STUDIES ON THE BLOOD PROTEINS OF HYPERIMMUNE HORSES. I.—The Effect of High Concentrations of Phenolic Substances on the Proteins of Plasma

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Work carried out on the combination of plasma proteins with higher concentrations of phenolic substances than those normally used for preservation has indicated the formation of easily dissociated complexes. The complex formed with albumin is very much less soluble in neutral salt solutions than the original protein. This observation has formed the basis for a new process for the purification of antitoxic and antibacterial sera, and this process is described. The bearing of the results on the formation of antibodies during the hyperimmunization of horses is discussed.

It has been customary for many years to use phenolic compounds, particularly Trikresol,† as preservatives for antitoxic products derived from the blood of hyperimmune animals. These compounds, when added undiluted to protein solutions, cause very extensive denaturation, and in practice various devices, such as mixing with diethyl ether or emulsifying with water, are used to overcome this undesirable property when they are added to antitoxin solutions.

It was reported by Glenny[1] that the addition of 0·35% of Trikresol to antitoxic sera leads to a loss in stability on storage. The Imray patent,[2] in which 10·0% phenol was used as a bactericide for digested serum, and experiments on the stability of antitoxin purified by pepsin treatment and heat denaturation[3] showed, however, that highly purified antitoxin solutions were as stable when antiseptics were added as when stored without preservative. It seemed possible, therefore, that the presence of proteins other than the antitoxic pseudoglobin might have some adverse influence on the stability, particularly as it had been observed that unconcentrated serum and antitoxic pseudoglobulin solutions (prepared by salt fractionation of plasma) containing traces of albumin always gave precipitates, often of a lipoidal character, on storage with antiseptic. The work of Luck *et al.*[4] has shown that albumin is a reactive protein capable of combining with, and acting as, a carrier for several substances in the blood. This indicated the possibility that Trikresol might combine with albumin to form a compound with different properties.

It was therefore decided to investigate the effect on plasma of concentrations of Trikresol higher than those normally used for antisepsis. The maximum concentration which it appeared possible to attain in a controlled manner at a high protein concentration was 1·0%, since a 2·0% solution of Trikresol is almost saturated, and it seemed that the most suitable method for the addition of Trikresol would be as an aqueous solution. It was expected that such a high concentration would induce precipitation, but it was surprising to find that after 48 hours at room temperature very little precipitate had formed and that the antitoxic value had not appreciably diminished. However, if a protein-precipitating salt were added at this stage, a dense protein precipitate formed at a very low salt concentration, and the filtrates showed practically no trace of colour, whereas filtrates obtained from plasma by the addition of low concentrations of similar salts were always brown in colour. An examination of the filtrate showed that it was devoid of albumin, but still contained a very large percentage of the antitoxin present in the original plasma. These observations led to the work reported in this paper, on which a preliminary communication has been made.[5] The investigation was carried out on three lines:

(A) The combination of Trikresol with plasma proteins.
(B) The effect of high concentrations of Trikresol on the precipitation of these proteins by ammonium sulphate.
(C) The development of a large-scale process for the purification and concentration of antibodies.

Under (B) other phenolic compounds and factors affecting the precipitation have also been studied.

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† Trikresol is the trade name for a liquid mixture of the three isomeric cresols and is marketed by British Schering Ltd.

Materials and methods

Plasma and serum.—These were taken from stocks kept for the routine issue of antitoxic sera.

Trikresol.—Solutions were made up by dissolving the required amount of Trikresol in distilled water. Trikresol estimations were made by Mr. A. J. Woiwod using his modification of the 4-aminoantipyrine method of Gottlieb & Marsh.[6] This method which can be used in the presence of protein needs the following reagents: sodium carbonate, 1·0% solution; sodium carbonate, 0·066% solution (prepared from 1·0% solution as required); potassium ferricyanide, 8·0% solution; 4-aminoantipyrine hydrochloride, 1·0% solution. Samples are diluted to contain approximately 50 μg. of Trikresol/ml. Samples (1 ml.) are pipetted into a 10-ml. graduated tube, 0·4 ml. of 1·0% 4-aminoantipyrine solution is added, and then 5 ml. of 0·066% sodium carbonate. The tubes are shaken and 0·1 ml. of 8·0% potassium ferricyanide is added. The tubes are shaken again, made up to the 10-ml. graduation, and mixed thoroughly. At the same time a reagent blank is prepared containing all the solutions in the first sample, except Trikresol, in the same quantities, and is made up to the same volume. All tubes (including blank) are kept in a dark cupboard for 10 minutes, and then read on a Spekker absorptiometer against a blank, using glass filters (Hilger No. 5). Drum readings are read against a standard curve calibrated 0–80 μg./ml. of Trikresol. The tubes after addition of potassium ferricyanide must be kept well away from bright sunlight.

Total protein.—This was estimated by a refractometric method.[7]

Antitoxin.—The antitoxic values have been estimated by the customary methods.

Degree of purification of antitoxic protein.—This was evaluated in terms of units/g. of protein. The increase in purity is denoted by R which represents the ratio of the final units/g. to the initial units/g.[7]

(A) *The combination of Trikresol with plasma proteins*

(1) Plasma from a horse immunized against diphtheria was mixed with an equal volume of 2·0% Trikresol solution. Samples were taken at intervals and Trikresol and antitoxin were estimated. The results shown in Table I indicated that all the Trikresol could still be estimated after 96 hours, and that the antitoxic content remained practically constant. Some precipitation occurred with plasma, but a similar experiment carried out with serum showed insignificant precipitation, although considerable opalescence developed. During the estimation of Trikresol the mixture is diluted considerably, and it is therefore possible that any combination of Trikresol with the plasma proteins taking place at a concentration of 1·0% is reversed by dilution.

Table I

Combination of Trikresol with plasma proteins

Original plasma: antitoxin 336 units/ml. diluted with an equal volume of 2·0% Trikresol solution

Time, hr.	Trikresol,* %	Antitoxin, units/ml.
0	1·00	162
1	1·02	168
2	0·94	162
4	1·06	162
24	0·92†	168
96	0·92†	168

* Determined as described in the text, when the high dilution used leads to the estimation of free and reversibly bound Trikresol

† Samples were centrifuged to remove the precipitate which had formed

(2) To investigate the reversal of combination a similar mixture was made up and allowed to stand for one hour. Samples were taken into centrifuge tubes and diluted with water as shown in Table II. After the addition of a few crystals of solid ammonium sulphate to promote coagulation (acetate buffer, pH 5·0, cannot be used as it interferes with the estimation of Trikresol), the tubes were stoppered and immersed for five minutes in a boiling-water bath. The tubes were then cooled and spun in an angle centrifuge. Trikresol was estimated in the supernatant liquid, and Table II shows the results obtained, together with those from similar experiments carried out with 7·0% solutions of albumin and mixed globulins (prepared by ammonium sulphate fractionation of normal horse plasma).

The results show no marked difference between plasma, albumin and mixed globulins, but do show that at a concentration of 1·0% Trikresol considerable combination has taken

Table II

Reversal of combination of Trikresol with plasma proteins

The following mixtures were made up and allowed to stand 1 hr. at room temperature:

A. Plasma + equal volume of 2·0% Trikresol
B. Globulin solution + equal volume of 2·0% Trikresol
C. Albumin solution + equal volume of 2·0% Trikresol

Composition of sample	Trikresol remaining in supernatant liquid, % of original
10 ml. A	56·0
5 ml. A + 5 ml. H_2O	71·8
2 ml. A + 8 ml. H_2O	72·1
1 ml. A + 9 ml. H_2O	100·0
10 ml. B	58·0
5 ml. B + 5 ml. H_2O	74·0
2 ml. B + 8 ml. H_2O	72·2
1 ml. B + 9 ml. H_2O	100·0
10 ml. C	56·0
2 ml. C + 8 ml. H_2O	84·7

place with protein. The combination, however, appears to be very loose and is markedly reversed by dilution. Similar results were obtained by Cooper,[8] who, in carrying out experiments to determine the nature of the germicidal action of phenols and cresols, investigated the absorption of phenol and *m*-cresol by gelatin, casein, egg-albumin and egg-globulin. He noted that both phenol and *m*-cresol have an irreversible precipitating action on egg-albumin.

(B) *The precipitability of the protein in antitoxic plasma by ammonium sulphate,* and factors affecting this precipitation*

(1) A comparison was made between antitoxic plasma diluted (*a*) with an equal volume of water, (*b*) with an equal volume of 2·0% Trikresol.

After allowing the mixtures to stand for one hour, increasing quantities of solid ammonium sulphate were added to 100-ml. quantities, and after standing for one hour and filtering, protein, antitoxin and Trikresol were estimated in the filtrates. The results given in Table III showed marked differences. In the presence of 1·0% Trikresol a very much lower concentration of ammonium sulphate was necessary to initiate the precipitation of protein and throughout series (*a*) the purities (units/g.) obtained were very much lower than those obtained when Trikresol is present.

The filtrates in the presence of Trikresol were almost colourless, whereas the filtrates from series (*a*) were coloured a deep brown. The rate of filtration in series (*b*) was slightly faster and filtration was complete in a much shorter time. Some combination of Trikresol and protein takes place throughout the precipitation range, but is greater with the protein precipitated by low concentrations of ammonium sulphate.

(2) Since such marked differences were shown by the two series, filtrates obtained by the addition of 150 g./l. of ammonium sulphate in series (*a*) and 90 g./l. in series (*b*) were concentrated by a further addition of solid ammonium sulphate. The precipitates thus obtained were separated and dialysed. The electrophoretic analyses given in Table IV show the very marked difference in composition of the two concentrates.

(3) The effect of pH on the course of precipitation of protein and antitoxin by ammonium sulphate in the presence of 1·0% Trikresol was investigated. Lowering the pH to various values from 7·6 to 5·0 by the addition of hydrochloric acid, before adding ammonium sulphate, was found to increase progressively the amount of both protein and antitoxin precipitated by the same concentration of ammonium sulphate as the pH was lowered. There was, however, no marked increase in purity, and filtration rates became slower as the pH was lowered. Below pH 5·0 filtration rates increased, but large amounts of antitoxin were precipitated.

(4) The effect of heating mixtures after the addition of ammonium sulphate was studied.

* Other protein-precipitating salts such as sodium chloride and sodium sulphate behave in a manner similar to ammonium sulphate

Table III

Comparison of the precipitation by solid ammonium sulphate of protein and antitoxin from plasma diluted: (a) with an equal volume of water or (b) with an equal volume of 2·0% Trikresol solution

Original plasma: protein, 6·99%; antitoxin, 470 units/ml. } 6720 units/g.

Ammonium sulphate concn., g./l.	Antitoxin remaining in filtrate, %	Protein remaining in filtrate, %	Units antitoxin/g. protein	Concn. of Trikresol in filtrate, %
Series (*a*)				
180	81·4	76·8	7110	—
200	67·3	60·8	7420	—
220	48·7	51·4	6330	—
240	21·7	39·6	3770	—
260	14·1	32·2	2940	—
280	5·4	27·3	1340	—
300	0	21·5	0	—
Series (*b*)				
80	90·4	55·8	10,900	0·65
100	83·5	53·2	10,500	—
120	80·4	43·7	12,300	0·61
140	73·8	32·6	15,200	—
160	49·1	20·8	15,800	0·53
200	7·4	2·9	17,200	0·45
220	1·0	1·0	—	—

A similar experiment carried out at a concentration of 0·75% of Trikresol showed similar results to those in series (*b*), but higher levels of ammonium sulphate were necessary to precipitate the same quantity of protein and all the filtrates had a brown or yellow colour, whereas those in series (*b*) were colourless.

Table IV

Composition of concentrates obtained from filtrates after precipitation of non-specific protein from antitoxic plasma by ammonium sulphate: (A) in the absence of Trikresol and (B) in the presence of 1·0% of Trikresol

	Protein, %	Antitoxin		*R**	Electrophoretic analysis, %			
		Units/ml.	Units/g. of protein		Globulins			Albumin
					α	β	γ	
Original plasma	7·94	530	6670	—	—	—	—	—
(A)	18·03	1560	8640	1·31	21·1	45·9	28·6	4·4
(B)	13·03	1590	12,200	1·82	—	64·1	35·9	—

* Ratio of final units/g. to the initial units/g.[7]

It is possible to heat for one hour at 37–40° without excessive loss of antitoxin. A slight increase in filtration rate occurs.

(5) Other compounds similar in structure to Trikresol have been used in place of Trikresol. These include *o*-, *p*- and *m*-cresols, phenol, *o*-chlorophenol and *p*-chlorophenol. All gave similar results to Trikresol at a concentration of 1·0% with the exception of phenol, for which a concentration of 2·0% must be used.

(6) The nature of the precipitate formed when 90 g./l. of ammonium sulphate is added to antitoxic plasma in the presence of 1·0% Trikresol was also investigated. The mixture as previously described was made and allowed to stand for one hour at room temperature. Solid ammonium sulphate (90 g./l.) was then added, and the suspension, after standing for one hour, was spun in an angle centrifuge. The supernatant liquid was poured off and the precipitate was successively washed, as shown in Table V, with ammonium sulphate solution equal in volume to the previous supernatant liquid. After thoroughly mixing the precipitate with washing solution, the suspensions were allowed to stand 24 hours to equilibrate and then again spun. The supernatant liquids were collected and protein and antitoxin were estimated. Part of each supernatant liquid was concentrated by adding an equal volume of saturated ammonium sulphate, centrifuging and dissolving the precipitate in a small quantity of distilled water. These concentrates were subjected to electrophoretic analysis and the results may be assumed to indicate the composition of the supernatant liquid. The precipitate left after the fourth washing with ammonium sulphate solution was also dissolved in distilled water, and the solution analysed electrophoretically and for protein and antitoxin. The results are given in Table V.

Table V

The effect of washing the first precipitate with ammonium sulphate solution

Soln.	Washing soln.	Protein, %	Antitoxin Units/ml.	Antitoxin Units/g.	Trikresol, %	Electrophoretic analysis,* % Globulins γ	Globulins β	Globulins α	Albumin
Original plasma	—	8·49	360	4240	0	31	40	14·5	14·5
1st supernatant liquid	—	2·64	160	6070	0·62	52 (82·3)	48 (58·8)		0
2nd supernatant liquid	$(NH_4)_2SO_4$, sp. gr. 1·097	0·626	21	3360	0·23	29 (10·9)	←49→ (10·5)		22 (18·0)
3rd supernatant liquid	$(NH_4)_2SO_4$, sp. gr. 1·097	0·403	6·6	1630	0·14	←10→ (1·3)			90 (46·3)
4th supernatant liquid	$(NH_4)_2SO_4$, sp. gr. 1·080	0·500	8·0	1600	0·11	25 (7·8)	17 (4·1)	18 (12·0)	40 (21·2)
5th supernatant liquid	$(NH_4)_2SO_4$, sp. gr. 1·080	0·210	2·4	1140	0·06	17 (2·0)	15 (1·0)	49 (12·3)	19 (4·9)
Soln. of final ppt.	—	1·67	4·5	270	0·04	50 (31·7)	50 (26·5)	0	0

* With the apparatus available for electrophoretic analyses, it was not always possible to estimate separately the various globulin constituents, and in all cases fibrinogen is included in the figure for β-globulin. The figures in parentheses give the percentages of the original component present in the fraction. The difficulty in estimating areas in the Schlieren diagrams is probably the reason for the total yield of γ-globulin being greater than the original

(C) *The development of a large-scale process for the purification and concentration of antibodies**

The methods generally available for the large-scale purification and concentration of antibodies fall into two main classes: (1) salt-fractionation methods and (2) enzyme-treatment methods.

The salt concentration methods, in which a neutral salt such as ammonium sulphate or sodium sulphate is used to fractionate the proteins present in antitoxic plasma or serum, involve two precipitations which must be carefully controlled in relation to antibody. The filtration after the first precipitation is generally very difficult, leading to large mechanical losses, and the final concentrates, unless laborious procedures, such as refractionation and electrodialysis, are employed, tend to be contaminated with albumin. On standing the serum becomes cloudy, and very often lipoidal protein precipitates form as a scum on the surface. These methods can be applied to the purification of antibacterial antibodies as well as true antitoxins, but a very low concentration of ammonium sulphate must be used for the first precipitation, since the antibacterial antibodies are associated with the lower globulin fractions. This filtration is generally extremely difficult, and the final concentrate is very viscous and cloudy.

The information obtained in the first part of this paper gave indications that a large-scale process might be evolved from the experimental data. The low concentration of ammonium sulphate necessary to precipitate most of the non-specific protein augured well for the retention of all the specific protein. Experimental concentrates appeared to remain remarkably clear, and since all the albumin is precipitated by the first addition of ammonium sulphate, the second precipitation need not be controlled by antitoxin tests.

For the preliminary work it was decided to carry out the initial precipitation of non-specific protein with the lowest concentration of ammonium sulphate which would give a reasonable filtration. A level of 90 g./l. was therefore adopted, and in practice has proved to be applicable to most batches, although a rather more rapid filtration with a slightly greater antibody loss may be obtained by using a level of 100 g./l.

On a large scale the process is conveniently carried out, as described in the experimental work, by allowing a mixture of equal volumes of plasma (or serum) and 2·0% Trikresol solution to stand for one hour, dissolving the required quantity of solid ammonium sulphate and again standing for one hour, to allow the precipitate to aggregate before filtering through cloth. The purified antibody protein is precipitated from the filtrate by the addition of a further quantity of solid ammonium sulphate, after dilution of the filtrate with an equal volume of water, in order to decrease the Trikresol concentration and so obviate the risk of denaturation. The precipitate is removed by filtration through cloth, and is then laid between sheets of blotting paper, but not pressed, to remove residual liquor. The precipitate is then dialysed against cold, running tap-water for 48 hours, and antiseptic is added to the resulting antibody solution, which has a protein content of approximately 9%, and may be ultra-filtered to a

* Covered by B.P. 605,687

concentration of 15% without becoming unduly viscous. Thereafter the solution is clarified, made isotonic and sterilized by filtration.

It is advisable, when adding antiseptic after dialysis, to adjust the pH to a value between 6·0 and 6·3 to ensure that the final sterilized solution has a pH between 6·3 and 6·7 when it has its maximum stability, which is greater than that of unconcentrated serum at its normal pH of 7·4–7·6, as shown in Table VI.

Table VI

Stability of purified lamb-dysentery serum compared with unconcentrated serum. Both sera were stored at 37° for 18 months (the antitoxins to the β- and ϵ-toxins of Cl. welchii *type B were estimated before and after storage)*

Serum	pH	Original units/ml.		Final units/ml.		% remaining	
		β	ϵ	β	ϵ	β	ϵ
Unconcentrated	7·60	1425	140	850	90	59·7	64·3
Purified	6·67	4750	525	3800	360	80·0	68·6

Both sera became cloudy and more viscous, but similar samples kept at room temperature for the same period retained their original viscosity. Whereas the unconcentrated serum became cloudy and formed a precipitate at room temperature, the purified serum remained perfectly clear

This process is applicable to plasma (or serum) containing no preservative, but in practice it is usual to preserve plasma (or serum) before processing, by adding 0·35% of Trikresol. This material should therefore be mixed with an equal volume of 1·65% Trikresol solution.

To plasma (or serum), containing 0·35% Trikresol, 0·85 volume of 2·0% Trikresol solution is added. The mixture is allowed to stand for 15 minutes before stirring in 0·30 volume (calculated on the original plasma volume) of 50% (w/v) ammonium sulphate solution. After a further 15 minutes, 3·0% (calculated on the total volume) of Hyflo Supercel (Johns Manville Corpn.) is stirred in, and stirring is maintained during filtration which is carried out by gravity (8-ft. head) in a 9-in. ebonite plate and frame-washing-type filter-press having 0·5-in.-thick frames. Under these conditions a 28-chamber press will filter 85 l. of suspension in 1–2 hours, giving a perfectly clear and almost colourless filtrate containing 80–85% of the original antibody. It is advisable to rest the filter-press in a frame so that, after filling up with suspension, it may be turned on its side to prevent blockage of the ports by filter-aid, which tends to settle. It is possible to recover a further 5% of the antibody by a limited washing of the cakes with approximately 20 l. of a 200-g./l. ammonium sulphate solution (sp. gr. 1·097). Further washing leads to solution of non-specific protein, as shown by the results obtained by processing a typical batch of lamb dysentery serum given in Table VII.

