on a water bath to approx. 12 l. so that the concentration of N was about 10 mg. N/ml. (total N = 118 g., fraction *C*, Table 1).

Electrodialysis of acid hydrolysate

After decolorizing the neutral acid hydrolysate (*C*) by boiling with a little activated charcoal, the hydrolysates were subjected to electrodialysis after the method of Cox, King & Berg (1929). The apparatus consisted of a Perspex box divided into three sections each of which had a tota

Table 1. *Stages in the isolation of toxic substance from wheat flour*

Process	Toxic dose	Total N available	No. of doses
Gluten	—	220 g.	—
Treated with 11 g. NCl_3	1·6 g. N	220 g.	140
After enzymic digestion (*A*)	1·5 g. N	Approx. 150 g.	101
After dialysis against distilled water (*B*)	1·5 g. N	125 g.	83
After hydrolysis with conc. HCl (*C*)	2·3 g. N	118 g.	50*
After electrodialysis	700 mg. N	35 g.	—
After removal of crystalline material (*D*)	500 mg. N	25 g.	50
After removal of aromatic amino-acids on charcoal (*E*)	460 mg. N	23 g.	—
After fractionation on Zeo-Karb†	—	—	—
Total of all active fractions	170 mg. N	5·5 g.	32
After removal of crystalline material	130 mg. N	4 g.	32
Most active fraction from column	96 mg. N	2·2 g.	23
After removal of crystalline material (*F*)	56 mg. N	1·3 g.	23
After fractionation on paper column‡ (*G*)	4 mg. N	64 mg.	16
Crystallization from aqueous ethanol	3 mg. solid	—	12

* Acid hydrolysis + electrodialysis caused a loss of one-third of the toxic substance. This is assumed to occur during acid hydrolysis during which stage there is little loss of N, thus the toxic dose goes up.
† For fractionation from Zeo-Karb column, see Table 3.
‡ For fractionation from paper column, see Table 4.

volume of 4 l. The most satisfactory membranes were prepared by treatment of animal parchment (plain skins from Witherby and Co., 326 High Holborn, London, W.C. 1) with 10% (w/v) formaldehyde for 5 hr. at 20°. These membranes had great mechanical strength and electro-endosmosis was minimal. The centre compartment of the box was fitted with a mechanical stirrer and the outer walls of the electrode compartments (platinum electrodes) were cooled (for a review of electrodialysis methods cf. Svensson, 1948). The hydrolysate (3–4 l.; 30–40 g. N) was poured into the centre compartment of the box and the end compartments were filled with distilled water. The electrodes were then connected to a 230 V., d.c. supply main with a 100Ω variable resistance in the circuit. The current rose rapidly to 2·5–2·8 amp., the pH of the anode compartment fell and that of the cathode compartment rose. The duration of the dialysis depended on the amount of N initially added to the centre compartment and on the membranes, but for 40 g. N there was usually a fall in current to about 1·8 amp. in 24 hr. At the end of this time the contents of the end compartments were withdrawn (all but about 200 ml.), and were replaced with distilled water. Within the next 4–8 hr. the current fell to 0·4 amp. and then remained steady. At this point electrodialysis was considered to be complete.

Table 2. *Distribution of nitrogen during electrodialysis of acid hydrolysate*

(Results expressed as g. N.)

	Compartment		
	Cathode	Centre	Anode
Mean values for four runs	9·6	5·0	14·2
Percentage of total N	33	17	50

While the major part of the toxic substance remained in the centre compartment during dialysis a small amount travelled to the cathode compartment so that the contents of the latter were collected, concentrated under reduced pressure and re-electrodialysed together with sufficient of the anode solution to bring the pH to 5·5. The anode solution was not toxic. The total amount of N in the combined neutral fractions from the electrodialysis was 35 g., the toxic dose containing 700 mg. N. The combined neutral fractions were concentrated under reduced pressure on a water bath until crystallization took place. The crystalline material was removed by filtration and the concentration repeated until no further crystallization took place. By this means the total N in the solution was reduced to 25 g. and the toxic dose to the equivalent of 500 mg. N (fraction *D*, Table 1). The distribution of N during four electrodialyses is shown in Table 2.