Table VII

Results obtained by processing lamb-dysentery serum containing 0·35% of Trikresol

Material	Vol., l.	Protein, %	Antitoxin Units/ml.†		Antitoxin Units/g. protein		*R* ‡		% of original		Electrophoretic composition, % Globulins			Albumin
			β	ϵ	β	ϵ	β	ϵ	β	ϵ	γ	β	α	
Original serum	35·0	7·50	420	268	5600	3570	—	—	—	—	31	40	15	14
Concentrate (includes 1st washing)	14·4	9·55	1020	500	10,700	5230	1·91	1·61	104·0	80·2	52	←48→		0
1st washing* ..	1·1	9·54	940	620	9830	6500	1·76	1·82	7·8	7·6	34	←66→		0
2nd ,, * ..	0·3	7·22	480	250	6640	3460	1·18	0·97	1·0	0·8	18	27	21	34
3rd ,, * ..	1·4	8·64	155	90	1790	1040	0·32	0·29	1·5	1·4	—	—	—	—

The washing of the precipitate was carried out as follows:

1st washing with 20·0 l. of 200 g./l. ammonium sulphate soln.;
2nd ,, ,, a further 56·0 l. of 200 g./l. ammonium sulphate soln.;
3rd ,, ,, ,, ,, 160·0 l. of water

* The values given are for concentrates prepared from the precipitate washings. The electrophoretic composition of the 3rd washing could not be determined as the solution was very opalescent

† The tests for β- and ϵ- antitoxins have an approximate accuracy of ± 7%

‡ See footnote to Table IV

The antibody protein is precipitated from the filtrate by adding 1·2 volumes of 50% (w/v) ammonium sulphate solution, and may be removed by gravity filtration through a filter-press similar to that used for the first filtration. The cakes of antibody protein can be dried with

compressed air at a pressure not exceeding 2 lb./sq. in., and are then dialysed and treated as previously described.

The filtrate after removal of antibody protein may be regarded as a practically pure solution of ammonium sulphate containing approximately 0·3% Trikresol. If it is boiled and evaporated to a concentration of 55% (w/v), the traces of residual protein are coagulated and Trikresol is distilled off. The resulting solution may be filtered, adjusted to 50% concentration and used again in the process. After using 20 times no undesirable impurities were found to accumulate.

Discussion

Although the results obtained lend little support to the original supposition that only albumin is concerned in producing the phenomena associated with the presence of Trikresol in antitoxic sera, some interesting observations on the various proteins present in the plasma of hyperimmune horses have been obtained.

The experiments carried out to investigate the combination of Trikresol with the plasma proteins show that a definite combination occurs when the concentration of Trikresol is of the order of 1·0%, but is readily reversed when the concentration is lowered. This occurs both with normal and immune plasmas, but for normal plasma Trikresol does not appear to combine selectively with any particular protein. A most striking alteration in the solubility of the proteins in salt solutions is, however, apparent from the results. If antitoxic plasma is diluted with an equal volume of water and 90 g./l. of solid ammonium sulphate is added, precipitation of protein does not occur, but when the same plasma is diluted with an equal volume of 2·0% Trikresol solution, the same concentration of ammonium sulphate produces a heavy protein precipitate containing all the albumin, the major portion of the α-globulin, approximately 40% of the β-globulin and approximately 30% of the γ-globulin (Table V). The residual β- and γ-globulins, which carry most (70%) of the antitoxin, remain in solution; this figure (70%) can be probably increased to over 80% if the antitoxin present in the precipitate, which occupies one-fifth of the volume of the original suspension, is included.

The results in Table III indicate that some Trikresol is combined with the protein remaining in solution, but it would appear that the solubility of the antitoxic protein–Trikresol complex does not differ appreciably from that of the original protein. The electrophoretic analyses and Trikresol estimations in Table V again show the reversibility of the combination between the proteins and Trikresol. As soon as the Trikresol concentration is decreased (second supernatant liquid), and in spite of the high concentration of ammonium sulphate (approximately 200 g./l.), the albumin complex, which had been completely precipitated, dissociates, and the albumin dissolves, whereas the globulins (or their complexes) remain precipitated. (The small concentration of globulins recorded is derived from the supernatant liquid retained by the precipitate.) Further lowering of the Trikresol concentration by repeated washing leads to some solution of the globulins, but the concentration of ammonium sulphate remains just within the range of normal globulin insolubility. It is interesting to note that these precipitated globulins are completely soluble in water, which indicates that no denaturation of protein has taken place.

A definite difference in behaviour between antitoxic globulins and non-antitoxic globulins appears, therefore, to be established, and may be attributed either to a difference in their combining power or to a difference in the solubilities of their Trikresol complexes. The antitoxic values of the various fractions (Table V) indicate that a small quantity of antitoxic protein has a lower solubility than the remainder, since the values are greater than could be attributed to supernatant liquid carried over by the precipitate. In addition, the more dilute ammonium sulphate solution used for washing the precipitate left after the removal of the third supernatant liquid, solubilizes some antitoxic protein. An experiment in which the filtrate obtained after the addition of 90 g./l. of ammonium sulphate to a mixture of equal parts of immune plasma and 2·0% Trikresol solution, was fractionated into two approximately equal parts by the addition of further ammonium sulphate, showed that the first fraction contained a preponderance of γ-globulin. It had a purity (units/g.) equal to less than half the purity of the second fraction, which contained a very high percentage of β-globulin. It has also been observed that as a horse is hyperimmunized and the antitoxic value of the plasma increases, the antitoxic content of the more insoluble globulin fractions increases at a much slower rate than that of the more soluble fractions. The work of Cohn and his collaborators[9] has shown that for human plasma, where there is no question of hyperimmunization, the γ-globulin fraction contains all the antibodies. It would be logical therefore to assume that in the early stages of immunization antibody is formed as γ-globulin, whereas in the later

stages, when hyperimmunization has taken place and large quantities of β-globulin have been produced, the antibody is associated with this globulin.

The results obtained from the experimental work recorded in this paper formed the basis for a large-scale process for the purification of antibodies. Successful large-scale purification of the plasma from horses hyperimmunized against the following organisms have been carried out: influenza virus type A, *Proteus* species O.X. 19, *Haemophilus pertussis*, bacillus of swine erysipelas, virus of dog distemper, *Corynebacterium diphtheriae* and *Clostridium welchii* type B.

The large-scale work has disclosed the fact that although the proteins are not denatured during the process, the application of pressure at any time when the proteins are present as Trikresol complexes will cause extensive denaturation. For this reason pumps have been avoided, and all filtrations are carried out by gravity, using very low pressure-heads. Apart from this precaution the process had proved to be extremely simple to run, since ammonium sulphate concentrations are standardized without reference to antibody tests. The process produces a high yield of purified serum which is stable and remains clear for long periods. An increase in purity of 1·5–2·0 is obtained, and concentration is of the order of 3–4 times the original, depending on the concentration of protein to which the final product is ultra-filtered. Clinical trials of diphtheria antitoxin and field trials of lamb-dysentery antitoxin have shown the purified sera to be potent therapeutic agents.

Acknowledgments

The author wishes to thank Mr. A. J. Woiwod, of the Media Production Unit at the Wellcome Research Laboratories, for estimating Trikresol, and Dr. C. L. Oakley and his staff for carrying out the immunological tests. He is indebted to Dr. E. Bidwell for all the electrophoretic analyses in this paper, and is very grateful to Dr. C. G. Pope for his criticism and suggestions.

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Received 8 July, 1952

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STUDIES ON THE BLOOD PROTEINS OF HYPERIMMUNE HORSES. II.*—The Effect of High Concentrations of Phenolic Substances on the Proteins of Whole Blood

By A. J. HARMS†

Based on the work on antitoxic plasma described in Part I, a study has been made of the conditions under which whole blood from hyperimmunized horses can be purified. From the information obtained a simple process, applicable to all antibodies, for the production in high yield of purified and concentrated antibodies direct from whole blood has been evolved and is described.

Practically all methods for purifying and concentrating antibodies used in the laboratory and for large-scale production include an initial separation of plasma or serum from the red

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blood-corpuscles. This may be accomplished by many different methods, such as settling and siphoning off the plasma, centrifuging or clotting. On a large scale these methods are generally tedious and entail losses of antibody of the order of 10% or greater. Purification of the plasma or serum obtained by the above methods leads to further losses of antibody, and it seemed profitable to investigate methods of purifying whole blood, so that these losses would be to some extent balanced by the inclusion of antibody lost during the initial separation process.

An investigation into the applicability of the enzyme treatment–heat denaturation method of purification[1] to whole blood had shown that no advantage was gained.[2] However, a new method of purifying plasma or serum by salt fractionation in the presence of high concentrations of Trikresol (Part I) gave some promise of a simple method for the production of clear stable concentrates from whole blood. Initial experiments on the lines of this process gave highly pigmented concentrates, even though a high proportion of pigment appeared to be precipitated. Further investigation, in which the dilution was increased and high concentrations of Trikresol were obtained, showed that it was possible to remove all but faint traces of pigment without excessive loss of antibody. A series of experiments was therefore carried out on whole blood, and will be reported together with the application of the results to produce a simple method for the large-scale purification of antibodies direct from the whole blood of hyperimmunized horses.

Materials and methods

These are exactly as described in Part I, except that instead of serum or plasma, whole blood collected in bottles, containing a small quantity of 10·0% potassium oxalate to prevent clotting, was used.

For the laboratory experiments blood from a single horse hyperimmunized against diphtheria has been used, and on a large scale, mixed blood withdrawn from several horses undergoing hyperimmunization for the routine production of the selected type of antibody, has been processed.

The original values for protein and antitoxin were estimated on plasma separated by centrifuging from the whole blood. For comparing yields of antibody from whole blood with those obtained by other processes, it has been necessary to have a standard for comparison. This has been taken from the normal method used in these Laboratories for preparing unconcentrated serum, i.e. allowing oxalated blood to settle, siphoning off the plasma, adding enough calcium chloride to give a slight excess of calcium ions, shaking to clot, and siphoning off the serum. Over a very long period the average yield of serum is 0·60 l./l. of blood, and this factor has been used in calculating yields from whole blood. Under these conditions it is therefore possible to obtain estimates of yields in some batches exceeding 100%.

Laboratory experiments

The method adopted for laboratory experiments designed to find conditions under which maximum yield could be obtained consistent with removal of pigment and non-specific protein (i.e. high purification) was generally as follows: (*a*) Whole blood was mixed with two volumes of Trikresol solution of various concentrations and allowed to stand at several temperatures for varying times. (*b*) Various amounts of solid ammonium sulphate were dissolved in the mixtures which were allowed to stand for varying times at different temperatures. At this stage the effect of the addition of alkylene chlorides on pigment removal was studied, together with further dilution with ammonium sulphate solution to increase filtration rates and yields of filtrate. (*c*) The suspensions were filtered and protein and antitoxin estimated in the filtrates.

Effect of Trikresol and ammonium sulphate concentration.—Preliminary experiments in which one volume of 2·0% Trikresol was added to one volume of blood showed that there was little likelihood of unpigmented filtrates. Therefore for the first experiment one volume of blood was mixed with two volumes of both 1·65% and 2·0% Trikresol solution. After allowing the mixtures to stand for four hours at room temperature, varying quantities of solid ammonium sulphate were dissolved and the suspensions remained overnight at room temperature before filtering. The results are given in Table I.

All the filtrates were pink in colour but those containing the smaller concentration of Trikresol were much darker. The concentration of ammonium sulphate had no influence on colour, and since initially it was desired to keep as much protein as possible in solution, 60 g./l. was used in all the following experiments. Also two volumes of 2·0% Trikresol were added to each volume of blood.

Table I

The effect of Trikresol and ammonium sulphate concentration

Original blood: * plasma protein, 9·50% antitoxin, 1350 units/ml. } 14,200 units/g.

Trikresol soln., %	Concn. of ammonium sulphate, g./l.	Yield of antitoxin %	Yield of antitoxin Units/g.
1·65	60	106·3	14,000
1·65	80	102·7	14,900
1·65	100	101·7	15,000
1·65	120	98·8	16,200
2·00	60	103·3	14,500
2·00	80	103·3	15,600
2·00	100	105·0	16,200
2·00	120	101·7	16,900

* Whole blood from a horse immunized against diphtheria was used

The effect of time of contact with Trikresol and with ammonium sulphate, and of temperature at the latter stage.—One volume of blood was mixed with two volumes of 2·0% Trikresol, and after standing at room temperature for varying times, 60 g./l. of solid ammonium sulphate was added and dissolved. The mixtures were again allowed to stand for varying times at several temperatures before filtering. The results obtained on the filtrates are recorded in Table II.

Table II

The effect of time of contact with Trikresol and with ammonium sulphate, and of temperature on the latter stage

Original blood: as for Table I

Time of contact with Trikresol, hr.	Contact with ammonium sulphate Time, hr.	Temp., ° c.	Yield of antitoxin %	Yield of antitoxin Units/g.
1	24	20	103·3	14,200
2	24	20	103·3	14,400
4	24	20	103·3	14,500
24	24	20	103·3	14,700
24	1	37	101·7	16,000
1	24	37	100·0	15,800
1	1	40	102·7	15,800
1	2	40	102·7	16,500

The results show that the time of contact has very little influence at either stage on yield or purity. Increasing the temperature, however, produced an increase in purity together with a diminution in the colour of the filtrates.

The effect of diluting and heating before filtering.—Since the suspensions obtained in the previous experiments were exceedingly thick, it was decided to investigate the effect of diluting with ammonium sulphate solutions before filtering, with the aim of making filtration easier and effecting the recovery of a greater volume of filtrate. One volume of blood was mixed with two volumes of 2·0% Trikresol and allowed to stand for one hour at room temperature. Solid ammonium sulphate (60 g./l.) was dissolved in the mixture, which was allowed to stand overnight. The suspension was divided up into several portions which were diluted with equal volumes of ammonium sulphate solutions. The solutions were heated for one hour at various temperatures before filtering, and the results obtained on the filtrates are given in Table III.

The colour of the filtrates decreased, and the rate of filtration increased, both with rise in temperature and rise in ammonium concentration. However, it appears unwise to exceed a temperature of 40°.

The influence of alkylene chlorides in colour removal.—Chloroform has been used for the removal of blood pigment, and it therefore appeared probable that the presence of small quantities of chloroform might assist in the removal of the last traces of colour from the filtrate.

One volume of whole blood was mixed with two volumes of 2·0% Trikresol and allowed to stand for one hour at room temperature. Solid ammonium sulphate (60 g./l.) was dissolved

Table III

Showing the effect of diluting and heating before filtering

Original blood: as for Table I

Concn. of ammonium sulphate soln., g./l.	Temp. ° c.	Yield of antitoxin %	Yield of antitoxin Units/g.
160	20	103·8	17,600
120	40	103·3	17,200
160	40	102·7	17,400
240	40	79·8	19,700
160	50	95·2	18,800
160	55	29·0	10,000

and the mixture again allowed to stand at room temperature overnight. To separate portions, equal volumes of 160 g./l. ammonium sulphate solution, together with varying quantities of chloroform, were added, and the mixtures were heated for one hour at 40° before filtering. Protein and antitoxin were estimated in the filtrates. The results are given in Table IV.

Table IV

The effect of chloroform

Original blood: as for Table I

Chloroform, %	Protein in filtrate, %	Antitoxin in filtrate, units/ml.	Yield of antitoxin %	Yield of antitoxin Units/g.
0	0·775	136	100·7	17,500
0·5	0·765	135	100·0	17,700
1·0	0·627	135	100·0	21,500
2·0	0·538	135	100·0	25,100

It will be observed that, without any significant loss in yield, a considerable increase in purity has been achieved. Furthermore a considerable removal of pigment is obtained, so that the filtrate from the mixture containing 2·0% chloroform was almost colourless.

A number of other alkylene chlorides have been used in place of chloroform. Carbon tetrachloride, trichloroethylene, dichloroethylene, tetrachloroethane, ethylene dichloride and methylene dichloride have not shown themselves to be more suitable than chloroform. The following phenolic compounds: *o*-chlorophenol, *p*-chlorophenol, 2 : 4-dichlorophenol and *p*-chloro-*m*-cresol have been used in place of Trikresol, but failed to give better results.

Sodium sulphate and sodium chloride have been used instead of ammonium sulphate but are not as suitable, nor has pH adjustment given any improvement in either yield or colour removal.

The effect of storing blood, with a small quantity of Trikresol or toluene as preservative, before processing has been investigated. Both compounds produce considerable haemolysis, and lead to some increase in colour of the filtrate after processing such stored blood. In practice it has been found that, provided blood is withdrawn from the horse under stringent aseptic conditions, it is possible to store whole blood at + 2° for three months without significant deterioration.

*Large-scale process**

Standard process.—The information obtained from the laboratory experiments reported above has been sufficient for the evolution of a large-scale process for producing purified and concentrated serum, containing substantially only β- and γ-globulins, direct from whole blood. As the time of contact with Trikresol, and afterwards with ammonium sulphate, is not critical, a time schedule suitable for large-scale production has proved simple to achieve. The high dilution required (finally 1 : 6) is a slight disadvantage, but by using vessels of 500-l. capacity it is possible to purify 80-l. batches of blood, so that the process is almost complete in 24 hours. The process is carried out as described below.

Blood† (80 l.) is weighed into a jacketed, spray-heated, stainless-steel vessel of 500-l.

* This process is covered by B.P. 633,958

† This blood has been stored at + 2° and in practice it has been found that better filtration is obtained if the blood is allowed to stand at room temperature for 24 hours before processing

capacity and fitted with a stirrer and thermometer. Trikresol solution (160 l. of 2%) in tap-water is added with vigorous stirring, followed after one hour by 14·4 kg. of solid ammonium sulphate. It is found convenient in practice to add 2·0% of chloroform while the ammonium sulphate is being dissolved rather than to wait until further dilution is carried out. Only half the quantity of chloroform is needed, and it produces an equal effect. The mixture is then allowed to stand at room temperature overnight.

The next morning 240 l. of 120 g./l. ammonium sulphate solution in tap-water is added, and the mixture is heated in about 30 minutes to 40° by spraying hot water at 55° over the outside of the stainless-steel vessel. The contents are held at 40° for one hour and then cooled to 37° before filtration.

Under the conditions described the precipitate is of a granular nature, and cakes 1 in. thick may be built up without difficulty. A filter-press is therefore suitable and filtration is accomplished in a 16-chamber, 19-in. washing-type ebonite filter-press having frames 1 in. thick, using chain cloth. As the precipitate is inclined to pack tightly in the press and therefore wash slowly, it is advisable to add 1% of asbestos filter-aid to the suspension before commencing filtration. The suspension may be pumped into the filter-press, but high pressure should be avoided and pressures of the order of 5 lb./sq. in. are adequate, except perhaps in the final stages, when the pressure may be raised to 7 lb./sq. in. without compressing the precipitate too tightly to wash.

A dilute solution of ammonium sulphate may be used to wash the precipitate. Approximately 30 l. of 90-g./l. ammonium sulphate solution will wash out most of the residual antibody, but if desired, the precipitate may be further washed with up to 100 l. of water to produce a weak washing liquor, which it is advisable to concentrate separately. The precipitate appears to be completely denatured, and washing with water does not remove either pigment or non-specific protein.

In order to recover the antibody, the filtrate and first washings are bulked and diluted with an equal volume of water to decrease the Trikresol concentration. Enough solid ammonium sulphate (generally 280 g./l.) to precipitate all the protein is then slowly added with stirring, and the suspension is filtered in a 9-in. ebonite filter-press. Dialysis, addition of antiseptic, adjustment of pH, ultra-filtration, clarification and sterilization are then carried out as described in Part I.

Modified process.—In order to bring the process into line with the project to recover waste ammonium sulphate liquors by reconcentrating, as described in Part I, several modifications in the process have been introduced. These modifications are designed to substitute all additions of solid ammonium sulphate by stock 50% (w/v) solutions (sp. gr. 1·212).

Thus after the addition of Trikresol, 30·0 l. of stock ammonium sulphate solution is added. To compensate for the increase in volume, 210 l. of 120-g./l. ammonium sulphate solution is added instead of 240 l. as described under the standard method. For the precipitation of antibody, the filtrate is not diluted with water, but 0·8–0·9 volume of stock ammonium sulphate solution is added instead of the solid. It is important that this addition should be made in two approximately equal parts, with an interval between the additions to allow the precipitate to aggregate. Otherwise bad filtration will result, and the cloths of the filter-press will fail to retain the precipitated protein. From this point the process proceeds as described under the standard method. The process appears to be universally applicable to antitoxins, and to antibacterial and antiviral antibodies. Successful large-scale production of concentrates from the blood of horses hyperimmunized against *Corynebacterium diphtheriae*, streptococcus (Scarlatina), *Clostridium welchii* type B, *Haemophilus pertussis* and the virus of dog distemper has been accomplished. Concentrates which have a light reddish-brown colour and remain clear and stable for long periods are obtained. They consist essentially of β- and γ-globulins, although occasionally traces of albumin (2–3%) are present, but do not appear to have any deleterious effect on the stability.

At 15·0% protein the concentrates are not unduly viscous. A considerable amount of lamb-dysentery serum has been prepared by this process and has successfully undergone field trials. The results of a typical batch given in Table V show that at 15·0% protein there is a threefold concentration of antitoxin. From a series of batches, yields of the order of 90% have been obtained. For comparison, the results obtained by separating plasma from the blood used in this batch and subjecting it to purification (*a*) by concentration in the presence of Trikresol (Part I), and (*b*) by enzyme treatment and heat denaturation, are also given in Table V.