Removal of aromatic amino-acids with charcoal

The aromatic amino-acids were removed by adsorption on activated charcoal. The charcoal was first treated with acetic acid according to the method of Tiselius, Drake & Hagdahl (1947) and then used as described by Schramm & Primosigh (1943). The filtrate (*D*) was treated in batches such that each batch contained approximately 4 g. N. Each batch was first diluted with 5% (v/v) acetic acid until the concentration of N was 1 mg. N/3 ml. Preliminary experiments on a pilot scale had shown that less than 20% of the N was in the form of aromatic amino-acids and this figure was used as a basis for the calculation of the amount of charcoal required; 1 g. of charcoal being required to adsorb 1 mg. of aromatic amino-acid N. The required amount of charcoal in a suspension in 5% acetic acid was poured into a Büchner funnel and washed with further quantities of 5% acetic acid. When the charcoal was nearly dry the solution of amino-acids was added and the effluent collected at a rate of approximately 5 l./hr. The charcoal was then washed with a volume of 5% acetic acid equivalent to 25 ml./g. of charcoal present, at a rate of approximately 10 l./hr. The effluent and washings were combined. The total amount of charcoal required to treat the 25 g. of N was 5 kg. and the total effluent amounted to 200 l. (fraction *E*).

Fractionation on Zeo-Karb 215 *column*

The effluent (*E*) from the charcoal was divided into three parts (approx. 10 g. N each) for fractionation (Partridge, 1949) on a cation-exchange column of Zeo-Karb 215 (obtain-

able from The Permutit Co.). The resin, after grinding, was sieved and 700 g. of the 60–80-mesh material was activated with HCl as described by Partridge & Westall (1949). The Zeo-Karb was poured into a column 5·8 cm. wide by 91·4 cm. long as an aqueous suspension and allowed to settle. After all the resin had been added, distilled water was forced up the column from the bottom so that the particles were freely suspended; the water flow was then reversed and the particles allowed to fall into position. By this means an evenly packed column is ensured. The column was washed with distilled water until the pH of the effluent had risen to 5·0–6·0.

The charcoal effluent (66 l.) was added to the column at such a rate that the outflow was approximately 5 l./hr. Ninhydrin tests were carried out on the effluent to check that the column was not being overloaded; the presence of a little glutamic acid in the effluent was ignored. When all the charcoal effluent had been added the amino-acids were displaced with 0·4 M-NH_3 solution added at a rate of approximately 1·5 l./hr. As soon as a significant amount of amino N appeared in the effluent it was collected in 20 ml. fractions by means of a fraction collector. A sample from every fifth tube was run on a one-dimensional paper chromatogram in butanol-water-acetic acid solvent (4:5:1) (Partridge, 1948) according to the method of Consden, Gordon & Martin (1944) in order that the amino-acid composition of the effluent might be followed. The elution with NH_3 was continued until the amount of N in the effluent became insignificant. The contents of the tubes were bulked into ten fractions according to their amino-acid composition. These were concentrated by evaporation under reduced pressure and N determinations (Kjeldahl) carried out. The distribution of N in a typical column effluent is shown in Table 3. Each of the last six fractions, in which proline was either absent or only present in very low concentration, was further concentrated and any crystalline material removed. The various fractions were then submitted to biological tests, the results of which are also shown in Table 3. It will be seen that although the activity was present in all of the last six fractions, it was concentrated in one fraction (*F*). The distribution of activity in the combined fractions from all three Zeo-Karb columns is shown in Table 1. It will be seen that for the most active fraction the toxic dose contained only 56 mg. N.

Before the Zeo-Karb was used again it was treated with 5% (w/v) NaOH solution at 55–60° to remove any aromatic amino-acids which may have been adsorbed and was then regenerated with 5 N-HCl (Newkirk & Handelman, 1949).