The weak washings form 2·5% of the main concentrate, and as shown in Table V have approximately the same purity.

Table V

Comparison of concentrates obtained by different processing methods

Original blood: mixed bleedings from horses immunized against *Cl. welchii* type B

Material	Protein, %	Antitoxin Units/ml. β	Antitoxin Units/ml. ε	Antitoxin Units/g. β	Antitoxin Units/g. ε	Yield of antitoxins, % β	Yield of antitoxins, % ε	Electrophoretic composition, % Globulins γ	Globulins β	Globulins α	Albumin
Original plasma	8·22	2800	220	34,000	2680	—	—	—	—	—	—
Main concentrate	15·00	8400	705	56,000	4700	86·0	92·3	33	←64→		3
Concentrate of weak washings	15·00	7950	710	53,000	4730	2·6	2·8	46	←51→		3
Concentrate from plasma* ..	15·00	9570	710	63,800	4730	87·1	72·8	40	53	7	0
Enzyme concentrate*	15·00	23,600	1700	157,000	11,300	69·3	55·2	—	—	—	—

* Small-scale concentrations involving some mechanical losses of antibody

Since the process produces a filtrate consisting essentially of β- and γ-globulins, a simple fractionation with ammonium sulphate can be employed to give a partial separation. If approximately 0·4 volume of stock sulphate solution is added to the first filtrate, a fraction of low purity containing a high γ-globulin content is precipitated. This may be filtered off, and the remaining fraction of high purity and high β-globulin content may be precipitated by further addition of ammonium sulphate solution to the filtrate. The results obtained by fractionating the first filtrate of a batch of blood from horses hyperimmunized against *C. diphtheriae* are shown in Table VI. Although the first fraction has low purity, its high γ-globulin content may render it more effective therapeutically.

Table VI

Fractionation of first filtrate

Original blood: mixed bleedings from horses immunized against diphtheria

Material	Protein	Antitoxin, units/ml. *In vitro*	Antitoxin, units/ml. *In vivo*	Yield of antitoxin *in vitro* %	Yield of antitoxin *in vitro* Units/g.	Electrophoretic composition, % Globulins γ	Globulins β	Globulins α	Albumin
Original plasma	9·08	237	250	—	2620	31	40	14	15
1st fraction	15·00	450	515	17·0	3000	65	29	6	0
2nd ,, ...	15·00	1210	1240	61·0	8070	14	74	12	0

Discussion

The experimental work described in this paper was not designed to investigate the action of Trikresol on whole blood, but rather to find conditions which would give a simple process for the purification and concentration of antibodies, direct from whole blood. There is no doubt, however, that the action of Trikresol on the proteins of blood must be similar to that described in Part I, when its action on plasma and serum was investigated. For whole blood a considerably higher concentration (1·66%), and a large quantity of Trikresol is needed to bring about the precipitation, at a low ammonium sulphate concentration, of non-specific protein of which there is a much larger quantity. In addition, the presence of 2·0% of chloroform is necessary to complete the precipitation of pigment and non-specific protein (Table IV).

Whereas the precipitate from plasma and serum was soluble on dilution, the precipitate of non-specific protein from whole blood appears to be completely denatured, even in the absence of chloroform, since it can be washed with water without dissolving. Raising the temperature to 40° is not the cause of denaturation, since the dilution before heating does not lead to any solubilization of protein. It is surprising that under these conditions practically the whole of the antibody protein remains in solution undenatured. In fact it is possible to heat the filtrate, after removal of denatured protein, for several hours at 40° without producing an appreciable precipitate of protein. It seems therefore probable that antibody protein has a different structure from normal plasma globulins, which renders it more difficult to denature by heat at low temperatures, although, as shown in Table III, it denatures very rapidly at 55°. However, unless the Trikresol concentration is decreased, leading to a breakdown in the protein–Trikresol complex, it is still readily denatured by pressure. The cause of the increased stability of antibody protein is not apparent, but may possibly be due to the fact that the active antibody groups occupy positions in the molecule to which Trikresol

would normally attach itself. The decreased combining power for Trikresol might be sufficient to account for the greater solubility and stability in ammonium sulphate solution at high Trikresol concentration.

The large-scale process described appears to be universally applicable to antibodies, and is simple in operation. A purification factor of between 1·5 and 2·0 is obtained together with a very high yield of antibody. With further experience of processing it may well be possible to obtain yields equivalent to those obtained by conversion of blood into unconcentrated serum. The purities obtained are not as high as those obtained by the enzyme processes, but the concentrate obtained from antitoxins may be further purified by pepsin treatment and heat denaturation. Since albumin has been removed, pepsin treatment will not produce such a large quantity of proteose as is obtained when plasma is treated with pepsin. It is also probable that higher yields of antitoxins would be obtained. Alternatively, the antibody present in the red cells, separated from plasma, may be recovered by subjecting the red cells to a slightly modified form of the process. In a trial batch, 5·0 l. of concentrate having an antibody concentration equal to twice that of the original plasma was obtained from 100 l. of cells, from which plasma had been previously separated by the method normally used in these laboratories.

Acknowledgments

The author is indebted to Dr. Oakley and his staff for carrying out the immunological tests, and to Dr. E. Bidwell for the electrophoretic analyses. He also wishes to thank Dr. Pope for his advice.

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Received 8 July, 1952

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STUDIES ON THE BLOOD PROTEINS OF HYPERIMMUNE HORSES. III.*—The Distribution of Antibodies to *Clostridium welchii* type B

By A. J. HARMS †

The antibodies present in various globulin fractions prepared from the blood of horses hyperimmunized with filtrates from *Clostridium welchii* type B have been estimated. Some observations on the distribution of the various antitoxins are made.

When horses are hyperimmunized for the routine production of lamb dysentery antitoxin by the intramuscular injection of filtrates from short-growth culture of *Clostridium welchii* type B, large quantities of β- and ε-toxins and only small quantities of α- and δ-toxins are injected. The same animal is therefore hyperimmunized mainly against β- and ε-toxins. Thus a typical course of injections will produce 2500 β-units and 500 ε-units, compared with 20 α-units and 7 δ-units/ml. of plasma. It seemed of some interest to investigate the distribution of the various antitoxins, in order to find out whether the antitoxins produced by hyperimmunization appeared in the same protein fractions as the antibodies produced by a much lower level of immunization. The method of producing purified solutions of antibodies in high yield direct from whole blood (Part I) appeared to give promise of a satisfactory starting material for fractionation, since practically all non-specific protein has been removed, whereas almost all the antibody is still present.

* Part II: preceding paper

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Materials and methods

The solution of specific protein.—This was prepared by the purification method outlined in Part I, from routine stocks of whole blood taken from horses hyperimmunized with the toxins of *Cl. welchii* type B.

Protein.—This was estimated by a refractometric method.[1]

Antitoxins.—These were estimated by the conventional methods.

Table I

Results obtained by fractionating the filtrate obtained by treating the mixed blood of horses, hyperimmunized against Cl. welchii *type B, with Trikresol, and heating with ammonium sulphate*

Sample	Percentage of total protein in fraction	Antitoxin		R*	Ratios of antitoxins					
		Units/ml.	Units/g.		β/α	β/ϵ	β/δ	ϵ/α	ϵ/δ	α/δ
Original plasma	—	α 28	344	—	75·2	11·70	221·0	6·40	19·00	2·95
		β 2100	25,800	—						
		ε 180	2210	—						
		δ 9·5	117							
1st fraction, 0–140 g./l.	8·0	α 46	590	1·71	39·5	16·90	117·7	2·12	6·98	3·04
		β 1650	21,200	0·82						
		ε 97·5	1260	0·57						
		δ 14	180	1·54						
2nd fraction, 140–170 g./l.	42·3	α 57·5	850	2·47	41·7	16·00	141·2	2·61	8·82	2·96
		β 2400	35,500	1·38						
		ε 150	2220	1·00						
		δ 17	251	2·14						
3rd fraction 170–200 g./l.	25·6	α 85	832	2·42	67·7	10·40	244·6	6·47	23·40	3·62
		β 5750	56,200	2·18						
		ε 550	5380	2·43						
		δ 23·5	230	1·96						
4th fraction, 200–230 g./l.	16·9	α 60	703	2·04	104·3	9·50	508·0	11·00	53·60	4·88
		β 6250	73,200	2·84						
		ε 660	7730	3·50						
		δ 12·3	144	1·23						
5th fraction 230–280 g./l.	7·3	α 23	375	1·09	147·8	10·30	708·0	14·30	68·80	4·78
		β 3400	55,300	2·14						
		ε 330	5370	2·43						
		δ 4·8	78	0·67						
Routine concentrate†	—	α 75	773	2·25	56·2	10·00	220·5	5·60	21·00	3·75
		β 4200	44,300	1·72						
		ε 420	4430	2·00						
		δ 20	206	1·76						

Sample	Electrophoretic analyses, %			
	Globulins			Albumin
	γ	β	α	
Original plasma	28	33	18	21
1st fraction, 0–140 g./l.	80	18	2	0
2nd fraction, 140–170 g./l.	67	30	3	0
3rd fraction, 170–200 g./l.	38	53	8	1
4th fraction, 200–230 g./l.	7	75	17	1
5th fraction, 230–280 g./l.	0	43	48	9
Routine concentrate†	46	42	12	0

* Ratio of final units/g. to the initial units/g.[1]

† This concentrate was prepared by the large-scale process using the same mixed blood

Experimental

The fractionation of the solution of specific protein was carried out by adding successive amounts of solid ammonium sulphate to the filtrate obtained after the removal of non-specific protein from whole blood. This filtrate contains approximately 90 g./l. of ammonium sulphate, and since the Trikresol concentration is high, it was diluted with an equal volume of water before fractionating. The level of ammonium sulphate was therefore reduced to 45 g./l., and it was necessary to add a further 140 g./l. of solid ammonium sulphate before a sizable precipitate was obtained. This precipitate was filtered off, pressed lightly between sheets of blotting paper, and dialysed against running tap-water for 48 hours to remove ammonium sulphate. A second fraction was obtained by adding a further 30 g./l. of ammonium sulphate to the filtrate from fraction 1 and treating as above. Three more fractions were similarly obtained, and each fraction was analysed electrophoretically, and for antitoxins and protein. The values obtained for the five fractions are shown in Table I. In this Table the ratios for all the possible pairs of antitoxins in each fraction are also shown.

Several striking observations can be made on the results in Table I:

(*a*) The small first fraction containing a very high percentage of γ-globulin has an extremely low purity (lower than that of the original plasma in the case of β- and ε-antitoxins).

(*b*) The first two fractions, comprising approximately 50% of the total protein of the original filtrate, have very high γ-globulin content and low purity.

(*c*) The remaining three fractions have high β-globulin content and high purity, and the purity appears to parallel the β-globulin content.

(*d*) The ratios of antitoxins arising from similar types of immunization (i.e. β/ε and α/δ) remain almost constant, whereas the ratios of those arising from dissimilar types of immunization (i.e. β/α, β/δ, ε/α and ε/δ) show very steep rises, indicating that the antibodies produced by a lower level of immunization are concentrated in the lower fractions, which have a high γ-globulin content.

(*e*) The ratios for the original plasma and routine concentrate are almost identical.

Discussion

The observations appear to fit the theory advanced in Part I, that antibodies are formed in the blood initially as γ-globulin which, as immunization proceeds, breaks down with an increase in antibody groupings to form β-globulin of higher purity. The lower purity of fraction 5 is probably due to the high α-globulin content, since no evidence exists which links antibodies with this globulin.

Further experiments, on the lines of the one reported, carried out on blood taken from horses at various stages of their course of immunization, should shed further light on the development of antibodies during immunization. Ethanol fractionation of the plasma from the same bleedings would probably give sharper separation into the constituent globulins, but cannot be reliably carried out without elaborate equipment.

It is of considerable importance therapeutically to determine whether these fractions have any qualitative differences in combating infection. With this in view, Mr. A. Thomson of these Laboratories is undertaking a series of experiments, using guinea-pigs infected with lamb dysentery by direct injection of cultures into the colon, to test the efficacy of each fraction.

Acknowledgment

The author is indebted to Dr. E. Bidwell, who carried out the electrophoretic analyses, and to Dr. Oakley and his staff for carrying out the immunological tests.

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Received 8 July, 1952

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THE BACTERIOSTATIC EFFECTS OF EXTRACTS OF CHICKEN DUODENUM ON CERTAIN MICRO-ORGANISMS. I.—Crude Extracts

By J. BARRETT and N. J. BERRIDGE

Aqueous extracts of the contents of the duodenum of chickens have been found on many occasions to be bacteriostatic. On some occasions such extracts were stimulatory. Stimulatory extracts were rendered inhibitory by removing the substances precipitated by alcohol. These and other properties suggest that the bacteriostatic activity was at least partly due to the presence of fatty acids. The inhibitory substances were present in the gut of the chicken before hatching.

The discovery that the growth of young animals may be accelerated by feeding antibiotics has increased interest in the microbiology of the animal gut. Observations on substances in the gut which stimulate or inhibit bacterial growth may assist in the eventual understanding of the complex ecology of this peculiar environment.

During attempts to measure the penicillin concentration in the gut of chickens an antibiotic activity was discovered in control birds which had not received penicillin. This has already been briefly reported.[1] Examples of the results obtained are given in Table I, where the natural antibiotic activity is expressed as apparent penicillin, according to a turbidimetric assay. Experiments with diffusion assays using penicillin cups showed phenomena which varied according to the organ taken ; inhibition, both partial and complete, as well as different degrees of stimulation with concentric zones of different density, were observed. It seems likely, therefore, that there is a variety of microbiologically active substances in the gut. The inhibitory effect of contents from the upper intestine was so marked, however, that it seemed worthy of the separate investigation described here.

Table I

The bacteriostatic activity of the gut contents of chickens

Organ	Units of inhibitory material/g. of contents (wet weight)		
	Experimental diet (40 μg. procaine penicillin/g.)	Control diet (no penicillin)	
		a	*b*
Crop	2·7	1·1	0
Gizzard	1·4	0·4	0
Upper intestine	Above range	Above range	27
Lower intestine	4·5	0·2	0·4
Caecum	0·4	0·4	—

Methods

Antibiotic assay

Serial dilutions of the extracts of intestinal contents in distilled water were incubated for 90 minutes with four volumes of a diluted, actively growing culture of *Streptococcus agalactiae* and the resulting turbidities were measured. These were compared graphically with tubes to which partially inhibiting concentrations of procaine penicillin had been added, and the resulting values were expressed in units such that, under the conditions of assay, one unit of the inhibitory material from chickens was equivalent to 1 μg. of procaine penicillin. Though the responses of the test organism to penicillin and to the extracts were not identical, penicillin was considered the best standard then available. This method was almost identical with that described by Berridge & Barrett[2] but, because of the small samples at first available, the volume of test solution was reduced to 5 ml., and optically denser suspensions were therefore necessary. Partly as a result of this and partly because *Strep. agalactiae* responded differently to this antibiotic, an incubation time of 90 rather than 30 minutes was preferred.

Preparation of extracts

Several methods have been used ; all have given qualitatively similar results. In most cases the birds were dissected within 2–3 hours of being killed. Except for the first few experiments in which a larger portion was taken, that part of the intestine extending from the gizzard to the entry of the bile ducts, which portion we have called the duodenum, was dissected from each carcass and the contents were removed at once. Alternatively the intestines were frozen

at — 16° until required. In some cases the contents were washed out with 5 ml. of distilled water per duodenum; in others they were extruded by squeezing and were then thoroughly mixed with 2–3 volumes of distilled water. From all the mixtures a milky liquid was obtained by centrifuging and this was the crude extract which was assayed for inhibitory activity. In the first experiments the centrifuging was done at 15,000 r.p.m. to remove micro-organisms, and the liquid remained opalescent and active. Later, high-speed centrifuging was found to be unnecessary. The contaminating organisms were relatively few and did not interfere with the assay because of the short incubation-period. In some experiments a buffer of 0·05N-potassium phosphates at pH 6·8 was used to prepare the extracts. When preparations from large numbers of birds were attempted, extracts were made by stirring the coarsely minced intestines with distilled water, straining through gauze and centrifuging. In the later experiments considerable increases in yield were obtained by extracting the debris from the centrifuging with boiling ethanol. Some of these results are illustrated in Table II. Finally, equally good yields were secured by omitting the centrifuging, adding four volumes of ethanol, boiling for 30 minutes under reflux, cooling at 5° overnight and filtering.

Table II

Bacteriostatic power of alcoholic extracts of chicken duodenal contents

Number and age of chicks	Total yield, units × F *	
	Aqueous extract	Subsequent alcoholic extract
3, removed from eggs just before hatching	163	864
3, 1-day-old, never fed	192	690
20, 4–6 weeks old	48,000	640,000

* Value of factor F not known owing to error in standard; values therefore comparable only among themselves

Results

Assays on extracts

Fig. 1 shows a comparison on a dry-weight basis between penicillin and an extract of intestinal contents. It is clear that under these conditions the crude mixture of substances dissolved in the extract had roughly 10^{-4} times the activity of procaine penicillin.

In Table III extracts from birds on special diets are compared, and the high dilution at which the extracts are still effective may be noted. Neither the huge doses of cyanocobalamin nor the supplement of ' fish solubles ' had any remarkable effect on the inhibitory power of the extract. Extracts from birds on a ' synthetic ' diet were also inhibitory. They provided less than half the activity obtained from control birds, but the experiment was on a small scale and the difference may not be significant.

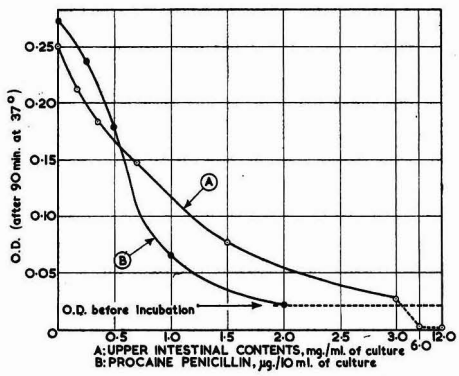

FIG. 1.—*Comparison of the effects of procaine penicillin and the bacteriostatic substances of the intestinal contents on the growth rate of* Strep. agalactiae

A Upper intestinal contents, mg./ml. of culture
B Procaine penicillin, µg./10 ml. of culture
O.D. (optical density) = log (I_0/I)

Table IV shows the effect of the age of the birds on the yield of inhibitory substances. They were present even in embryo birds just before hatching, in day-old chicks which had not received any food (see also Table II) and in one adult bird examined. Here, however, the concentration was low.

Some of the birds used had been reared on diets containing penicillin. It must therefore be emphasized that inhibitory extracts were obtained whether the birds had received penicillin or not, and that the inhibition due to penicillin in the gut contents of birds which had received it was insignificant at the dilutions used to assay the indigenous substance. This was proved by determining the penicillin concentration by the usual cup-plate assay, in which the indigenous substance does not interfere because of its much lower rate of diffusion.

The results so far given were obtained between September, 1951, and March, 1952.

Table III

Optical densities × 100 of assay cultures containing inhibitory extracts from chickens on different diets

Diet *	*a*	*b*	*c*	*d*	*e*	*f* †
Dilution of extract present in culture						
1 : 80	0	0	0	0	0	2
1 : 320	7	6	8	5	8	10
1 : 1280	19	17	21	20	18	22
1 : 2560	21	21	19	21	21	—
Growth control (no extract)			25			

* Diet *a*: All-vegetable basal diet + animal-protein factor
b: *a* + 4 mg. cyanocobalamin/100 g.
c: *a* + 10 mg. ,, ,,
d: *a* + 20 mg. cyanocobalamin/100 g.
e: *a* + fish solubles
f: National Baby Chick meal

† These are results from a different experiment

Table IV

Effect of age of bird on yield of inhibitory substance

No. of birds used	Age, days	Mean weight, g.	Yield,* units/bird
12	1	30	27
60	21	150	166
100	21–28	200	213
14	42–49	600	268
1	> 365	4000	83

* By coarse mincing and aqueous extraction only

After April, 1952, the previous results could no longer be repeated, in fact the test organism was now stimulated. Between 1 and 21 May three groups of 60, 60 and 90 laboratory birds respectively, gave aqueous extracts which were stimulatory. On 30 June 11 birds were taken from eggs just about to hatch. Aqueous extracts from these were slightly stimulatory. No change in the diet or breed of chickens had been made, neither was there any change in the test organism, for a culture freshly obtained from the freeze-dried stock behaved in the same way as the original. The only change coincident with this one was some slackening of the response of the chickens themselves to diets containing penicillin.[3] However, when the stimulatory extracts obtained at this period were treated with 80% ethanol, as described at the end of Methods (see above), the alcohol extracts showed the usual inhibitory activity. The possibility of seasonal variation remains to be examined.