Fractionation of effluent from Zeo-Karb 215 on a paper column

The use of a column consisting of powdered paper was originally suggested by Consden *et al.* (1944). In the present case a powdered paper known commercially as solka floc (200-mesh grade; obtainable from Johnsen, Jorgensen and Wettre, 26 Farringdon Street, London, E.C. 4) has been used. The paper was washed thoroughly with distilled water, boiled for 30 min. with 5% acetic acid, washed with water, boiled with absolute ethanol, filtered, washed with ether, and dried in an oven at 110°. Approximately 1 kg. of the dried paper was then suspended in about 6 l. of acetone and loaded into a glass column 120 cm. long by 6·5 cm. diameter. The paper suspension was added in a continuous stream and the acetone allowed to flow freely from the column. After all the suspension had been added the column was washed with a further 6 l. of acetone. The level of the acetone was allowed to fall until it was just above the top of the paper and a solvent consisting of a mixture of butanol, water and acetic acid added. The composition of this solvent was similar to the 4:5:1 mixture described by Partridge (1948), but it was more economically prepared by mixing *n*-butanol 63%, water 27% and acetic acid 10% by volume together and allowing it to stand for 48 hr. before use. During this time esterification takes place and equilibrium is established; if the solvent separated into two layers then only the upper layer was used. The solvent (15 l.) was passed through the column at as fast a rate as possible until the paper had become saturated with water. This could be checked by adding a drop of water to 10 ml. samples of the effluent, since, when the solvent is saturated with water, a cloudy solution is produced under these conditions. The solvent level was then reduced to the level of the paper and the column was ready for fractionation.

Only the most active fractions (*F*, Table 1; 194–205, Table 3) from the Zeo-Karb column were fractionated on the paper column, the less active fractions being combined and first refractionated on a further Zeo-Karb 215 column. The solution of amino-acids to be fractionated (300 mg. N) was evaporated to dryness *in vacuo*, a little ethanol added and the evaporation repeated twice. The residue was dissolved in about 300 ml. of the butanol-acetic acid solvent to which 6 ml. of glacial acetic acid and 10 ml. of ethanol had been added. This usually resulted in a quite clear solution, but in the event of the solution being cloudy it was first cleared by centrifugation and only the supernatant solution used for fractionation. The clear solution was then added to the

Table 3. *Fractionation on Zeo-Karb* 215

Fraction tubes no.	N from column (mg.)	N after removal of crystalline material (mg.)	No. of toxic doses	N/toxic dose (mg.)
76–123	1930			
124–159	1700	—	Not toxic	Not toxic
160–171	547			
172–188	700			
189–193	216	162	1	162
194–205	618	375	8	47
206–213	245	173	1·5	115
214–220	212			
221–239	174	183	1	183
240–261	18			
Total N in mg.	6360	893	—	—

column without disturbing the surface. This solution was allowed to percolate into the paper before the developing solvent was added. The flow rate was adjusted to 1 drop/sec. and the effluent was collected in 18 ml. fractions on a fraction collector, approximately eighty fractions being collected in 24 hr. Samples from every fifth tube were run on single-dimensional paper chromatograms (Consden *et al.* 1944) in butanol-acetic acid in order to determine the amino-acid composition of the effluent. The fractions were then grouped according to the composition of their ninhydrin-positive substances as shown in Table 4. All the groups were biologically tested, but only one (*G*, Table 1, 355–475, Table 4) possessed any activity; a typical fit being produced by material containing as little as 4 mg. N. The ninhydrin-reacting substance in (*G*) gave initially a yellow colour with ninhydrin, but on heating the colour changed through brown to the usual purple.

Table 4. *Fractionation on paper column*

(300 mg. N on 1 kg. paper. Each tube contained 18 ml. of solvent.)

Tube no.	Total N (mg.)	Amino-acid composition
150–180	68	Leucine + trace valine
181–209	53	Valine + trace leucine
210–229	22	Unidentified, purple ninhydrin
230–255	56	Proline
256–269	18	γ-Aminobutyric acid
270–282	6·5	Traces of various unidentified ninhydrin positive substances
283–304	25	
305–315	8·2	
316–340	14·6	
341–354	5·6	
355–475	16	Toxic substance
476–		Histidine positive Pauly reaction

When the amount of ninhydrin-positive material in the fractions fell to an insignificant amount the solvent was changed to an 85% ethanol-water mixture in order to remove the last traces of substances with a very low R_F. 2 l. of 85% (v/v) ethanol followed by 6 l. of 50% (v/v) ethanol were added and the effluent collected. After concentration under reduced pressure these fractions were tested biologically, but failed to give any positive reaction. The solvents were then used in the reverse order. After the addition of 2 l. of butanol-acetic acid solvent the column was again ready for further fractionations.

Treatment of the active fraction from the paper column

The fractions (*G*, Table 1; 355–475, Table 4) from the paper column were pooled and evaporated to dryness under reduced pressure. The acetic acid was removed by the addition of successive small quantities of distilled water and evaporation to dryness. The residue was dissolved in the minimum amount of distilled water and any insoluble material was filtered off. After the addition of a little absolute ethanol to the solution the toxic substance crystallized in the form of small colourless beads. The toxic dose of this crystalline material, m.p. 226–232° (decomp.), when fed to a rat over a period of 5 days was 3 mg. of solid. The total number of toxic doses obtained from 17·7 kg. of flour was about 12, giving an overall yield of 8·5%. A phase-contrast photograph of the recrystallized material is shown in Fig. 1.

Alternative methods of isolation

While this work was in progress, Bentley, McDermott, Pace, Whitehead & Moran (1949*a*) reported the purification of a toxic substance from agenized zein. In many respects the methods used by these workers corresponded to those employed in the present work. It seemed possible that some of their methods might also prove useful in the extraction of a toxic substance from flour.

Butanol extraction. A sample of an active fraction from a Zeo-Karb 215 column equivalent to 380 mg. N (two toxic doses) was made up to 50 ml. with distilled water and extracted with *n*-butanol at 60° for 22 hr. as described by Dakin (1918). On examination of the two extracts by paper-partition chromatography in butanol-acetic acid-water it was found that the butanol fraction contained most of the amino-acids (357 mg. N). The water fraction was completely lacking in valine and leucine and contained 21·5 mg. N. Biological tests showed that both fractions were of approximately equal activity. Although this method was attractive in that the total N for one toxic dose was reduced from 190 to 21·5 mg. N the loss of 50% of the toxic material rendered the process uneconomic. The appearance of 50% of the activity in the butanol extract was contrary to the experience of Bentley *et al.* (1949*a*). This discrepancy must now be attributed to some small difference in technique as it appears that the toxic substance from wheat flour is identical with that from zein.

Partition between phenol and acid. A sample of the concentrated toxic factor from Zeo-Karb 215 was partitioned between equal volumes of phenol and 0·1 N-HCl. The amino-acids were distributed almost equally between the two layers and the method was not considered useful in the present case.

Degradation of crystalline toxic substance

As the quantity of crystalline material available after biological testing was about 10 mg. it was not possible to isolate and characterize degradation products. When the identity of a degradation product had been indicated by chromatography on paper it was mixed with an authentic specimen and an attempt made to separate the substances by partition chromatography in two dimensions on paper (phenol/NH_3 followed by acetic acid/butanol/water). If no separation was achieved the unknown was assumed to be identical with the known specimen.

With this reservation, the following substances were identified as the products of acid hydrolysis (6 N-HCl for 24 hr.) followed by treatment with H_2O_2; homocysteic acid, methionine sulphoxide, methionine sulphone, α-aminobutyric acid and homoserine. In addition, a small quantity of undegraded toxic substance was identified. The relative positions of these substances on a chromatogram is shown in Fig. 2.

A sample (2 mg.) of crystalline toxic substance was desulphurized by treatment with Raney nickel by the method of Fonken & Mozingo (1947). After removal of the solid nickel a small quantity of NiS was precipitated when H_2S was passed into the filtrate. After removal of NiS the filtrate was divided into four portions. One quarter was chromatographed on paper using collidine/lutidine as solvent. The R_F indicated that the degradation product was an aminobutyric acid. One quarter was now mixed with synthetic α-aminobutyric acid and one quarter with γ-aminobutyric acid. Each mixture was chromatographed on paper. The

mixture of unknown substance and γ-aminobutyric acid separated into two ninhydrin-positive spots; the mixture of unknown and α-aminobutyric acid could not be separated. Methionine gave α-aminobutyric acid under similar conditions.