Various experiments

It was at first considered possible that a colicine or some other antibiotic of bacterial origin could be present. A simple examination of the flora of the upper intestine revealed relatively few micro-organisms, so few that the contents had to be plated directly without previous dilution. Micrococci, staphylococci, a few spore-bearing rods, Gram-negative rods, and lactobacilli were found. The lactobacilli were most numerous. None of the cultures isolated produced an inhibitory substance *in vitro*.

Some of the results in Tables II and IV show that the inhibitory activity is not derived directly from ingested food. To supplement this evidence a bird was deprived of food for 24 hours immediately before being killed. The expected yield of inhibitory material was obtained. This was 60 units in the expressed duodenal contents, and a further 146 units were extracted from the mucosa removed by scraping.

In order to discover whether bile was responsible for the inhibitory activity of intestinal contents, bile taken from the gall bladder of chickens was assayed. Its inhibitory power was insignificant at dilutions greater than 1 in 160 and it was thus much less than that of intestinal contents. Moreover, contents taken solely from above the entry of the bile duct were subsequently found to be strongly inhibitory.

The duodenal secretion of pigs is known to contain lysozyme.[4] *Strep. agalactiae*, however, was slightly stimulated by crystalline lysozyme (supplied by Armour Laboratories, London) at a concentration of 250 μg./ml.; furthermore no lysis could be observed when the chicken intestinal extract was added to a suspension of *Micrococcus lysodeikticus*.

Catalase had no effect on the inhibitory activity of the extract.

Certain intact proteins, when added to the assay medium, prevented the inhibition. If

they were added to the inhibited culture, growth was eventually resumed. This was observed with bovine plasma-albumin (0·1%) (Fraction V, supplied by Armour Laboratories) (heated at 50° for 10 minutes and centrifuged), horse serum [2% (v/v)] and chicken serum [2% (v/v)]. This prevention of inhibition did not occur with corn-steep liquor from which the coagulable protein had been removed, but which remained rich in growth factors.

A few micro-organisms were examined for sensitivity with the results shown in Table V.

Table V

Diffusion zones produced by the inhibitory extracts with various test-organisms. Cylinder-plate assay. Diffusion at 5–8° for 48 hours followed by incubation

Organism	Diameter of clear zone, mm.
Streptococcus agalactiae	16
Mycobacterium phlei	16
Mycobacterium smegmatis	16
Staphylococcus aureus	10
Bacillus subtilis	10
Bacterium coli	No zones

Properties of the substance

Extracts retained their activity at room temperature and at pH values from 1–4 for at least 24 hours, and at pH 10 for over a week. Between pH 6 and 9 loss was caused by the growth of insensitive micro-organisms. On shaking with chloroform, 75% of the activity was destroyed, the remainder being found in the gelatinous precipitate. On attempting extraction with light petroleum at pH 9 the activity was destroyed, although it had been stable in the absence of the organic solvent. No loss in inhibitory power occurred on heating an extract for various times up to two hours at 60°, but as a precipitate, presumably of coagulated protein, appeared later in increasing amounts, an increasing proportion of the activity disappeared from the solution.

The rate of diffusion of the inhibitory substance was slow. Thus with diffusion assays using the penicillin-cup method with immediate incubation, the zone diameter was equal to that of the cup. If, however, diffusion was allowed to proceed at 5° for 48 hours before incubating, zones of a convenient size were produced. This was a possible alternative method of assay. The low rate of diffusion was confirmed in other experiments, when extracts were dialysed for short periods in cellulose-film tubing without significant loss.

The precipitate which appeared when the aqueous extracts were adjusted to pH 3 removed most of the inhibitory activity, as did also the precipitate obtained on saturation with ammonium sulphate. The smaller quantities of precipitate produced by half saturation with ammonium sulphate or full saturation with sodium chloride removed only a portion of the activity.

Discussion

The results show that for a certain period all the chicks examined contained a bacteriostatic substance in the lumen of their duodenum, and that this was not derived immediately from the food nor from bacterial action. It seems most likely that the substance was a product of the epithelium. Indeed it was found that epithelium removed by scraping duodena after slitting contained twice as much inhibitory material as the contents previously removed by expression. Whether the inhibitory material was liberated by actual secretion, by autolysis or by damage to the epithelium during the extraction it is impossible to decide from the present evidence, for the epithelium is very easily detached, and the cells themselves may be fragile. The phosphate buffer solutions would be less damaging than distilled water, and as they gave the same results it seems unlikely that the inhibitory substances were liberated from burst cells. However, experiments with isotonic solutions were not carried out until the later period and then the aqueous washings of all kinds were found to be stimulatory. Intact cells were observed in deposits obtained by centrifuging these washings, and the centrifuged solutions could be made inhibitory by treatment with ethanol. Since no cells were present, the ethanol was not a means of extracting inhibitory substances from the cells. Several of the other experiments show that the inhibitory substance is very readily adsorbed by some proteins and it follows that if, as seems possible, the inhibitory substance in the alcoholic extracts is the same as that observed in the aqueous extracts of the first period, the function of the alcohol is merely to release the inhibitory substance from proteins. The failure of the aqueous extracts of the second period to inhibit would then appear to be due to a preponderance of adsorbing proteins. This behaviour is reminiscent of that of the lipids which cannot be completely removed from proteins until the mixture has been treated with hot alcohol. In this and many other ways the inhibitory

material behaves like a fatty acid (see, for example, Pollock,[5] Kodicek[6] and Laser[7, 8]). Later results on concentrates and purified material have confirmed the belief that fatty acids are at least partly responsible for the phenomena. It is hoped to include these results in another communication.

Acknowledgment

The authors wish to thank Dr. M. E. Coates and Dr. J. W. G. Porter for their assistance in obtaining chickens.

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Received 31 October, 1952

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MANUFACTURE OF ALGAL CHEMICALS. VI.—Laboratory-scale Isolation of L-Fucose from Brown Marine Algae

By W. A. P. BLACK, W. J. CORNHILL, E. T. DEWAR and F. N. WOODWARD

Methods for the isolation of crystalline L-fucose from both dried milled brown seaweed and fucoidin have been worked out on the laboratory scale, with a view to the ultimate development of a process suitable for large-scale production. Preparation from the original seaweed involves hydrolysis with acid, formation of fucose phenylhydrazone, and subsequent decomposition of the phenylhydrazone. The isolation from fucoidin involves hydrolysis of the polysaccharide by heating a 16% (w/w) solution in 0·25N-hydrochloric acid at 135° in an autoclave for two hours, removal of salts and acid with ion-exchange resins, purification with ethanol and charcoal, and direct crystallization of the fucose from ethanol.

Introduction

L-Fucose (L-galactomethylose, 6-deoxy-L-galactose) was first obtained in crystalline form by Günther & Tollens,[1] who isolated it from the products of hydrolysis of *Fucus* species through the intermediate phenylhydrazone. It was obtained in microscopic needles which had the composition $C_6H_{12}O_5$.

Widstoe & Tollens[2] isolated L-fucose from several samples of gum tragacanth; the white varieties on hydrolysis yielded fucose and arabinose, and brown tragacanth gave fucose and xylose. The existence of L-fucose in tragacanth was confirmed by James & Smith,[3] who isolated 2 : 3 : 4-trimethyl-α-methyl-L-fucoside from the hydrolysis products of methylated tragacanthic acid.

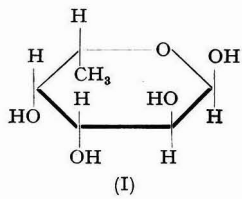
L-Fucose has been shown to be present in the specific blood-group substances. This was first demonstrated by Bray, Henry & Stacey[4] in their methylation studies on the group-A specific substance from commercial pepsin. They showed that the polysaccharide part of this glyco-polypeptide was composed of D-galactose, D-mannose, D-acetylglucosamine and L-fucose, the residues of acetylglucosamine and fucose being present as terminal groups in a highly branched structure. The existence of fucose in the blood-group substances has since been confirmed by a number of workers, such as Bendich, Kabat & Bezer[5] and Partridge.[6] Aminoff, Morgan & Watkins[7] have shown that the carbohydrate part, which makes up about 80% of the whole, of the human blood-group A substance from ovarian-cyst fluid contains one L-fucose, one D-galactose and two hexosamine (acetyl-D-glucosamine and acetyl-D-chondrosamine) residues, there being about 280 such units in each molecule. The polypeptide part, constituting about 20% of the whole, contained at least 11 amino-acids. Recently, Watkins & Morgan[8] have found that α-methyl-L-fucopyranoside, and to a lesser extent L-fucose, when mixed with the H-agglutinin in eel serum, neutralize completely the power of the antibody to agglutinate group O cells. This is the first instance of the inhibition by a simple sugar of a specific antigen–antibody reaction, where both components occur naturally.

Frog-spawn mucin also contains L-fucose.[9] The polysaccharide part (42% of the whole) of this mucin contains L-fucose, D-mannose, D-glucose, D-xylose, D-galactose, D glucosamine and D-chondrosamine, and the peptide portion contains some 18 amino-acids.

Vasseur,[10] and Vasseur & Immers,[11] showed that the jelly substance from certain sea-urchin eggs was composed of a polyfucose sulphate in combination with protein. The polysaccharide had high ash (over 40%) and sulphate (up to 32%) contents, and was considered to be esterified by one sulphate group per monosaccharide residue. Some idea of the structure of the polysaccharide has been obtained by oxidation with periodate.[12] This carbohydrate obviously shows some resemblance to fucoidin.

The presence of L-fucose has also been reported in the gum from *Acacia sieberiana*, a Belgian Congo tree,[13] and in the cell-wall of the flax fibre.[14]

L-Fucose crystallizes from water in fine microscopic needles (m.p. 145°), which mutarotate in aqueous solution from $[\alpha]_D^{20}$ — 152·6° (initial reading) to — 75·9°.[15, 16] The crystalline form is therefore α-L-fucose (I, α-L-fucopyranose).



(I)

Fucose is present in the brown seaweeds as the cell-wall constituent fucoidin, which is believed to be essentially a polyfucose monosulphate,[17, 18] e.g. $(C_6H_9O_3SO_4Ca_{0.5})_n$. Hydrolysis of fucoidin with acid, followed by the removal of excess acid and inorganic salts, should yield an aqueous solution of fucose, from which the sugar should crystallize directly on evaporation. The isolation of fucose in this way has been investigated in this paper. The production of fucoidin from brown marine algae has been investigated by Black, Dewar & Woodward.[19]

Because of the early difficulties in obtaining quantities of fucoidin, previous workers[15, 20, 21] have used the seaweed itself as a source of fucose. The method employed is essentially that described originally by Tollens and co-workers,[1, 2] whereby the seaweed (*Ascophyllum nodosum*) is treated first with cold dilute hydrochloric acid, and then boiled with dilute sulphuric acid to effect hydrolysis. After separation of the weed residue, excess acid is removed either with barium or calcium carbonate, the filtrate evaporated to small volume, impurities precipitated by addition of methanol and ether, and the fucose isolated as the crystalline phenylhydrazone. The hydrazone is readily decomposed with benzaldehyde, and the free sugar crystallized from absolute ethanol. Bates and associates[15] fermented the filtrate, after neutralization with calcium carbonate, with baker's yeast acclimatized to ferment galactose, which was supposed to remove the mannose and galactose present in the solution. This treatment, however, would appear to be unnecessary, as galactose has not been identified in quantity in the products of hydrolysis of the Phaeophyceae, and only one reference appears in the literature to the presence of mannose in the brown seaweeds.[22]

Hockett, Phelps & Hudson[21] quote a yield of 39–60 g. of crystalline fucose from 1000 g. of anhydrous, hydrochloric acid-treated *A. nodosum*. On the assumption that the original untreated weed had a fucose content of 7%, and that the preliminary acid wash has removed 33% of the original weed,[23] this yield works out at 37–57% of the total fucose present. Clark[20] obtained 38–40 g. of crystalline fucose from 1000 g. of acid-treated *A. nodosum*, and Bates and associates[15] quote a yield of 30 g. from 1000 g. of water-washed *A. nodosum*.

Experimental; discussion of results

Preparation of L-*fucose from dried milled* Pelvetia canaliculata

The weed was collected at Atlantic Bridge, Seil Island, Argyllshire, in March, 1949, dried on a rack at 25–30° for 48 hours, and ground in a Christy & Norris mill fitted with a $\frac{1}{64}$-in. mesh screen. Fucose was estimated in the weed colorimetrically,[24] and the other constituents by the methods previously employed by one of the authors.[25] The analysis of the weed is shown in Table I.

The milled weed (1018 g.) was hydrolysed with 4% (w/v) sulphuric acid (8 l.) at 100° for three hours, the hydrolysate was worked up, and the crystalline fucose prepared as described

Table I

Percentage chemical composition (dry basis) of Pelvetia canaliculata

Fucose (as $C_6H_{12}O_5$)	Ash	Mannitol	Laminarin	Organic nitrogen
11·2	24·1	8·5	2·48	1·55

by Hockett, Phelps & Hudson.[21] The preliminary hydrochloric acid washing of the weed was omitted because cold acid treatment is known to extract appreciable quantities of fucoidin, e.g. when this weed was stirred with 10 volumes of 0·16N-hydrochloric acid at pH 1·5 in the cold for six hours, 19·8% of the total fucose present was extracted.[19] On the other hand, preliminary cold acid treatment removes the bulk of the mineral matter, which is therefore eliminated before hydrolysis and does not contaminate the fucose phenylhydrazone precipitate. Again, cold acid extracts most of the mannitol in the weed, thereby rendering the concentrated hydrolysate less viscous, and the hydrazone is more readily filtered from the mother liquor. It is recommended, therefore, in spite of the loss of fucose, that the weed be washed initially with cold hydrochloric acid to remove the major portion of the ash and other soluble material.

In the experiment, 126·7 g. of fucose phenylhydrazone was obtained, but the precipitate was badly contaminated with mineral matter (ash, 24·7%). After decomposition of the hydrazone with benzaldehyde, the fucose crystallizing out still had a high ash content (ash, 20·6%). Most of the mineral matter was eventually removed by dissolving the fucose in hot absolute ethanol, and filtering off the salts. The filtrate, on evaporation *in vacuo*, gave a syrup (42·5 g.) which was taken up in hot absolute ethanol (43 ml.), and the solution was allowed to crystallize in the refrigerator at 5° for two days. The yield of crystalline fucose A, after filtration, washing with ethanol and drying *in vacuo* over phosphorus pentoxide, was 31·5 g. (Found: ash, 1·9%). The yield, based on the total weight of fucose in the weed, is 28%, which is considerably less than that obtained by Hockett, Phelps & Hudson.[21]

The sugar A (30·8 g.) was recrystallized by dissolving in water to give an approximately 80% solution, diluted with absolute ethanol (2 ml. for each gram of sugar), and placed in the refrigerator to crystallize.[15] The fucose B was filtered off, washed with absolute ethanol, and dried. The mother liquor was evaporated to dryness, the syrup dissolved in absolute ethanol (2 ml. per gram of syrup), allowed to crystallize in the refrigerator and the fucose C isolated. The analysis of the two fractions is recorded in Table II.

Table II

Recrystallization of fucose A

Recrystallized fucose	Wt. as % of fucose A	Ash, %	M.p., ° c.	Equilibrium $[\alpha]_D^{17}$ in water (*c.* 4·1)
Fucose B	.. 39·0	0·29	137–8	− 75·0°
Fucose C	.. 36·2	0·18		− 74·6°

Hockett, Phelps & Hudson[21] quote a 70% recovery for the recrystallization of fucose from absolute ethanol.

Preparation of L-*fucose from fucoidin*

The three samples of fucoidin used in these investigations were prepared by the method of Black, Dewar & Woodward,[19] and gave the analysis shown in Table III.

Table III

Analysis of fucoidin samples

Sample	Species extracted from:	Fucose (as $C_6H_{12}O_5$), %	Ash, %	$[\alpha]_D^{20}$ in water (*c*, 0·992; 2-dm. tube)
Fucoidin A	*Fucus vesiculosus*	39·9	35·6	− 118°
Fucoidin B	*F. vesiculosus*	34·9	36·3	
Fucoidin C	*P. canaliculata*	41·1	32·0	

Rate of hydrolysis of fucoidin with acids

The rate of liberation of L-fucose from fucoidin A was followed by estimating the reducing power in the hydrolysate with Shaffer & Somogyi's method.[26] The reducing power of fucose solutions, when estimated with Shaffer & Somogyi reagent 50, is linearly related to the fucose content,[27] although L-fucose has a much lower reducing power than D-glucose (1 mg. of fucose = 4·37 ml. of 0·005N-$Na_2S_2O_3$; 1 mg. of glucose = 9·02 ml. of 0·005N–$Na_2S_2O_3$).

The results are recorded in Table IV. In Expt. 1 the fucoidin A was heated under reflux on a boiling-water bath with the hydrochloric acid, 2-ml. portions of the hydrolysate were withdrawn at intervals and allowed to cool. Solution (1 ml.) was then transferred by pipette to a weighed beaker, the weighed solution diluted with water (20–30 ml.), neutralized with

0·1N-sodium hydroxide (phenol red as indicator), and made up to 100 ml. The reducing power of 5-ml. portions of this solution was then estimated with Shaffer & Somogyi reagent 50 against a 5-ml. water blank as described by Cameron, Ross & Percival.[28]

$$\% \text{ Conversion to fucose} = \frac{(\text{Titration diff.}) \times 100 \times (\text{total wt. of soln.}) \times 100 \times 100}{4{\cdot}37 \times 5 \times (\text{wt. of 1 ml. of soln.}) \times 39{\cdot}9 \times 1000}$$

In Expt. 2, the hydrolysis was carried out under the same conditions, but the rate of liberation of fucose was followed by estimating the acetaldehyde liberated on treatment with periodate. Hydrolysate (1 ml.) was weighed, made up to 10 ml. in a standard flask, and 5 ml. of this solution was neutralized with solid sodium bicarbonate and the fucose determined by the standard procedure.[24] In this way, the results obtained by the reducing power method can be calibrated against the true fucose figures. The results obtained by the reducing power method are invariably too high in the final stages of hydrolysis, due to the presence of small quantities of galactose, xylose and uronic acid in fucoidin,[17] which exert a reducing action on the copper reagent but which do not liberate acetaldehyde on treatment with periodate.

In Expts. 3 and 4 the fucoidin was dissolved in hydrochloric acid in a Lintner pressure bottle fitted with a rubber stopper, and the hydrolysis was carried out at 135° in a Pentecon autoclave. The reducing power was estimated as before.

The results in Table IV show that the liberation of fucose from fucoidin with 0·57N-hydrochloric acid at 100° is complete after two hours, whereas with 0·25N-hydrochloric acid at 135° hydrolysis is complete after one hour.

Table IV

Rate of hydrolysis of fucoidin A with acids

Expt. No.	Wt. of fucoidin A hydrolysed, g.	Acid used	Concn. of fucoidin, % (w/w)	Temp. of hydrolysis, °C.	% conversion to fucose after time (hr.) 0·25	0·50	1·0	1·5	2·0	3·0	5·0	7·5
1	1·962	0·57N-HCl, 20·41 g.	8·77	100		62·2	81·0		106·3	118·8	125·0	134·1
2	1·962	0·57N-HCl, 20·41 g.	8·77	100		70·5	90·1		93·5	92·5	86·4	89·4
3	3·296	0·05N-HCl, 17·20 g.	16·07	135	4·3	8·5	14·3					
4	3·292	0·25N-HCl, 17·14 g.	16·12	135	41·1	61·6	112·7	134·6	132·9			

The concentration of acid in the solution increases during hydrolysis owing to the splitting of the ethereal sulphate grouping, and this was readily detected in neutralizing with 0·1N-sodium hydroxide before estimating reducing power.

$$\left[\begin{matrix}C_6H_9O_3SO_4^- \\ C_6H_9O_3SO_4^-\end{matrix}\right]Ca^{2+} \xrightarrow{\text{complete hydrolysis } (4H_2O)} 2C_6H_{12}O_5 + H_2SO_4 + CaSO_4$$

In a concentrated solution, such as Expt. 4, the normality of sulphuric acid liberated is considerable and may often exceed that of the original hydrolysis acid. The slow rate of hydrolysis in Expt. 3 is probably due to the presence of some sodium alginate in fucoidin A, which on treatment with hydrochloric acid will give insoluble alginic acid, and hydrogen ions will be removed from solution. The hydrolysis after one hour was found to be almost neutral, indicating that insufficient hydrochloric acid had been added. In all these experiments, an insoluble residue was formed during hydrolysate. Percival & Ross[17] found 3·3% of uronic acid in the hydrolysis products of their highly purified fucoidin from *Himanthalia lorea*.