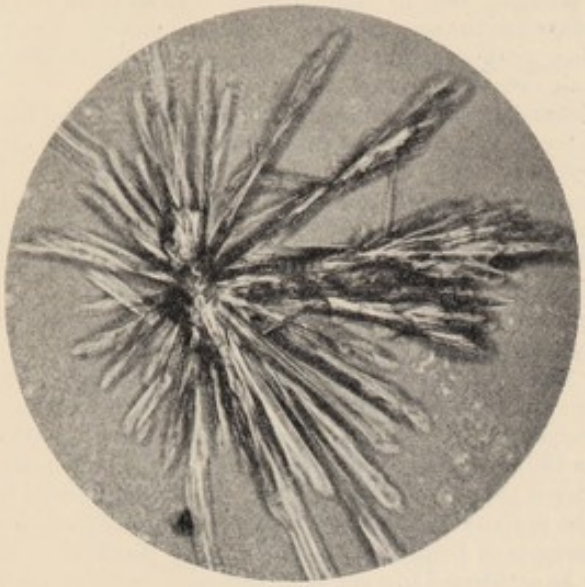

Fig. 1. Phase-contrast photograph of the crystalline toxic substance from agenized wheat flour (× 180).

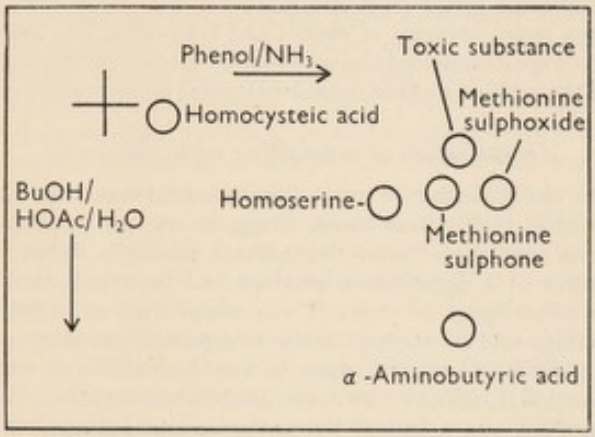

Fig. 2. Diagrammatic representation of the position of the toxic substance and its degradation products (acid hydrolysis) in a two-dimensional chromatogram.

Treatment of some methionine peptides with NCl_3

The following peptides were treated with NCl_3 in $CHCl_3$: methionylglycine, carbobenzyloxymethionylmethionine amide, methionylmethionine, carbobenzyloxymethionylmethionine, carbobenzyloxy-L-methionine amide, carbobenzyloxyglycyl-L-methionine and carbobenzyloxy-L-methionylglycine. A sample (20 mg.) of each was suspended in approximately 10–15 ml. of $CHCl_3$. Air containing NCl_3 from approximately 50 ml. of Cl_2 was passed into each. The solutions were left at room temperature for about 1 hr. when the remaining NCl_3 was removed by sucking a vigorous stream of air through the solutions until no further $CHCl_3$ remained. Each specimen was dissolved in 6N-HCl (0·2–0·3 ml.) and heated in a sealed tube for 11 hr. at 100–105°. The amino-acid composition of each hydrolysate was studied by two-dimensional paper-partition chromatography using butanol acetic acid and phenol/NH_3 as solvents. Wherever a component was detected which resembled the toxic factor in R_F values a second chromatogram was run with a mixture of peptide hydrolysate and crystalline toxic substance. In every case it was possible to separate the toxic substance from all the peptide degradation products and it was, therefore, concluded that in no case had the NCl_3 treatment of a peptide given rise to the toxic substance.

RESULTS AND DISCUSSION

Isolation

In order to isolate the toxic substance from wheat flour it was obviously desirable to obtain a flour possessing the maximum activity. As a result of many experiments it was concluded that it was not practicable to treat wheat flour with more than about ten times the amount of nitrogen trichloride which was normally used commercially. In the later stages of the work commercial gluten itself was treated with nitrogen trichloride, since the toxic substance is associated with the gluten fraction of the flour, but the product was only slightly more active than that obtained when the flour itself was treated with nitrogen trichloride. The digestion of agenized gluten with pepsin and trypsin resulted in a very considerable reduction of the average molecular weight, for about 80 % of the nitrogen was dialysable at this stage. Since on a nitrogen basis the non-dialysable material was as toxic as the dialysable it was apparent that the toxic substance was an integral part of the protein and was not a low-molecular-weight impurity.