Preparation of L-*fucose from fucoidin by the phenylhydrazone procedure*

Fucoidin C (10·07 g.) was hydrolysed with 0·5N-sulphuric acid (400 ml.) at 100° for four hours, neutralized with barium carbonate, filtered, the barium salts were thoroughly washed with boiling water, and the filtrate and washings evaporated *in vacuo* at 50°. The yellow glass was dissolved in water (40 ml.), ethanol (400 ml.) was added, the brown sticky precipitate was centrifuged off, and the centrifugate evaporated to a syrupy glass (5·15 g.). The glass was dissolved in water (7 ml.) and ethanol (45 ml.), phenylhydrazine (5·5 g., i.e. 2·0 mol.) and glacial acetic acid (1·0 ml.) were added, and the solution was maintained at 5° for two days. The crystalline fucose phenylhydrazone was filtered off, washed with ethanol (2 × 10 ml.),

and dried *in vacuo* over phosphorus pentoxide to a yellowish-white solid (3·202 g.). Yield, 49·9%; m.p. 170–172°.

The phenylhydrazone (3·175 g.) was decomposed by suspending in water (65 ml.), benzaldehyde (1·68 g., i.e. 1·3 mol.) was added, and the mixture was heated at 90° for one hour with frequent stirring. After cooling to 5°, the benzaldehyde phenylhydrazone was filtered off, the filtrate extracted with chloroform (3 × 10 ml.) to remove benzoic acid and excess benzaldehyde, and evaporated *in vacuo* to a pale-yellow syrup (2·17 g.). This was dissolved in absolute ethanol (3 ml.), when the fucose began to crystallize almost immediately. After three days at 5°, the crystals were filtered, washed with ethanol (2 × 1 ml.) and dried *in vacuo* over phosphorus pentoxide to a white powder (1·824 g.). Details of the product are shown in Table V.

Table V

Crystalline L-*fucose from hydrolysed fucoidin C*

Yield from phenylhydrazone, %	Yield from fucoidin C, %	Ash, %	M.p., ° c.	Equilibrium $[\alpha]_D^{17}$ in water (*c*, 2·04)	Purity (calc. from rotation), %
89·0	44·4	3·7	131–3	− 73·0°	96·1

The percentage purity was calculated assuming the $[\alpha]_D^{20}$ of L-fucose at equilibrium to be − 76·0° in water.[21] The product was still slightly contaminated with mineral matter, and this is reflected in the low melting-point. From the results it is apparent that the most serious loss of yield is at the formation of the phenylhydrazone, where 50% of the fucose in the hydrolysate is lost. In a similar experiment using methylphenylhydrazine (3·3 mol. per mol. of fucose present), fucose methylphenylhydrazone was formed in 90·4% yield, which was increased to 96·8% when corrected for a 93·4% yield of the methylphenylhydrazone from pure fucose.[24]

Preparation of L-*fucose from fucoidin by direct crystallization*

Since in the phenylhydrazone procedure a serious loss occurs at the formation of the hydrazone, an attempt has been made to crystallize fucose directly from the hydrolysate after removal of salts and acid with ion-exchange resins. Zeo-Karb 225 and Amberlite 1R-4B-OH were found to be satisfactory as cation- and anion-exchanger respectively. Amberlite 1RA-400-OH, however, was found to absorb fucose almost quantitatively, and the solution after treatment contained little or no sugar. This strongly basic resin must react with fucose in some way, perhaps to form a *N*-glycoside, e.g.:

$$\underset{\text{Fucose}}{CH_3{\cdot}[CH{\cdot}OH]_4{\cdot}CHO} + \underset{\text{resin}}{R{\cdot}NH_2} \rightarrow \underset{N\text{-glycoside}}{CH_3{\cdot}[CH{\cdot}OH]_4{\cdot}CH{:}NR} + H_2O$$

In neutralizing fucoidin hydrolysates, care must be taken to add sufficient Amberlite 1R-4B-OH to deal not only with the acid used for hydrolysis but also with the sulphuric acid liberated from the ethereal sulphate group in fucoidin. A typical hydrolysis was carried out as follows:

Fucoidin C (5·030 g.) was hydrolysed with 0·5N-sulphuric acid (400 ml.) at 100° for four hours, and the solution centrifuged to remove a small brown precipitate. The colourless centrifugate was passed through a Zeo-Karb 225 column, the effluent neutralized by adding Amberlite 1R-4B-OH and stirring for about 30 minutes, and the resin filtered off and washed thoroughly with water. The filtrate and washings were evaporated *in vacuo* at 50° to a syrup, which was dissolved in water (20 ml.), ethanol (200 ml.) was added, and the light-brown precipitate removed at the centrifuge. Further impurities were removed by evaporating the centrifugate again to dryness, extracting the syrup with absolute ethanol (50 ml.), and centrifuging from the undissolved material. The alcohol was evaporated to give a yellow syrup which was dissolved in water (50 ml.), decolorized with charcoal (2 g.) at 100° for 30 minutes, filtered, and the filtrate evaporated *in vacuo* to dryness. The colourless syrup (2·32 g.) was dissolved in absolute ethanol (4 ml.) and the solution placed in the refrigerator, when crystals formed immediately. After two days at 5°, the crystalline fucose A was filtered, washed with ethanol (2 × 1 ml.), and dried to a white powder (1·170 g.).

The mother liquor was evaporated to a syrup, which was dissolved in water (25 ml.), decolorized with charcoal (1 g.) as before, and the resulting syrup (0·99 g.) crystallized from ethanol (1 ml.). After three days at 5°, crystalline fucose B was isolated (0·409 g.). The analysis of these two products is shown in Table VI.

The yield of fucose A (56·6%) is an improvement on the yield (44·4%) obtained by the phenylhydrazone procedure, and this method also gives considerable saving in time and

Table VI

Crystalline fucose fractions from hydrolysed fucoidin C

Fraction	Yield from fucoidin C, %	Ash, %	Equilibrium $[\alpha]_D^{17}$ in water (*c*, 2·02)	Purity (calc. from rotation), %
Fucose A	56·6	0·4	− 72·3°	95·1
Fucose B	19·8	0·2	− 63·1°	83·0

materials. The fucose B obtained from the mother liquor is rather impure, and would require further purification.

Recrystallization of L-*fucose*

Fucose can be readily purified by treatment with charcoal and recrystallizing from three volumes of ethanol.[21] L-Fucose (1·405 g.; equilibrium $[\alpha]_D^{17}$ in water, − 71·6°) was dissolved in water (25 ml.), the solution was heated with charcoal (1 g.) at 100° for 30 minutes, filtered, and the filtrate evaporated to dryness. The colourless syrup (1·327 g.) was dissolved in absolute ethanol (4 ml.), allowed to crystallize at 5° for three days, and the fucose isolated as a pure-white powder (1·139 g.). Yield, 81·1%; m.p., 135–137°; equilibrium $[\alpha]_D^{17}$ in water, − 76·1° (*c*, 2·05).

Large-scale preparation of L-*fucose from impure fucoidin*

In this large-scale experiment with impure fucoidin B, optimum conditions of hydrolysis were employed (Table IV; Expt. 4). Fucoidin B (91·7 g.) was dissolved in 0·25N-hydrochloric acid (478 ml.) to give a 16·1% (w/w) fucoidin solution, and the solution hydrolysed at 135° for 2 hours. The hydrolysate was centrifuged to remove a brown residue, cations were removed with Zeo-Karb 225 and the acids absorbed with Amberlite 1R-4B-OH, and impurities removed with alcohol treatment as described previously. The resulting brown syrup was dissolved in water (340 ml.), decolorized with charcoal (36 g.), and the filtrate evaporated *in vacuo* at 40° to dryness. The colourless syrup (33·3 g.), which was dried thoroughly *in vacuo* over phosphorus pentoxide, was dissolved in ethanol (30 ml.), seeded with crystalline L-fucose, and kept at 5° for four days with occasional stirring. The crystalline fucose A was filtered, washed with ethanol (3 × 20 ml.), and dried *in vacuo* over phosphorus pentoxide to a white solid.

The mother liquor on evaporation gave a glass (12·8 g.) which was dissolved in water (12 ml.), and the solution treated with ethanol (80 ml.), phenylhydrazine (17 ml.) and glacial acetic acid (2·5 ml.), and kept at 5° for one day. The fucose phenylhydrazone was isolated (6·50 g.), and this was decomposed with benzaldehyde as described previously to give crystalline fucose B as a white solid. The analysis of these two fractions is shown in Table VII.

Table VII

Crystalline fucose fractions from hydrolysed fucoidin B

Fraction	Dry wt., g.	Yield from fucoidin B, %	Ash, %	Equilibrium $[\alpha]_D^{17}$ in water	Purity (calc. from rotation), %
Fucose A	18·90	54·2	0·8	− 71·1° (*c*, 4·22)	93·6
Fucose B	2·85	·8·2	± 0·0	− 73·4° (*c*, 2·36)	96·6

The total yield of crystalline fucose therefore amounts to 62·4% of that present in fucoidin B. With larger quantities, the fucose would be crystallized from concentrated aqueous solutions.[15]

Summary

Crystalline L-fucose has been prepared in 28% yield from dried milled *P. canaliculata* by the method described by Hockett, Phelps & Hudson.[21] It is recommended that the seaweed be given a preliminary wash with cold dilute hydrochloric acid before hydrolysis to remove the major proportion of the inorganic salts and other soluble material which otherwise contaminate the fucose phenylhydrazone precipitate and render its separation a difficult problem.

The preparation of L-fucose from fucoidin has also been investigated. The complete hydrolysis of fucoidin is effected by heating a 16% (w/w) solution of the polysaccharide in 0·25N-hydrochloric acid at 135° in an autoclave for two hours. When the course of the hydrolysis is followed by estimating reducing power, the results indicate 'over-hydrolysis' in the final stages owing to the presence of small quantities of sugars other than L-fucose in the fucoidin molecule. Results obtained by the reducing power method must therefore be 'calibrated' against the true fucose figures obtained by estimating the acetaldehyde liberated on treatment with periodate.

Fucoidin, on hydrolysis, neutralization with barium carbonate and treatment with phenylhydrazine, gave fucose phenylhydrazone (yield 49·9%), from which crystalline fucose was obtained in 44·4% yield and 96·1% purity.

Fucose has been crystallized directly from fucoidin hydrolysates without the preparation of the intermediate phenylhydrazone. After hydrolysis, salts and acids are removed by ion-exchange resins, further impurities are eliminated by ethanol precipitation and treatment with charcoal, and the fucose crystallized from absolute ethanol. Fucoidin from *P. canaliculata* gave crystalline fucose in 56·6% yield and 95·1% purity; the yield, calculated on the weight of original weed, is 35·8%. The mother liquor gave a second crop of crystals in 19·8% yield and 83·0% purity.

In a hydrolysis involving 92 g. of impure fucoidin (fucose, 34·9%) from *F. vesiculosus*, crystalline fucose has been obtained in 54·2% yield and 93·6% purity by direct crystallization. The mother liquor gave a further 8·2% yield (purity, 96·6%) by the phenylhydrazone procedure, making a total yield of 62·4%.

Pure L-fucose can be obtained from the products described above by treating with charcoal and recrystallizing from three volumes of ethanol.

Acknowledgment

The work described in this paper forms part of the programme of research and development on seaweed undertaken by the Institute of Seaweed Research, and the authors are indebted to the Institute for permission to publish.

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Midlothian

Received 24 October, 1952

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THE REDUCTION OF NITRATE IN MIXTURES OF MINCED GRASS AND WATER *

By A. J. G. BARNETT

Nitrate is partially reduced in grass/water slurries to nitrite, which, in the presence of the lactic acid produced in simultaneous fermentation, is partially converted into nitric oxide. The initial reduction is brought about by strains of *Escherichia coli* (*Bact. coli*) and it has been further noted that ascorbic acid, normally undergoing 60% decomposition, is destroyed more rapidly and to a greater extent if nitrate is present in the fermenting mixture.

The disease known variously as 'oat-hay poisoning' or 'grass tetany' has attracted attention in different parts of the world, notably in America and in the Netherlands. The disease is characterized by the production of methaemoglobinuria in the animal and is much more common in cattle than in horses or sheep, although Winks, Sutherland & Salisbury[1] have shown that pigs are prone to the same condition. It is further known that the condition is due to the ingestion of nitrate which in itself is not toxic to the animal but that the nitrite which may result from it is extremely toxic.

Sjollema[2, 3] has shown that some Dutch pastures may contain as much as 2% of potassium nitrate on a dry-matter basis, and that oat straw may contain, on the same basis, as much as 7%.

The disease has been produced experimentally in cattle (Newson *et al.*,[4] Thorp,[5] by introducing potassium nitrate into the animals' diet, and Bradley *et al.*[6-9] and Davidson *et al.*[10] have shown that samples of oat hay, known to be initially toxic, lost this toxicity on removal of the original nitrate content with water. Seekles & Sjollema[11] have demonstrated, by recovery experiments, that some 10% of added nitrate is decomposed to nitrite in the animal intestine. It has been suggested by Howell[12] that the lower toxic limit of potassium nitrate in the animals' diet is 1·4% and that the condition of methaemoglobinuria induced by the ingestion of higher amounts may be relieved by injection of methylene blue, which reverses the reaction nitrate → nitrite.

The reduction of nitrate to nitrite has been brought about *in vitro* by Olson & Moxon[13] who, using red root (*Amaranthus retroflexus*) which had been moistened, demonstrated the formation of nitrite from initial nitrate and isolated as the active organism *Bacillus subtilis*, which indeed may well have been present under their experimental conditions.

Nitrate is the chief form in which nitrogen is taken into plants because ammonia or ammoniacal nitrogen in the soil are usually converted into this form by soil micro-organisms. Nitrate may accumulate in the plant as a result of many factors including, it is believed, a lack of the necessary light to produce sufficient carbohydrate to provide the energy required for the latter part of the change nitrate → nitrite → ammonia. Such conditions prevail during dull warm weather in early spring and it is suggested by Wilson[14] that one form of bloat is due to nitrate formed under these conditions. At the same time neither Sjollema[2, 3] nor Bradley, Beath & Eppson,[6] working on the disease in Wyoming, have been able to correlate either the incidence of the condition or the nitrate accumulation in the plant with soil, climatic or manuring effects.

The question of the formation of nitrite in silage arose in this Laboratory partly out of experiments which have already been described (Barnett[15]) and partly because no nitrite has ever been noted in samples of field silage examined here.

Apparatus

The apparatus described in a previous paper (Barnett[15]) was used in these experiments.

Method

Nitrite.—Nitrite was determined by the usual Griess–Ilosvay technique. Of the filtered effluent from the aspirator concerned, 1 ml. is diluted to 500 ml. or to 1000 ml., depending on the concentration of nitrite involved, and 50 ml. of the resultant solution is used for each test.

Nitrate.—5 g. of the dried-grass sample is boiled with 50 ml. of distilled water and to 5 ml. of the cooled filtrate are added, in the order given, 5 ml. of saturated silver sulphate solution, 5 ml. of saturated lead subacetate solution and 5 ml. of alumina cream. The mixture is shaken mechanically for 15 minutes and filtered. 5 ml. of the filtrate is evaporated, in a porcelain basin,

* Some of the results submitted were the subject of a communication read at the IInd International Congress of Biochemistry, Paris, 1952

to dryness on the water bath ; after cooling, 1 ml. of phenoldisulphonic acid reagent is added, the mixture being stirred almost continuously with a glass rod for 10 minutes. This procedure is similar to that described by Allport[16] for nitrate in meat products. The actual nitrate determination was made by the customary colorimetric technique due to Sprengel.[17]

Ascorbic acid.—Ascorbic acid was estimated by the 2 : 6-dichlorophenolindophenol method as modified by Bessey.[18] 20 ml. of the effluent is agitated in a blender with 50 ml. of the metaphosphoric acid–acetic acid solution and, after dilution to 100 ml., the filtrate is titrated against the indicator in the usual way, the results being compared with those derived by titration with a standard ascorbic acid solution.

Preparation of slurries.—1400 g. of fresh minced grass of known dry-matter content is initially mixed with a solution of 5 g. of potassium nitrate dissolved in 2 l. of distilled water. A similar amount of minced grass is mixed with 2 l. of distilled water and the two slurries are placed in aspirators set up as previously described. By passing in air or nitrogen according to the details of the earlier communication the conditions of fermentation may be altered. In the ascorbic acid experiments 0·8 g. of ascorbic acid was added to each of the slurries.

Media

(*a*) *Nitrate/peptone water :*—1% (w/v) of peptone with 0·4% (w/v) of sodium chloride and 0·5% (w/v) of potassium nitrate in distilled water was sterilized and tubed in the usual way after adjusting the pH to 7·0.

(*b*) *Nitrate/peptone/glucose water :*—This medium was prepared as (*a*) with the addition of 1% (w/v) of glucose.

Experimental

The formation of nitrite from nitrate

Three aspirators were set up in the manner already described, two charged with minced grass and nitrate, and one without nitrate as a control. One of the nitrate-containing mixtures was continuously aerated with sterile CO_2-free air and the other, along with the control, was kept under anaerobic conditions by passing in a steady stream of sterile O_2-free nitrogen. The results of nitrite determinations are indicated in Figs. 1 and 2. The actual amounts of nitrate converted into nitrite are about 26% in the aerobic mixture and 13% under anaerobic conditions. The disappearance of nitrite is signalized by the change in pH, the measurement of nitrite loss and the smell of N_2O_4 produced in the atmosphere near the exit tube from the slurry if that is momentarily opened.

It is possible to estimate the N_2O_4, produced *via* NO, from the aerated slurry by passing the emergent gases through a known volume of dilute sodium hydroxide solution and performing

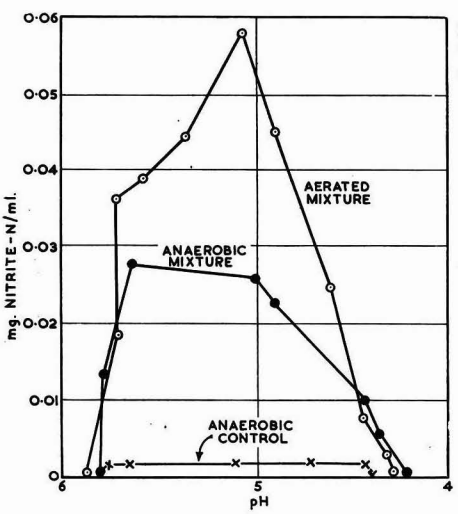

FIG. 1.—*The formation and decomposition of nitrite from nitrate under aerobic and anaerobic conditions in grass/water slurries plotted against pH change (temp., 18°)*

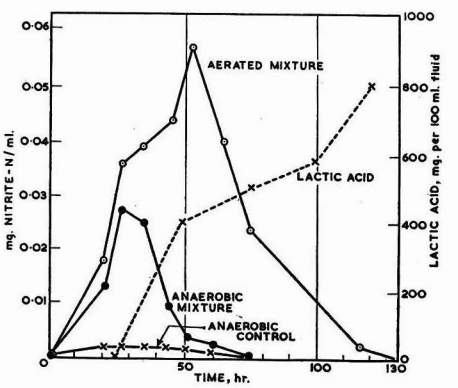

FIG. 2.—*The formation and decomposition of nitrite from nitrate under aerobic and anaerobic conditions in grass/water slurries plotted against time. The corresponding curve for lactic acid production is also shown (temp., 18°)*

nitrite determinations on the aliquot portions. On the basis that 3 mol. of HNO_2 give 1 mol. of nitrous acid, it is possible to calculate the recovery of NO as nitrite which was found to be about 70%. The difference between this and the expected theoretical is probably due to the formation of gases other than NO during the main reaction, which consists in the decomposition of nitrite by lactic acid simultaneously formed in the fermentation.

The loss of ascorbic acid in slurries containing added nitrate

Four aspirators (A, B, C, D) were set up in this experiment. Each contained the usual amounts of minced grass and water and to each was added 0·4 g. of ascorbic acid/l. of fluid. In addition the contents of aspirators A and B contained 5 g. of KNO_3. Aspirators A and C were aerated continuously while B and D were kept under anaerobic conditions. The disappearance of ascorbic acid was measured as described above for seven days and the results obtained are indicated in Table I. It is clear that under anaerobic conditions a 75% loss of ascorbic acid occurs in the mixture containing added nitrate while the loss in the control is 62%. Under conditions of aeration the loss of ascorbic acid in the nitrate mixture occurs much more rapidly and is of the order of 95%, the figure for the control being 75%.