Although it is possible that the non-dialysable residue from the enzymic digestion could have been satisfactorily hydrolysed by acid, it was felt that the elimination of possibly 'resistant' non-dialysable peptides at this stage was an advantage.

The acid hydrolysis caused a destruction of about 30 % of the toxic substance (see Table 1), but the proportion of amino nitrogen to total nitrogen was raised from 25 % to approximately 60 %; thus a further substantial reduction in the average molecular weight was achieved. It was felt that fractionation would be so simplified by this reduction in molecular weight as to make an acid hydrolysis an essential step in spite of the ensuing loss of activity.

Electrodialysis achieved a very considerable purification of the toxic substance with negligible loss. Table 1 shows that the toxic dose contained 2300 mg. nitrogen before electrodialysis and 500 mg. nitrogen after electrodialysis and removal of crystalline material from the neutral dialysate. About 36 % of the total amino-acids in hydrolysed gluten consist of glutamic acid. Thus most of the nitrogen in the anode compartment was glutamic acid. The basic amino-acids, arginine, lysine and

histidine, together account for about 10% of the total solids and, of course, a very much higher percentage of the N. Thus it is not surprising that even though only a crude fractionation was achieved by this process a great reduction in the ratio nitrogen/toxic dose was achieved.

The removal of aromatic amino-acids by charcoal was found to be a necessary step before fractionation of the mixture on a Zeo-Karb column, since we, like Partridge (1949), found that in the presence of aromatic amino-acids fractionation on Zeo-Karb 215 is adversely affected.

The toxic substance was found in the fractions from the Zeo-Karb column immediately following proline. Although activity was spread over rather a large number of fractions, the maximum activity appeared in a relatively small fraction. Only this fraction was used for the next step in purification and the other active fractions were combined and refractionated on Zeo-Karb.

Single-dimension paper chromatography of the various active fractions from Zeo-Karb indicated the presence of at least two major abnormal components. One of these, eventually identified as the toxic substance, had R_F in butanol/acetic acid of 0·07 and in phenol/ammonia of 0·64. The second abnormal component was provisionally identified, in the first instance by paper chromatography, as γ-aminobutyric acid; it was then isolated, analysed and found indistinguishable from an authentic specimen of γ-aminobutyric acid. A yield of 46·7 mg. of thrice recrystallized γ-aminobutyric acid, m.p. 193°, was obtained from 1800 g. of gluten.

Since it has not been possible to isolate γ-aminobutyric acid from unagenized gluten by the methods which were successful in the case of agenized gluten, it must be concluded that γ-aminobutyric acid arises as a result of the action of nitrogen trichloride on the protein. Since all fractions from the paper column other than the one containing the toxic substance were biologically inactive, it is concluded that the toxicity of agenized wheat flour cannot be attributed in any way to the presence of γ-aminobutyric acid. Further, Keil (1932) did not detect any toxic action arising from the subcutaneous injection of the sodium salt of γ-aminobutyric acid to human subjects.

The most active fraction from Zeo-Karb was still a complex mixture, but a considerable quantity of neutral amino-acid was removed by concentration and crystallization. The activity remained entirely in the mother liquors and these were found, by two-dimension paper chromatography, to contain at least sixteen ninhydrin-positive substances.

Fractionation on a column of powdered paper was a most efficient method. The degree of fractionation depended on the amount of material put on the column. When a column containing 1 kg. of paper was loaded with a mixture containing 100 mg. nitrogen, fractionation was complete and every substance appeared in the effluent unmixed with substances of similar R_F values. When the load was increased to 300 mg. nitrogen the substances with high R_F values were not completely separated; when the load was increased to 500 mg. nitrogen even the components with low R_F values were mixed. The progress of fractionation of a 300 mg. nitrogen batch on a paper column is shown in Table 4.