Table I

Decomposition of ascorbic acid in anaerobic and aerated slurries containing added nitrate. The concentrations of ascorbic acid are expressed in g./l. of aspirator fluid

Time, days	Anaerobic slurries		Aerated slurries	
	+ KNO_3 (B)	Control (D)	+ KNO_3 (A)	Control (C)
0	0·40 g.	0·40 g.	0·40 g.	0·40 g.
1	0·40	0·40	0·40	0·40
2	0·35	0·35	0·02	0·12
3	0·20	0·30	0·02	0·12
4	0·15	0·25	0·02	0·12
5	0·10	0·20	0·02	0·10
6	0·10	0·15	0·02	0·10
7	0·10	0·15	0·02	0·10

The organisms responsible for the reduction of nitrate to nitrite

At peak nitrite-formation, that is after about two days, coliform organisms are present in the mixture in large numbers. After preliminary culture in glucose broth and MacConkey's agar, it was possible to isolate and type, using sugar, citrate and nitrate media, three coliform organisms. Two of these were of an intermediate type and one was of faecal origin. The actual naming of these organisms is of little importance in the present context but it is of importance to note that in all experiments of this type the same three organisms were invariably isolated although the grass-source varied. One of the intermediate types, when inoculated aerobically in glucose/nitrate/peptone water at 37°, gave only a small nitrite formation but the other organisms examined were extremely active, and at the end of seven days the pH of the medium had in no case fallen below 6·0. The two intermediate types grew well at 27° and the nitrite former was equally active at this temperature.

It is a matter of speculation as to why only three organisms could be isolated in this work, as many coliforms occur in silage; in this connexion we received seven strains (A1.1, A1.2, A2, A3, A4, B2 and B3) through the courtesy of Dr. T. Gibson of Edinburgh. All these had been isolated from silage and were of common occurrence in it. Six of these were strong reducers of nitrate in nitrate/peptone water both under aerobic and anaerobic conditions, whereas the seventh (A3) was inactive. Fig. 3 indicates the results obtained under aerobic conditions. The peculiarity of the curves for B3 and A4 which indicate a high initial formation of nitrite and partial subsequent decomposition are explained when the results in Fig. 4 are studied. These indicate the results of incubating the seven coliforms in nitrate/glucose/peptone water under aerobic conditions.

It will be seen that as well as A3, A1 and A2 are inactive, but A1.2 and B.2 form nitrite which is not further decomposed. A4, on the other hand, forms nitrite which is reduced to ammonia, whereas B3 forms nitrite which is decomposed because acid is formed after nitrite production. It would appear, therefore, that in media containing nitrate and carbohydrate some coliforms are not capable of performing the traditional reaction nitrate → nitrite. In this reaction, lactate or malate may act as hydrogen donators;[19, 20] Aubel[21] has shown that *E. coli* can reduce nitrate to ammonia in the presence of glucose as hydrogen donator. In our *in vivo* experiments, lactic acid reaches an 0·05M-concentration within 48 hours and malic acid and hexoses are present in grass. Again, *in vitro*, glucose is present in the peptone/glucose/

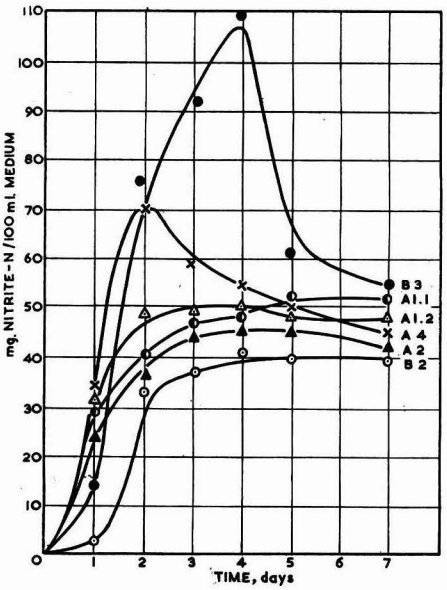

FIG. 3.—*The formation of nitrite in nitrate/peptone media by strains of* E. coli *under aerobic conditions. Strain A*3 *(see text) is inactive (temp.,* 37°)

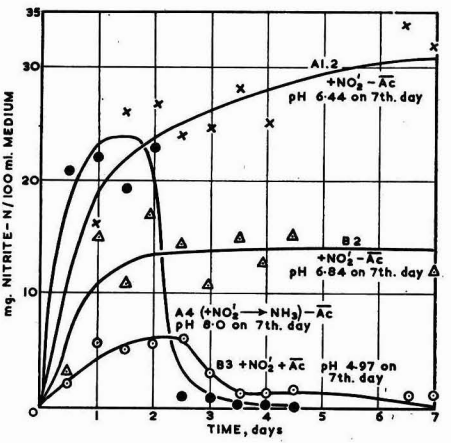

FIG. 4.—*The formation and decomposition of nitrite from nitrate in nitrate/peptone/glucose media by strains of* E. coli *under aerobic conditions. Strains A*3, *A*2 *and A*1.1 *(see text) are inactive (temp.,* 37°)

nitrate medium. It follows, therefore, that in all the experiments described above an abundance of hydrogen donators is present; this should enable the conversion of nitrate to ammonia to take place before the production of lactic acid is sufficiently great to decompose the intermediate nitrite. We have not been able to demonstrate in the experimental slurries that greater amounts of ammonia or simple bases are formed in those containing added nitrate than in the corresponding controls. That an ample supply of carbohydrate is necessary for whatever course the decomposition of nitrite is to take, however, can be readily seen if a simple aqueous extract of minced grass, containing added nitrate, is inoculated with an active strain of *E. coli*. In a corresponding control the small amount of nitrate initially present in the crop is reduced to nitrite which disappears, but in the nitrate-enriched extract only a small amount of the nitrate formed is decomposed.

Nitrate in fresh grass

As has been stated,[12] the lower toxic limit of nitrate concentration in the animal's diet may be supposed to be of the order of 1·4%.

Four plots of a standard grass mixture were each subjected, at different periods, to the same fertilizer treatment. In each plot one-third of the area was left untreated and the other two-thirds were given a dosage of Nitro-Chalk at the rates of $1\frac{2}{3}$ cwt. and $3\frac{1}{3}$ cwt. per acre respectively. At the selected time of cutting, the mixed crop from each sub-area was sampled and the nitrate analysis done in quadruplicate. The results obtained are shown in Table II and it will be seen that there is no correlation between (*a*) the different seasons of cutting, (*b*) the amount of Nitro-Chalk added, and (*c*) the intervals between spreading the fertilizer and harvesting. Lastly, the maximum nitrate-nitrogen concentration in the dry matter is equivalent only to about 0·15% KNO_3. Although no firm conclusions may be drawn from these experiments, it is suggested, as indeed is found in practice, that nitrate poisoning, in so far as fresh grass crops are concerned, is unlikely to be a serious menace in North-East Scotland.

Summary

1. The reduction of nitrate to nitrite has been shown to occur in laboratory silage as a result of the action of strains of *E. coli*. The nitrite formed is subsequently decomposed (partially at least to nitric oxide) by the lactic acid formed in the main course of fermentation.

2. Of seven strains of *E. coli* typically found in silage, only six reduced nitrate in nitrate/peptone water, both under aerobic and anaerobic conditions. Under similar conditions, but using nitrate/peptone/glucose water, only four of the strains were active; of these two produced

Table II

The concentration of nitrate in dried grass, the original crop having been treated with Nitro-Chalk at different periods

Plot	Nitro-Chalk applied	Crop harvested	Average NO_3-N content, %
1	9.5.51	10.6.51	Areas 1 0·021; 2 0·019; 3 0·021
2	23.6.51	10.7.51	Areas 1 0·021; 2 0·015; 3 0·026
3	23.6.51	5–10.8.51	Areas 1 0·022; 2 0·019; 3 0·020
4	15.8.51	7.9.51	Areas 1 0·017; 2 0·023; 3 0·021

Average of analyses of all areas	1 (no Nitro-Chalk)	0·020% NO_3-N
,, ,, ,, ,, ,, ,,	2 ($1\frac{2}{8}$ cwt. Nitro-Chalk/acre)	0·019% ,,
,, ,, ,, ,, ,, ,,	3 ($3\frac{1}{8}$ cwt. Nitro-Chalk/acre)	0·022% ,,

nitrite without further change, one reduced nitrate to nitrite which was further reduced to ammonia, and with the last, the nitrite formed was decomposed by the acid simultaneously formed.

3. The need for ascorbic acid in the diet of adult ruminants is not entirely established, but in good silage-making it is destroyed to about 60% of the original content. In the presence of nitrate, particularly under aerobic conditions where 98% of it is decomposed, the losses are considerably increased.

4. A systematic analysis of a number of crop samples obtained from grass plots subjected to different fertilizer treatments and harvested at different periods failed to yield substantial correlation between the factors of time of cutting, dosage of Nitro-Chalk and the interval between spread of fertilizer and cutting.

Acknowledgments

The author wishes to thank the Agricultural Research Council for a grant in aid of technical assistance, Dr. T. Gibson of the Edinburgh and East of Scotland College of Agriculture for bacterial cultures, and Mr. J. F. Young of the Department of Agriculture, University of Aberdeen, for grass samples.

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Received 19 September, 1952

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RATE OF EXTRACTION OF GROUND PYRETHRUM FLOWERS IN CONTACT WITH DILUTE MISCELLAE *

By M. G. EDWARDS

A very fine state of subdivision is necessary to obtain a high percentage extraction of pyrethrum flowers, but very fine powders present difficulties in orthodox apparatus, and a method of extracting material finer than 200 mesh is outlined. This method is assumed to give complete extraction and is utilized to assay the residues of pyrethrum flowers normally ground for industrial and for analytical purposes, which have been extracted in stages over a period of time, and an empirical equation is deduced enabling percentage extraction at any time to be calculated from constants determined for such powders.

Introduction

A very considerable amount of work has been done on the determination of the pyrethrin contents of pyrethrum flowers, but although all the methods of analysis evolved depend upon first extracting ground flowers with a solvent, very little is revealed in the literature concerning this preliminary process. For large-scale production, manufacturers of extracts have realized that as fine a powder as possible should be used, consistent with the operation of the plant, which, as a rule, precludes the use of any very fine powder. Here again, practically no information is available regarding actual methods and rates of extraction, perhaps because, in this case, they would constitute trade secrets.

The object of this investigation was to determine the rates of extraction of pyrethrum ground to a fineness suitable for industrial or for laboratory extraction. This means determining pyrethrin I and pyrethrin II in every case, as both these and the non-insecticidal substances are all extracted at different rates, and it is not possible to attack the problem by simply evaporating and weighing the dry material extracted. Consequently, the investigation is somewhat limited, owing to the time-consuming methods of analysis which are necessary.

All assays have been made by the Association of Official Agricultural Chemists' mercury-reduction method, with some minor modifications. The term ' pyrethrin ' is employed throughout to include pyrethrins and cinerins as determined by analysis.

Materials

The rates of extraction of pyrethrum powders of three different grades of fineness are considered, the mechanical analyses of which are given in Table I.

Table I

Mechanical analyses of pyrethrum powders

I.M.M. Sieve No.		Size of powder		
Passing	Retained	Coarse	Medium	Fine
—	5	2·3	—	—
5	10	14·9	—	—
10	20	51·6	3·7	—
20	30	13·5	24·0	0·9
30	40	9·1	22·6	7·8
40	60	4·8	40·6	48·2
60	80	2·7	3·7	21·4
80	120	1·0	3·1	9·8
120	—	0·1	2·3	11·9

The pyrethrum powders have been selected as follows:

Coarse.—This is a sample of flowers as used in a large-scale batch plant. In a plant of this type employing a large static mass of flowers, it is essential that the solvent has easy access and reasonably quick movement through the mass; consequently the flowers are ground to an optimum size consistent with this.

Medium.—These represent flowers ground in the disintegrator to a size suitable for extraction in the Soxhlet apparatus for analytical purposes, which is practically the minimum

* From part of a thesis ' Extraction of Pyrethrum Flowers ', 1951, for Ph.D. degree, London University

below which difficulties arise through fine particles compacting and preventing access of the solvent.

Fine.—These are so finely ground that it is possible that difficulty may arise owing to agglomeration of the fine particles restricting access of the solvent, and to avoid this a quantity of filter aid was admixed. It was hoped to get practically complete extraction. This grade was obtained by repassing the medium grade through the disintegrator fitted with a finer screen. For analytical purposes this method of grinding would present a concomitant difficulty in the possible losses of pyrethrins owing to decomposition caused by exposure of the fine particles to the air.

Complete extraction

It is shown below, however, that even this fine mesh powder is not completely extracted, and 24 hours' Soxhlet extraction yields 98·4% of the pyrethrin I and 96·9% of the pyrethrin II.

It is interesting to note that after this length of time the rate of extraction becomes so slow that it would take 96 hours and 2206 hours respectively to extract 99% of the insecticidal material, calculating from the extraction equation derived below.

It seems to have been generally assumed that if a pyrethrum powder, finely ground for analysis, has been extracted for a length of time and yields no appreciable pyrethrins on re-extraction, then it was completely extracted in the first place. This is now shown to be incorrect; for example, in the case mentioned above, an additional 24 hours' extraction would yield only 0·3% of the original pyrethrin I and 0·5% of the pyrethrin II, by calculation from the same equation, or amounts barely detectable by the normal assay.

Consequently an entirely different method of extraction was developed which obviates any losses during grinding, and is based on a method used by the author to extract undried flowers.[1]

The flowers are extremely finely ground to dimensions ranging from — 200 mesh (0·0063 cm.) down to very small particles which settle only very slowly in light petroleum. Briefly, the method is as follows: 10 or 12·5 g. of the flowers, according to their pyrethrin contents, roughly ground for sampling purposes, is introduced into the planetary-mill jar, 50 ml. of the solvent is added and the mixture ground for one hour. The mixture is then washed by decantation in the mill jar with several lots of 50 ml. of solvent, the extracts being passed through a filter to collect any fines, which are afterwards returned to the mill. After removing the bulk of the pyrethrins in this way, the residue is reground for a further six hours. The mill jar is then placed in a hot-air bath and fitted with a reflux condenser in a similar manner to that previously described,[1] and the contents refluxed for six hours, or alternatively the contents may be transferred to a flask for treatment. It has been pointed out[2] that additional non-insecticidal materials which assay as apparent pyrethrins are extracted by hot solvents, but the refluxing was carried out in all cases in this work for the purposes of comparison with extractions of undried flowers not reported here, and any such apparent pyrethrins were afterwards separated from the concentrated extracts by refrigeration overnight, followed by filtration with a filter aid. After standing overnight, the residue is washed by decantation with 50–ml. lots of solvent, as before, until no more coloured solution is obtained. The residue is in such a fine state of subdivision that some of the solid is bound to be carried over into the extract, and although this is too fine to be removed easily by filtration, it can afterwards be readily separated by filtration of the concentrated extract in conjunction with a filter aid.

In its final state the residue should all pass a 200-I.M.M. sieve; it is assumed that for practical purposes extraction is complete, as it has not been found possible to remove any pyrethrins from the extracted residue by any means.

Any type of porcelain ball-mill may be used, but the planetary type is particularly suitable, as it is quite light and uses only two balls, which can be removed and washed off readily; the mill jar can then be handled almost as easily as a beaker, with considerable advantage, as all operations are carried out while the bulk of the sample remains in the mill jar.

Experimental

At room temperature.—Extractions were carried out by stirring the ground flowers with the solvent in a cylindrical tinned-iron vessel with a conical base fitted with a tap. The flowers were enclosed in a 100-mesh brass-wire basket fitting closely inside the vessel, to prevent an undue proportion of the powder running through the tap.

The solvent used throughout was light petroleum, b.p. 55–65°, freed of aromatic hydrocarbons by treatment with sulphuric acid.

The general method adopted was to extract in several stages. The powder (500 g.) was

weighed into the extraction vessel and solvent was added to cover it, and the mixture was well stirred with a stout glass rod. In the first stage, after a few minutes, the solvent was run off through the tap in the base of the vessel *via* a large filter to catch the fines escaping through the wire-mesh basket into a 5-l. flask. This operation was repeated once more so that the bulk of the easily extractable pyrethrins was removed and the succeeding solution remained dilute. The fines were returned to the vessel from the filter at the end of each operation. After this preliminary operation the treatment was the same for each stage. About 10 minutes before the time interval, the miscella was run out and the vessel quickly refilled with solvent five or six times, so that practically pure solvent was running out at the hour. Thus at the end of each stage the whole of the dissolved pyrethrins had been washed out; no allowance had to be made for any pyrethrin-containing miscella in the vessel, as would be necessary if a continuous extraction was attempted. This was the main reason for extracting in stages in this manner.

This method was accurate for periods of two hours and upwards, but for shorter initial intervals, when more appreciable quantities of pyrethrins are dissolving during the final 10-minute period of washing, a different method had to be used. In this, 50-g. aliquots were extracted in a similar manner in the body of a Seitz filter, and the final washing period was reduced to about one minute, during which a large quantity of light petroleum was rapidly drawn through the mass.

At higher temperatures.—Near the boiling point of the solvent, the extraction was carried out on 50 g. of powder in a large Soxhlet extractor, the flask being changed at each time interval; immediately before changing the flask a large quantity of hot solvent had been poured into the extractor to wash out all the extract.

In all these methods the residue, after completion of the number of stages decided upon, was completely extracted by grinding in the planetary mill and treating as outlined above. All the extracts from each stage and the extracts of the residues were evaporated and assayed.

Results

The percentages of pyrethrins, R, remaining unextracted, plotted against the time, T, on double logarithmic paper are found to approximate to straight lines very closely in all cases, as shown in Fig. 1, for values at least down to $T = 24$ minutes. Below this the straight-line law must break down, as the curve must pass through $R = 100\%$ at $T = 0$.

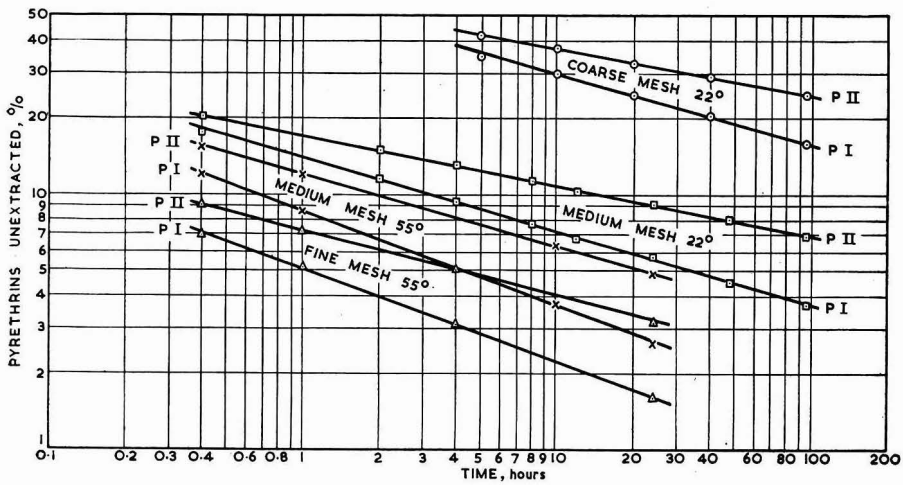

FIG. 1.—*Pyrethrins remaining unextracted in powders of different grades after varying periods of contact with dilute light-petroleum miscellae* (PI, *pyrethrin* I; PII, *pyrethrin* II)

It is possible to develop an equation for each case as follows: Taking medium-mesh flowers at room temperature, Table II shows the results for pyrethrin I obtained in this case. The figure at 24 minutes was obtained from a separate Seitz-filter extraction and the remainder from the multi-stage extraction of 500 g. of powder.

Table II

Percentage of pyrethrin I remaining unextracted (R) at each stage in the extraction of medium-mesh flowers at room temperature

T, hr.	% of pyrethrin I/100 g. of dry material			R	log R	log T
	In extract	Total extracted	% extracted			
0·4	0·7780	0·7780	82·40	17·60	1·2455	− 0·3979
2	0·8359	0·8359	88·53	11·47	1·0595	0·3010
4	0·0187	0·8546	90·51	9·49	0·9773	0·6021
8	0·0160	0·8706	92·21	7·79	0·8915	0·9031
12	0·0100	0·8806	93·26	6·74	0·8287	1·0792
24	0·0100	0·8906	94·32	5·68	0·7543	1·3802
48	0·0099	0·9005	95·37	4·63	0·6656	1·6812
96	0·0084	0·9089	96·26	3·74	0·5729	1·9823
Residue	0·0353					

The equation to the straight-line curve may be written:

$$n \log R + \log T = \log a \quad .. \quad .. \quad .. \quad .. \quad .. \quad (1)$$

where n and a are constants, or

$$R = a^{1/n}/\sqrt[n]{T} = C/\sqrt[n]{T}$$

where C is a constant $= a^{1/n}$.

From Table II log R and log T may be grouped as follows:

log R	log R	log T	log T
1·0595	0·8287	0·3010	1·0792
0·9773	0·7543	0·6021	1·3802
0·8915	0·6656	0·9031	1·6812
0·8287	0·5729	1·0792	1·9823
3·7570	2·8215	2·8854	6·1229

Applying equation (1)

$$3{\cdot}7570n + 2{\cdot}8854 = 4 \log a$$
$$2{\cdot}8215n + 6{\cdot}1229 = 4 \log a$$

Solving,

$$n = 3{\cdot}4607$$
$$\log a = 3{\cdot}9718$$

or

$$C = 14{\cdot}05$$

The extraction equation for pyrethrin I therefore becomes $R = 14{\cdot}05/\sqrt[3{\cdot}46]{T}$.

The degree of scatter of the points about the lines, as shown in Fig. 1, is small in every case, and consequently the method described above is sufficiently accurate for the purpose of fitting the lines. In other cases, however, the degree of scatter may be much greater, when it would be necessary to apply statistical methods to fit the line, that is, the line of linear regression. The statistic required to fix this line may be calculated from Table III.

Table III

Data for calculating regression equation

	$y(=\log T)$	$x(=\log R)$	$(x-\bar{x})$	$(x-\bar{x})^2$	$y(x-\bar{x})$
	0·3010	1·0595	+ 0·2381	0·05669	+ 0·07167
	0·6021	0·9773	+ 0·1559	0·02430	+ 0·09387
	0·9031	0·8915	+ 0·0701	0·00491	+ 0·06331
	1·0792	0·8287	+ 0·0073	0·00005	+ 0·00788
	1·3802	0·7543	− 0·0671	0·00450	− 0·09261
	1·6812	0·6656	− 0·1558	0·02427	− 0·26193
	1·9823	0·5729	− 0·2485	0·06175	− 0·49260
Sum ..	7·9291	5·7498	—	0·17647	− 0·61041
Mean ..	1·1327 ($\bar{y}$)	0·8214 ($\bar{x}$)			

$$b = \text{regression coefficient} = \frac{\Sigma y(x-\bar{x})}{\Sigma (x-\bar{x})^2} = -\frac{0{\cdot}61041}{0{\cdot}17647} = -3{\cdot}4590$$

The regression equation is:

$$y = \bar{y} + b(x - \bar{x})$$

or $\log T = 1{\cdot}1327 - 3{\cdot}4590(\log R - 0{\cdot}8214)$
or $3{\cdot}4590 \times \log R + \log T = 3{\cdot}9739$
i.e., $n = 3{\cdot}4590$
$\log a = 3{\cdot}9739$
$C = 14{\cdot}09$

Constants for other grades and for pyrethrin II calculated by the first method outlined above are given in Table IV.

Table IV

Extraction constants for different grades of flowers

Grade	Temp., ° c.	n (PI) *	n (PII) †	C (PI)	C (PII)
Fine	55	2·75	3·90	5·11	7·22
Medium	55	2·53	3·59	8·62	11·94
Medium	22	3·46	5·16	14·05	16·88
Coarse	22	3·60	5·47	55·90	56·43

* PI = pyrethrin I
† PII = pyrethrin II

The constants apply to all the cases where the miscella is dilute ; the maximum concentration in the experiments, excluding the initial part of the first stage, was of the order of 0·5 g. of total pyrethrins per litre.

In stronger concentrations, for example if the original solvent is left in contact with the powder for the whole time, the concentration would rise to about 5–10 g./l. and the rate of extraction would be slower. The same extraction equation appears to apply but the constants are larger.

The free chrysanthemumcarboxylic acids are included in all these determinations, but further experiment has shown that they may be much more slowly extracted than the pyrethrins themselves, although they appear to obey fairly closely the same general extraction-equation when the constant n for the monocarboxylic acid, for example, is nearly double what it is for pyrethrin I. Thus, the free acid in the later extracts continually increased from, for example, 2% of the pyrethrin I in the first two hours' extraction to over 30% in an extract made at 48 to 96 hours. There are, however, several unknown analytical factors that render it difficult to interpret results. Thus, the amount of monocarboxylic acid determined by the Seil method in the later stages is only about half that given by the mercury-reduction method and an even smaller fraction of that determined colorimetrically, though this does not apply to the initial extracts ; owing to this uncertainty, no results are recorded here.

Generally, the process taking place when pyrethrum powders are extracted with dilute light-petroleum solutions appears to consist in the very rapid solution of pyrethrins in the initial stages, the amount depending on the fineness of the grinding. A measure of the extent of this initial solution is given by the constant C, the percentage of pyrethrins left unextracted at one hour.

After this initial period, the rate of extraction becomes progressively slower, being inversely proportional to the nth root of T, where n varies with temperature but is not appreciably affected by the fineness of the powder. The rate of the extraction of pyrethrin II is found to be less than that of pyrethrin I.

The extraction equation should prove of particular value in analysis when only a short extraction, say four hours, would be necessary to evaluate a normally ground powder for which the constants are known. It will also be of use in industrial plant where dilute miscellae are used, and can be extended to include more concentrated miscellae by the determination of constants applicable to particular concentrations which can no longer be considered dilute.

Acknowledgments

The author is indebted to Mr. H. B. Stent, Director of East African Industrial Research, for permission to publish this paper, and to Mr. I. G. Edwards for correcting the proofs.

East African Industrial Research Board
Nairobi

Received 25 August, 1952

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TAINT IN POTATOES GROWN ON LAND TREATED WITH TECHNICAL γ-BENZENE HEXACHLORIDE OR PURE γ-BENZENE HEXACHLORIDE

By H. R. JAMESON and F. C. PEACOCK

It is shown that, on a γ-benzene hexachloride basis, pure γ-BHC and technical (90%) γ-BHC tainted potatoes to about the same degree when applied just before the crop was planted. On a total BHC basis, technical (90%) γ-BHC and a BHC concentrate containing 56% γ-BHC, when compared indirectly with the results of earlier and more numerous trials[1] showed about the same degree of taint as crude BHC (13% γ-BHC); the decrease in tainting with time after treatment for technical γ-BHC seemed to be rather more rapid than for crude BHC.

Introduction

Experiments in recent years have shown that where potatoes are to be grown on land previously treated with a crude BHC product, recommendations for the safe use of the latter must be strictly followed to ensure that the crop will not be tainted. Misuse of BHC has, however, produced tainted crops, and some recommendations now forbid the use of crude BHC on land intended for planting at any time with potatoes.

Experiments were begun in 1947 to see whether BHC with a high γ-BHC content could be used to reduce risk of taint, so as to provide a direct means of controlling wireworm on the potato crop.

Experimental

Field work

The chemicals were applied as dusts containing 0·2–2·0% γ-BHC, in the form of (*a*) 56% γ-BHC product made by partially purifying crude (13% γ-) BHC, (*b*) technical-grade (90%) γ-BHC or (*c*) the highly purified γ-isomer; these dusts were worked into the soil in the spring, after the application of 3–5 cwt. of potato fertilizer/acre and shortly before Arran Banner potatoes were planted. The plots were four yards long and three rows wide, and the treatments were replicated four times.

About five months after planting, the crops were lifted and a representative sample of 15–20 lb. was removed from each plot.

An initial trial, Expt. A, laid down in 1947 and cropped annually with potatoes until the 1949 crop was lifted, was designed to examine the taint potentials of BHC products containing approximately 56% and 90% γ-BHC (i.e. technical γ-BHC); these chemicals were used at rates ranging from 0 to 4 lb. of γ-BHC/acre. In two similar trials, Expt. B and C, started in 1948, the technical γ-BHC was compared at application rates ranging from 0 to 1 lb. of γ-BHC/acre with corresponding rates of the highly purified product. In the three trials which were laid down on medium loam soil at Jealott's Hill and at Plant Protection Ltd.'s experimental farm at Fernhurst, nearly 200 samples of potatoes were examined in about 6600 tastings.

Assessment of taint

The degree of taint in potato samples was assessed by the Hawthorndale technique described in a previous paper.[1] When samples from all replicates of a given treatment were negligibly tainted, lower application rates were not examined; when high taint was similarly recorded, higher application rates were not proceeded with.

For comparison purposes the taint indices y' and y'' for corresponding rates of technical γ-BHC and the product containing 56% γ-BHC used in the same trial have been inserted on the surface in Fig. 1. This surface is part of that derived from previous work with crude BHC[1]; it is obtained from the equation:

$$y = (0{\cdot}15 \pm 0{\cdot}06) + (0{\cdot}14 \pm 0{\cdot}02)x_1 - (0{\cdot}0033 \pm 0{\cdot}0005)x_1x_2$$

and employs the following notation

y = taint index (i.e. taint grading for treatment minus that for the corresponding untreated controls)

x_1 = lb. of crude BHC/acre.

x_2 = time in months elapsed between application of chemical and the lifting of the crop.

In Figs. 1 and 2, x_1 is entered appropriately with lb. of crude BHC, lb. of technical γ-BHC or lb. of 56% γ-BHC product/acre.

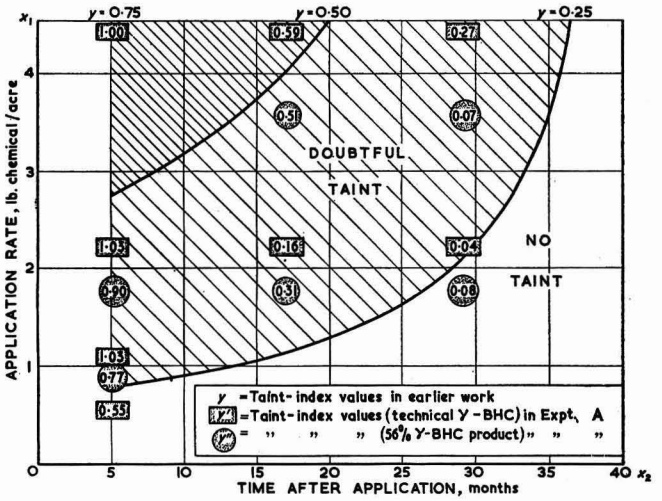

FIG. 1.—*Relationship between taint indices for γ-BHC products in the present work and those for crude BHC in earlier work*

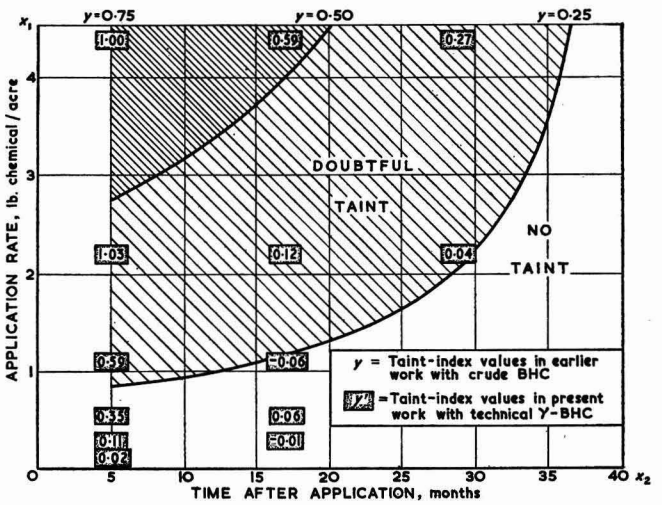

FIG. 2.—*Relationship between taint index, application rate and time after application for tainting potatoes by technical γ-BHC*

Comparison of the values for y' and y'' with those estimated for y at the points indicated showed that, weight for weight, the 56% γ-product and technical γ-BHC were no less taint-productive than crude BHC, and might even be more so, with potatoes lifted five months after soil treatment. To reduce taint risks while maintaining high insecticidal activity, products incorporating γ-BHC of high purity were needed to minimize the total BHC applied. In 1948

two trials were therefore begun to compare technical γ-BHC with a pure form of the isomer. The chemicals were applied at 2, 4, 8 and 16 oz. of γ-isomer/acre just before the potatoes were planted; in 1949 a second crop was taken from the same plots without further treatment. Statistical analysis of the results showed that there was no significant difference between the taint indices resulting from soil treatment with equal weights of γ-BHC in the form of the technical grade or the pure isomer.

In view of this finding the more numerous data for technical γ-BHC from all trials were pooled, and compared with data for crude BHC. The mean taint indices (y') obtained for the various rates (x_1) in trials A, B and C during the three years 1947–1949 are inserted in Fig. 2. Comparing the values for y' with the curves for y, it would seem that at 1–4 lb./acre technical γ-BHC taints the potato crop rather more than crude BHC in the first season after application; in the second and third years its taint potential falls off more rapidly. Statistical analysis, however, showed no consistent significant differences between the indices for technical γ-BHC and those derived from the equation for crude BHC. The results of these experiments indicate that on loam soils in this country up to about $\frac{1}{2}$ lb. of γ-BHC or technical γ-BHC can be applied per acre just before a potato crop is planted, without risk of subsequent taint complaint, and that similar freedom from taint may be expected with 2 lb./acre applied 12 months before planting a crop.

Acknowledgments

Acknowledgement is made to Mr. P. A. Collier and Mr. P. Roberts of Imperial Chemical Industries Ltd., Statistical Section, Jealott's Hill Research Station, who carried out the statistical analysis of the pooled data.

Imperial Chemical Industries Ltd.
Hawthorndale Laboratories
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Received 24 October, 1952

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THE EFFECT OF AGGREGATION ON DIFFUSION OF GASES AND VAPOURS THROUGH SOILS

By P. B. FLEGG

The effect of aggregate size upon the relationship between diffusion coefficient of gases through porous media and the porosity has been investigated. Over the range of aggregate sizes used, no differences were found. The presence of moisture in the soil was found to exert an influence directly related to the amount of water present, irrespective of the degree of aggregation of the soil. When the reduction in porosity due to the presence of water is calculated, the observations made fit the curve for air-dry soils with fairly good agreement.

The relationship between the diffusion of a gas through a porous medium, D, the diffusion coefficient of the gas through air, D_0, and the porosity of the medium, S, is now fairly well established. Buckingham[1] investigated the relationship experimentally and found that $D/D_0 = S^2$. His results were obtained with carbon dioxide, and show a great scatter. He concluded that the rate of diffusion was not greatly dependent upon soil texture and structure, but was determined mainly by the porosity of the soil. Efforts to repeat his work to obtain a more precise relationship have shown that if biologically active soils are used, reliable figures cannot be obtained because of the evolution of carbon dioxide.[2] Also, a method which does not incur the possibility of differences in pressure is preferable. Penman[3] used a method which avoided this difficulty. Using carbon disulphide and acetone vapour over a wide range of total porosities, he found that the relationship $D/D_0 = 0{\cdot}66S$ held for porosities up to 0·65. Soil porosities much higher than this are unlikely to be encountered in agricultural practice. He confirmed this relationship later with carbon dioxide.[4]

The materials used by Penman were generally in a relatively fine state of division, but when glass beads of diameter up to 3 mm. were used, rates of diffusion were higher than the general curve would suggest. Only three such determinations were recorded, and the increased rate of diffusion may have been due to particle shape and packing, and not particle size. Penman attributes the increased rate of diffusion to the comparatively large spaces at the edge of the container when such large particles are used. More recently Bavel,[5] using a similar but more accurate method, has found the ratio between D/D_0 and S to be of the order of 0·6, confirming the work of Penman. This value was obtained with alcohol and sand–soil mixtures. When sand–glass bead mixtures were used, a lower result was obtained. De Vries,[6] by applying a formula built up from the theory of electric conductivity, has shown that particle shape will alter the relationship. The experimental results obtained by Penman and Bavel with glass heads and mica do not agree with the relationship found for granular media. De Vries also shows that a soil with aggregates of spherical appearance will show a greater rate of diffusion than the same soil with no aggregation, the total porosity being the same in each case. Thus the effect of aggregate size upon the diffusion of gases through soils is uncertain. The following work represents an attempt to obtain data on this point, using soils in the air-dry state and when moist. It seemed possible that in a wet soil made up of small aggregates a continuous film of water joining each adjacent particle or aggregate might cause blockages and considerably reduce the rate of diffusion.

Experimental

The method used is, in principle, the same as that of Penman,[3] and is briefly described here.

The soil container was a 40-mesh sieve of diameter 10 cm. This was fitted tightly into a Petri-dish lid, and the joint sealed by a wide rubber band which was checked and renewed from time to time. The soil container was much larger than that used by Penman. This was to diminish errors due to packing when large soil aggregates were used.

The depth of material used was 2·5 cm., and the porosity was calculated from the weight, apparent volume of the material and its specific gravity. For soils, sand and gravel this was assumed to be 2·65.

Ether and acetone were used, and 15 ml. of the liquid chosen was placed in the Petri-dish lid. The sieve containing the soil was then fitted into the dish lid and the joint sealed. The rate of loss in weight due to diffusion of vapour through the soil was determined by weighing at approximately 20- to 30-minute intervals. The front of the balance was removed to allow the diffusing vapour to escape. No readings were used to calculate D until steady-state diffusion had been achieved, which took about one to two hours. Thus the effect of adsorption of vapour by the material was eliminated.

Corrections for the impedance of the sieve and holder were determined for ether and acetone. D_0 for acetone was taken as 0·095 ;[3] D_0 for ether was taken as 0·089.[7] The method of calculating the results was that used by Penman.[3]

In order to investigate the effect of aggregate size, three air-dry soils (of the texture of clay loam and silt loam), sand and gravel were used, and sieved into several fractions ranging from 25–12·5 mm. in diameter down to less than 2 mm.

The effect of the presence of moisture in materials of different aggregate size was also investigated. The sieve was filled with soil and the total porosity and diffusion coefficient were determined as described above. Water was then carefully applied to the top of the soil until it was well soaked. It was then left overnight for the water to soak in evenly. The reduction in porosity due to the presence of water and the actual percentage of moisture on a dry-soil basis were calculated from the weight of water present. A diffusion experiment using ether was then carried out, making allowance for the loss in weight during the duration of the experiment due to the evaporation of the water. Several such determinations were made as the soil dried out, the porosity and moisture content being calculated each time.

The Yaxley clay loam, in a coarse and fine state, and sand were used in this series of experiments.

Results

The temperature fluctuations during each experiment were greater than in Penman's experiments, and this, together with errors in calculating S, are probably the main reasons for the scatter of the results.

Table I shows the range of aggregate sizes used and the experimental results. Small and large aggregates of each soil, and in some instances mixtures of small and large aggregates,

were used. The results have been arbitrarily grouped into those from soils with small aggregates (< 2 mm. in diameter), and those from soils with larger aggregates (> 2 mm.). When these are plotted together with previous work (Fig. 1), it can be seen that, despite the spread of the results, there is no apparent difference due to aggregate size. Generally the results are lower than those found by Penman and are more in accordance with those of Bavel. It is difficult to decide whether this is an exaggeration of the dip in Penman's curve found between the values of S from 0·45 and 0·60, or not. If the values obtained from soils are ignored in Penman's results this dip does not occur. De Vries[6] has indicated from theoretical considerations that the occurrence of blocked pores in the soil would produce such a lowering of the value of D/D_0. Of the materials used, soil is the most likely to exhibit discontinuous pore spaces.

Table I

Soil types and sizes of aggregates used

Aggregate size, diameter in mm.	S	D/D_0	Aggregate size, diameter in mm.	S	D/D_0
Yaxley clay loam subsoil			Rhayader clay loam		
25–6	0·55	0·35	25–12·5	0·71	0·44
25–6	0·65	0·37	25–12·5	0·71	0·42
< 25	0·55	0·35	25–6	0·75	0·67
< 25	0·65	0·38	< 2	0·62	0·33
< 6	0·61	0·38	< 2	0·60	0·35
< 6	0·64	0·36			
< 6	0·59	0·32	Sand and gravel		
< 2	0·52	0·26	< 1	0·37	0·26
< 2	0·48	0·30	< 1	0·35	0·23
			3–1	0·36	0·23
Wisbech silt loam			3–1	0·36	0·21
25–12·5	0·69	0·49			
25–12·5	0·66	0·42	Vermiculite		
12·5–2	0·66	0·51	< 5	0·99	0·57
< 2	0·69	0·47	< 5	0·99	0·59

Some results were also recorded for vermiculite, but the points obtained fell well below the Penman line. This was also found by Penman when using mica. It is not to be expected that such materials with a plate-like structure will behave in the same way as more granular materials. Although falling below the relationship found by Penman, these points lie on the extrapolation of the Bavel relationship. The Bavel line is, however, based on a small number of accurate measurements between porosities 0·35 to 0·45, and not over the complete range of porosity, as is Penman's line. It cannot be assumed, therefore, that the points found for vermiculite have any bearing on the relationship found by Bavel.

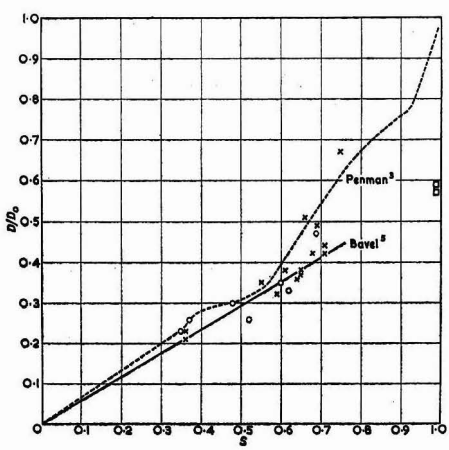

FIG. 1.—*Results obtained from soils of different aggregate sizes compared with previous work*

× Soils containing aggregates > 2 mm.
○ " " " < 2 mm.
□ Vermiculite " < 5 mm.