In assessing the value of a paper column as a method of fractionation of a particular amino-acid mixture, it was of great convenience to find that a column and a paper strip behaved in a similar fashion. There were, however, small but significant differences, thus proline appeared in the effluent from the column before γ-aminobutyric acid, whereas on a paper strip, in the same solvent mixture, the order was reversed. The loss of about 30% of the toxic substance on the paper column cannot be explained.

Some difficulty was experienced in the crystallization of the toxic substance from the paper column. There was contamination with a small quantity of carbohydrate, presumably the product of slow decomposition of the very large quantity of paper in contact with acetic acid. Similar difficulties have been reported by Moore & Stein (1949) when using starch columns with acidic solvents.

Degradation and comparison with other toxic factors

With the reservation made in the appropriate experimental section, the identification of α-aminobutyric acid as a product of desulphurization with nickel suggested that the toxic substance was derived from methionine. The production of methionine sulphoxide and methionine sulphone on acid hydrolysis supported this view. The occurrence of homoserine, α-aminobutyric acid and homocysteine as products of acid hydrolysis suggested in the first instance that we were dealing with a peptide, but the failure to identify any product of desulphurization other than α-aminobutyric acid was contrary to this conclusion.

In the earlier stages of this investigation we were of the opinion that the toxic substance was a peptide and we believed that our substance differed from that which was produced from zein by treatment with nitrogen trichloride. This belief was occasioned by the suggestion of Bentley *et al.* (1949*a*) that a toxic factor which they had obtained in a highly concentrated form from zein did not give a reaction with ninhydrin. However, when Bentley *et al.* (1949*b*) crystallized their toxic substance from zein and degraded it to α-aminobutyric acid by hydrogenolysis (Bentley, McDermott, Pace, Whitehead & Moran, 1950) it became obvious to us that the toxic substance which we had isolated from wheat flour

was closely similar to and probably identical with that already isolated from zein. Identity was confirmed when Dr L. Reiner supplied us with a sample of the crystalline substance isolated from zein in a parallel, but independent investigation (Reiner, Misani, Fair, Weiss & Cordasco, 1950). Dr Reiner's material could not be separated from our own by two-dimension partition chromatography on paper, and on acid hydrolysis it gave rise to the same five ninhydrin-positive substances.

We have been unable, through lack of sufficient material, to obtain a full elementary analysis of our material; an analysis has, however, been published by Bentley *et al.* (1950) and the deduction has been drawn by these authors that the molecule $C_5H_{12}O_3N_2S$ can be regarded as being derived from methionine sulphoxide by addition of NH or from methionine sulphone by replacement of O by NH. All the hydrolytic degradation products which we have 'identified' by chromatography could be envisaged as possible degradation products of such a structure.

It seemed reasonable to expect that treatment of a single methionine peptide with nitrogen trichloride would give rise to the toxic substance, but, as indicated in the experimental section, we were unable to detect any formation of the toxic substance in the reaction between nitrogen trichloride and six different methionine peptides. It may be that the chromatographic method used to detect formation of the toxic substance was insufficiently sensitive and that a very low yield of toxic substance was obtained; it seems more probable, however, that the reaction conditions were not analogous to those which exist in the agenization of flour.

Although it has not been possible to produce typical epileptiform fits in all animals by feedin agenized flour, it cannot be assumed that the tox factor is harmless in those animals which show n nervous response. In view of the very serious an eventually fatal outcome of the treatment of dog ferrets, rabbits and cats with the toxic substanc the use of nitrogen trichloride as an ageing accelerat in the manufacture of flour seems highly undesirabl

SUMMARY

1. The isolation of a toxic substance from agenize wheat flour is described.

2. The probable identity of this substance wit a substance having similar physiological properti from agenized zein is indicated.

3. The toxic substance was isolated from an ac hydrolysate of the gluten fraction of flour and regarded as part of a flour protein.

4. Large-scale partition chromatography paper-pulp columns was developed as a method fractionation of complex mixtures.

5. Degradation experiments suggested that t toxic substance was a derivative of methionine. T probable structure is given.

6. Methionine peptides treated with nitrog trichloride did not give rise to the toxic substance

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