The effect of the presence of water in the soil is shown in Fig. 2. The calculated porosities (S) of the soil when air dry, and at its wettest, are shown. With the exception of the Yaxley soil (< 6 mm. aggregates), the relationship between D and moisture content is linear within the limits of experimental error. The deviations in the Yaxley soil smaller than 6 mm. were undoubtedly due to the soil shrinking away from the edge of the container and cracking as it dried out (Fig. 2*b*). This allowed a free passage, about 1–2 mm. wide, for the ether vapour around the edge of the sieve. Two separate experiments were carried out with this soil to confirm this. Differences in porosity and moisture content of the soil in these two experiments are due to packing. In Expt. 1 the porosity of the air-dry soil was 0·55, whereas in Expt. 2 it was found to be 0·59.

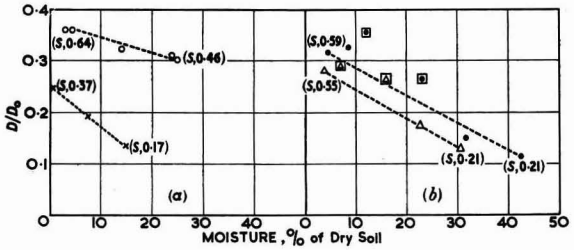

FIG. 2.—*The lowering effect on D/D_0 of the presence of moisture*

○ Yaxley soil, 19–6 mm.
× Sand < 2 mm.
△ Yaxley soil < 6 mm., Expt. 1
● ,, ,, ,, ,, ,, 2
□ Cracks developed in soil while drying out

In Expt. 2 with this soil (Fig. 2*b*), the cracked soil was removed from the sieve, broken up gently and repacked into the sieve, and D was measured. The difference between this point ($D/D_0 = 0{\cdot}326$, water 8·6%) and the point obtained with the air-dry soil at the beginning of the experiment ($D/D_0 = 0{\cdot}318$, water 4·5%) can be attributed to experimental error. When the results shown in Fig. 2 are compared with Fig. 1 the agreement is quite reasonable. This is shown in Fig. 3, where the points affected by soil cracking are omitted. Some errors are to be expected from the method of calculating the porosity of the wet soil, as any swelling which may have occurred when it was wetted was not accounted for.

Conclusions

1. Using ether and acetone the diffusion coefficient D of gases through soils has been determined over the range of soil porosities to be expected in agricultural practice, and a relationship between D and S was obtained which is in good agreement with previous work (Penman and Bavel).

2. No differences due to aggregate size over the range smaller than 2 mm. to 12·5–25 mm. in diameter were found.

3. The effect of wetting soils of three aggregate-size ranges has been investigated, and the reduction in D was related directly to the moisture present, irrespective of aggregate size, except where the original soil packing was disturbed by relatively large cracks in the soil caused by shrinkage during drying.

4. When the reduction in soil porosity caused by the presence of water is calculated, the results obtained from wet soils agree fairly well with the curve obtained for air-dry soils. Thus over the range covered by the experiment, the reduction in D due to the water in the soil was approximately proportional to the reduction in porosity due to the volume of water present.

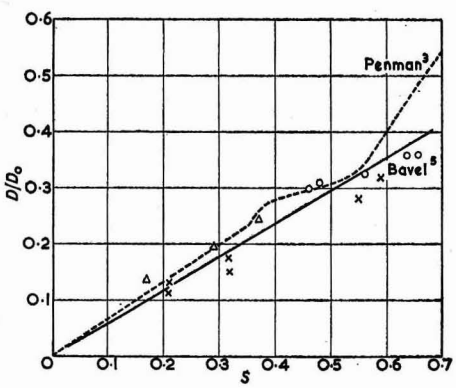

FIG. 3.—*Comparison of the results from wetted soils (Fig. 2) with the D/D_0–S relationship found for dry soils (Fig. 1). Points affected by soil cracking are omitted. Porosity, S, calculated from the moisture content of the soil*

○ Yaxley soil, 19–6 mm.
△ Sand < 2 mm.
× Yaxley soil < 6 mm.

Acknowledgments

The author wishes to thank Dr. R. L. Edwards for his help and valuable suggestions,

Miss I. Warman for help in carrying out the diffusion experiments, and the Directors of the Mushroom Research Association for permission to publish this paper.

Mushroom Research Station
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Peterborough

Received 30 September, 1952

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SOUTH AFRICAN TOBACCOS. I.—Preliminary Identification of Amino-acids and other Constituents

By H. L. PEARSE and L. NOVELLIE

Methods of preparing tobacco-leaf extracts for paper chromatography are described. Preliminary work has been carried out on the free amino-acids of three varieties of tobacco grown in South Africa, and normally used for flue curing. The following amino-acids have been detected in tobacco leaf: aspartic acid, asparagine, glutamic acid, glutamine, serine, threonine, alanine, tyrosine, proline, valine, γ-aminobutyric acid, leucine, *iso*leucine, phenylalanine, tryptophan, β-alanine, histidine and lysine. Several substances, as yet unidentified, which give a ninhydrin reaction are also present. The paper-chromatographic method also appears promising for the study of sugars and polyphenols in tobacco leaf.

The ultimate quality of tobacco doubtless depends to a very considerable extent on the chemical composition of the leaf. There have been numerous studies of this,[1–8] and some progress has been made in defining tobacco quality in terms of quantitative amounts and ratios of various groups of compounds, e.g. carbohydrates, polyphenols, alkaloids and proteins, but very little work has yet been done to identify and estimate the individual constituents of each class. Such studies may well lead to a much clearer understanding of the relationship between composition and quality than has been possible so far.

Paper partition-chromatography appears to be a particularly suitable method for making a preliminary survey of the composition of tobacco leaf, and also for studying the changes which take place during growth and during the process of curing. Roberts & Wood[9] have published some details of chromatograms obtained using the extracted juice of cured samples of Pennsylvania Seed Filler tobacco leaf, and also the juice of fresh and cured leaf of an unnamed Indian variety. These authors give a composite map of the amino-acids and polyphenols detected by paper chromatography, using the solvents phenol and butanol/acetic acid.

In this paper, preliminary investigations of the preparation of tobacco leaf extracts for paper chromatography, and some observations of the amino-acid composition of tobacco leaf are reported.

Material

Three varieties of tobacco were used, (1) 219 (a cross between Amarelo and Piet Retief Swazie), (2) Amarelo and (3) Yellow Mammoth. The Amarelo varieties are high yielding, but the flue-cured leaf has a characteristic pungent aroma which is not favoured on the world's tobacco markets. Yellow Mammoth is an Orinoco variety and is one of the widely grown flue-cured types. Samples of bottom leaf, middle and top leaf were obtained from tobacco grown on noritic-turf soil at the Central Tobacco Research Station, Rustenburg. Extracts for chromatography were prepared from the green leaf as received (i.e. approximately 12 hours after harvesting), and from leaf which was coloured by keeping it at 90° F. and at approximately 80% R.H. in a controlled cabinet for 1–2 days (colouring is the first stage of tobacco curing, where the normal green colour changes to a bright yellow). Parallel samples at each stage were dried rapidly in a forced-draught oven at 75° F.

Methods of extraction and preparation

1. As a starting point the method used by Roberts & Wood[9] was tried. The fresh green or coloured leaf was first minced in a domestic meat mincer to form a brei. This was then squeezed by hand through two thicknesses of cloth of the madapollam type; the resulting juice was boiled for about five minutes, cooled and centrifuged for 20 minutes. The resultant clear-brown liquid was preserved by adding a few crystals of thymol and keeping it in a refrigerator.

Attempts to use this juice directly for paper chromatography failed; interference by salts and other compounds caused such bad streaking on the chromatograms, that very few distinct spots could be detected. Juice prepared by adding water to ground, dried leaf gave similar results.

2. *Purification with ethanol*

Sufficient absolute ethanol was added to 20 ml. of juice to bring the concentration of ethanol to about 80%. With the juice from fresh, green leaf-samples, a voluminous precipitate of water-soluble protein occurred; this was much less evident with the juice from the coloured leaves. Removal of this precipitate by filtration or centrifugation gave a clear liquid which was concentrated *in vacuo* to approximately 5 ml. From 10 to 30 μl. of this concentrate was used for two-dimensional chromatograms. Results were somewhat better than those obtained with the untreated juice, but bad streaking still occurred, and the results were far from satisfactory.

3. *Purification by Neuberg's methods*

Both Neuberg's mercuric acetate method[10, 11] and his copper method of purifying amino-acids[12] were tried. The original extract (10 ml.) was used, and after purification concentrated to 2 ml. Fairly good chromatograms were obtained, but the technique was too tedious for dealing with large numbers of samples. There also appeared to be a danger of losing at least a portion of the amino-acids.

These methods were therefore abandoned in favour of the more convenient ones described below.

4. *Purification using ion-exchange resins*

(*a*) *Zeo-Karb* 215.—The resin was conditioned using the method of Partridge & Westall.[13] Juice (10 ml.) was diluted with distilled water to 250 ml. and then run slowly on a resin column (1 cm. × 20 cm.). The effluent from the column was only slightly less coloured than the original diluted juice, but gave a negative ninhydrin test, showing that the amino-acids had been satisfactorily held on the column. After washing until the effluent was quite colourless, the column was eluted with 0·2N-ammonia solution. The fractions containing amino-acids were collected, bulked and evaporated to approximately 2 ml.

This technique was used throughout for obtaining purified amino-acid extracts for paper chromatography. Preparations obtained by this method invariably gave exceptionally clear and well-defined chromatograms. The main disadvantage of the method is the loss of arginine,[14] which is not removed from the resin by ammonia.

(*b*) *Dowex* 50.—Juice (10 ml.) was diluted as before and run on a Dowex 50 column. The amino-acids were then eluted with a saturated solution of barium hydroxide; the usual precautions were taken to prevent carbonate formation on the resin. The fractions containing amino-acids were combined, and the barium precipitated with 2N-sulphuric acid, care being taken to avoid excess acid. Although numerous tests have not been carried out, there appeared to be considerable loss of glutamine and glutamic acid when this method was used.

For the same resin, the amino-acids were also driven off with 0·2N-ammonia solution. This method appeared to be very satisfactory, as both arginine and lysine were recovered, although it has not yet been determined whether the recovery is quantitative.

5. *Extraction of amino-acids and sugars from the dried material*

The technique used followed that of Laidlaw & Reid.[15] Finely powdered tobacco leaf (5 g.) was extracted with 80% ethanol. After removal of the ethanol, the extract was made up to 200 ml. with distilled water, and clarified with cadmium sulphate and barium hydroxide. The solution was then passed through a Dowex 50 column (1 × 20 cm.) and then through a column of Amberlite IR4B resin prepared with 0·2N-ammonia solution. The eluent, which was almost colourless, was concentrated to 5 ml. and used for chromatographic separation of the sugars. Amino-acids were recovered from the Dowex resin with 0·2N-ammonia solution.

Paper-chromatographic methods

Amino-acids.—For comparative experiments approximately 15 μl. of the concentrated solution from the resin was used for two-dimensional chromatograms. These were run first in phenol/water [70 : 30 (w/w)] ascending for 48 hours, and then in *n*-butanol/acetic acid/water (40 : 10 : 23 by vol.) for 18 hours, both at 30° c. After drying, the papers were sprayed with ninhydrin (0·2% in water-saturated butanol), followed by drying for 5 minutes at 130° c. Fig. 1 shows the average positions taken up on the chromatograms by the amino-acids and other ninhydrin-reactive substances in the extracts.

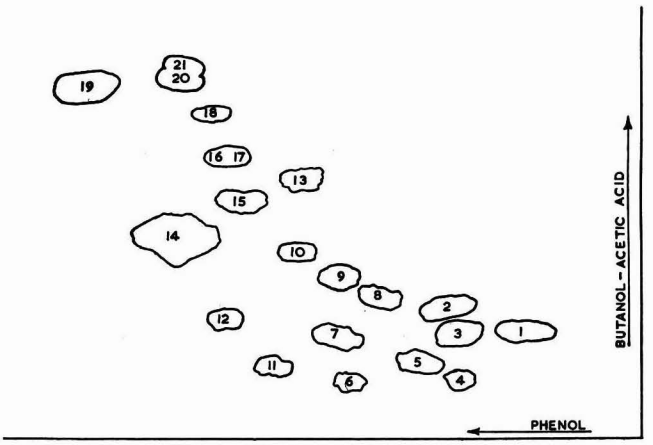

FIG. 1.—*Map of amino-acids obtained from tobacco extracts on two-dimensional chromatograms in phenol and butanol/acetic acid*

1. Aspartic acid
2. Glutamic acid
3. Serine
4. Spot 4[9]
5. Asparagine
6. Lysine
7. Glutamine
8. Threonine
9. Alanine
10. β-Alanine
11. Histidine
12. Spot 12[9]
13. Tyrosine
14. Proline
15. γ-Aminobutyric acid
16. Valine
17. Methionine
18. Tryptophan
19. Spot 19[9]
20. Phenylalanine
21. Leucines

The leucines, methionine and phenylalanine were sought in some of the extracts by running one-dimensional chromatograms in methyl ethyl ketone/pyridine/water (70 : 13 : 17 by vol.) for 24 hours at 30° c. This solvent mixture gave fairly good separation and better definition of phenylalanine than the original 70 : 15 : 15 mixture described by Wieland & Bauer.[16]

Sugars.—The sugar extracts have so far only been run in phenol and *n*-butanol/acetic acid. Other solvents would probably be more suitable.[17]

For detection of the sugars, the aniline phthalate spray of Partridge,[18] the β-naphthylamine spray described by Novellie[19] and the *p*-anisidine phosphate spray of Mukherjee & Srivastava[20] were used.

The methods of drying and spraying were similar to those described by these authors.

Polyphenols.—The methods were similar to those of Roberts & Wood.[9]

Results

Amino-acids in tobacco leaf

The results obtained for free amino-acids in the various extracts are shown in Tables I, II and III. Strictly quantitative results were not aimed at in this preliminary work, and only a rough indication of the relative intensities of the various amino-acids present is possible. Methods of extraction and of preparation of the solutions for chromatography were, however, kept as uniform as possible.

The outstanding features of the chromatograms were:

1. The following free amino-acids have been detected, although not all are present in all the extracts: aspartic acid, asparagine, glutamic acid, glutamine, serine, threonine, alanine, tyrosine, proline, γ-aminobutyric acid, valine, phenylalanine, leucine, tryptophan, β-alanine,

Table I

Yellow Mammoth : free amino-acids in leaf extracts

	Bottom leaves		Middle leaves		Top leaves	
	Green	Coloured	Green	Coloured	Green	Coloured
Aspartic acid	+++	++	+++	+++	++++	++
Asparagine	+	+++	+	+++	++	++++
Serine	++	+++	+++	+++	++++	++
Glutamic acid	+	++	+++	+++	++	++
Glutamine	+	+++	+	+++	++	++++
Threonine	++	++	++	++	++	++
Alanine	++	+++	++	++	++	++
Tyrosine	+	+	+	++	+	++
Proline	+	+++++	+	+++++	++	+++++
γ-Aminobutyric acid	+++	+++	+++	+++	++++	++++
Valine	++	++	++	++	++	++
Phenylalanine	+	++	+	+++	+	+++
Leucines	++	+	++	+	++	+
Tryptophan				+	+	++
β-Alanine				+	+	+
Histidine				+		++
Lysine		+	+	+	+	+
Spot 19		+	+	+	++	+++
Spot 12	+	+	+	+	+	+
Spot 4			+	+	+	+

Table II

219 : free amino-acids in leaf extracts

	Bottom leaves		Middle leaves		Top leaves	
	Green	Coloured	Green	Coloured	Green	Coloured
Aspartic acid	+++	++	+++	+++	++++	++
Asparagine	+	++++	++	++++	++	++++
Serine	+++	+++	+++	+++	+++	+++
Glutamic acid	+	++	+++	+++	++	++
Glutamine	+	++++	++	+++	++	+++
Threonine	++	++	++	++	++	++
Alanine	++	++	++	++	++	++
Tyrosine	+	++	+	++	++	++
Proline	+	+++++	++	+++++	+++	+++++
γ-Aminobutyric acid	+++	+++	+++	+++	++++	++++
Valine	++	++	++	++	++	++
Phenylalanine	+	++	+	+++	+	+++
Leucines	++	+	++	+	++	+
Tryptophan		+		+	+	++
β-Alanine		+		+	+	+
Histidine		+		+		++
Lysine		+	+	+	+	+
Spot 19	+	+	+	+	+	+
Spot 12	+	+	+	+	+	+
Spot 4			+	+	+	+

Table III

Amarelo : free amino-acids in leaf extracts

	Middle leaves			Middle leaves	
	Green	Coloured		Green	Coloured
Aspartic acid	+++	+++	Valine	++	++
Asparagine	++	++++	Phenylalanine	+	+++
Serine	+++	+++	Leucines	++	+
Glutamic acid	+++	+++	Tryptophan		++
Glutamine	++	+++	β-Alanine		+
Threonine	++	++	Histidine		++
Alanine	++	++	Lysine		+
Tyrosine	+	++	Spot 19	++	++
Proline	++	+++++	Spot 12	+	+
γ-Aminobutyric acid	+++	+++	Spot 4	+	+

histidine and lysine. Arginine was detected in juice hydrolysates, and several unidentified ninhydrin-positive substances were present in some of the extracts.

Roberts & Wood[9] did not find histidine and β-alanine in the varieties examined by them. Spot 11 of these authors may possibly have been histidine from its position. The presence

of histidine in the present extracts was confirmed by carrying out the Pauly test on the chromatogram.[21, 22] Spots 10 and 13 of Roberts & Wood were not found. Spot 19 was very evident in most samples, and numbers 4 and 12 were also noted.

2. In the green leaf the most abundant amino-acids were usually aspartic acid, serine, glutamic acid and γ-aminobutyric acid.

3. In general, the quantity of the free amino-acids increased from the bottom to the top leaves.

4. The leaf samples of the 219 and Amarelo varieties contained more free amino-acids than the samples of the Yellow Mammoth variety.

5. Greater quantities of free amino-acids were invariably present in the juice after the leaves had been coloured.

6. In the coloured leaf of all samples proline was invariably by far the most abundant amino-acid. Asparagine, glutamine and phenylalanine were all present in considerably greater quantities than in the freshly harvested green leaf.

Sugars

Four sugars have been so far detected in dried-leaf extracts: sucrose, glucose, fructose and an unidentified sugar running faster in phenol than either of the other three, and giving a brown colour with the *p*-anisidine phosphate spray.

Polyphenols

In chromatograms of the unprocessed juice, spots giving a yellow reaction with ammonia were observed, and all the spots mapped by Roberts & Wood,[9] with the exception of spot A, have been noted. We confirm these authors' identification of spot F as chlorogenic acid. The preparation of extracts for the special study of polyphenols will be the subject of future work, since it appears from the work of Koenig & Dörr[23] that these substances are of considerable importance in determining the quality and aroma of tobaccos.

Acknowledgments

One of the authors (H. L. P.) wishes to thank the Director of the National Chemical Research Laboratory for his interest and encouragement in the project, and for permission to work in the National Chemical Research Laboratories.

This paper is published with the approval of the Secretary for Agriculture, and the Council for Scientific and Industrial Research.

Central Tobacco Research Station
Rustenburg, Transvaal

and

National Chemical Research Laboratory
South African Council for Scientific and Industrial Research
Pretoria

Received 19 August, 1952

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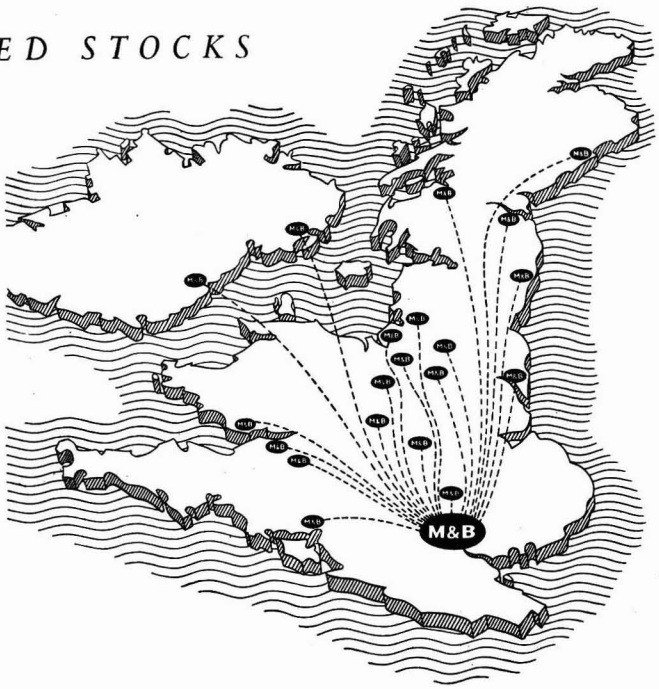

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J. Sci. Food Agric., 4, February, 1953

JOURNAL OF THE SCIENCE OF FOOD AND AGRICULTURE

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