SOME GENERAL CHARACTERISTICS OF ENZYME FOAM FRACTIONATION*

by

MORRIS LONDON, MARTIN COHEN** AND PERRY B. HUDSON***

Departments of Biochemistry and Urology, Francis Delafield Hospital, and Institute of Cancer Research, Columbia University, College of Physicians and Surgeons, New York, N.Y. (U.S.A.)

The studies presented here were designed with a two-fold purpose: one, to start the development of a simple technique to aid in the purification of enzymes, and two, to determine a set of principles to be applied in a foam technique so that denaturation may be kept to a minimum.

That foaming can be used in an enzyme fractionation has already been demonstrated in two other laboratories[1,2] and in this one[3,4]. The underlying principle of this process seems to be that proteins partition themselves between surface region and bulk liquid of an aqueous system. There are differences among proteins in the way they partition themselves, and the foaming process is a method of removing the surface region from the bulk liquid.

MATERIALS AND PROCEDURES

Materials

Two enzymes were foamed, singly or in combination:

1. "Arlco" urease (Jackbean). 47.5% protein: carbohydrate and water also present.
2. "Armour" catalase 10 (pork liver). 1000 Keil units/g. 73.5% protein. Kat-f 2400[5].

These enzyme preparations were dissolved in 0.2 *M* acetate buffer (or 0.05 *M* phosphate buffer at values higher than pH 5.6) and filtered. "Concentrations" expressed in % were defined as grams of preparation per 100 ml of solution. Cation-exchanged water was used in all solutions that came in contact with urease.

Apparatus

The apparatus first used (Fig. 1) consisted of gas washing bottles (Corning 31760, 250 ml and 125 ml) fitted with fritted glass discs. The gas was supplied from a bottle partially filled with chips of dry ice. The foam was led from the top of the apparatus by glass tubing and a rubber hose into graduated receivers.

This apparatus was used successfully for the purification of acid phosphatase[3] and urease[4]. In order to investigate variables, it became necessary to modify the apparatus (Fig. 2). The dry ice bottle was replaced with a CO_2 tank 1. (Pure nitrogen was tried, but

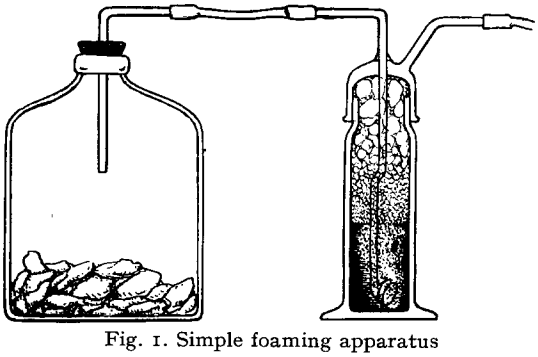

Fig. 1. Simple foaming apparatus

* This investigation was supported by a grant from the American Cancer Society, upon recommendation of the Committee on Growth, the National Research Council.

** Part of this paper is from the Master's Thesis of M.C., Columbia University, 1953.

*** Damon Runyon Senior Clinical Research Fellow.

References p. 120.

abandoned because of poor foaming properties with the enzyme systems.) A rubber hose led from the regulator to a gas reservoir 2 (a 2-gallon bottle). It was partially filled with water, since the dry gas tended to clog the gas disperser. The bottle was connected with the foaming apparatus through manometer 3 and flowmeter 4. The gas supply (Fig. 2A) was connected to either of the foam units (Fig. 2B) by the hose endings 2 and by different grades and types* of gas dispersers 1. For a number of runs the glass tube was cemented to a suction flask (Fig. 2B-a). In those cases the gas disperser was placed inside the suction flask and connected to the flowmeter by a rubber hose through the sidearm of the suction flask.

The vertical distance 8 of the column used was called "foam height". The vertical distance 7 of the flask (or tube) was called "liquid height".

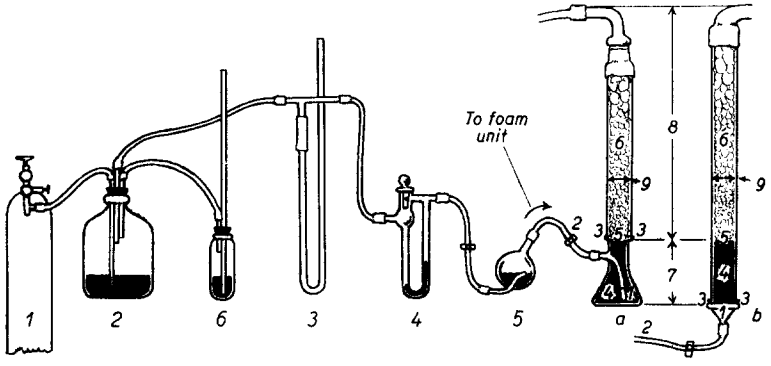

Fig. 2. Foaming apparatus for the investigation of variables

A

Pressure and rate regulation
1. Gas tank (with two-stage automatic regulator)
2. Pressure reservoir
3. Manometer (mercury)
4. Orifice-flowmeter (Brodie's fluid, Density 1.033)
5. Liquid trap
6. Safety valve (mercury)

B

Alternate foaming apparatus
1. Gas disperser
2. Connection to pressure and rate regulating apparatus
3. Joint (de Khotinsky cement)
4. "Liquid column"
5. Liquid surface
6. "Foam column"
7. "Liquid height"
8. "Foam height"
9. "Column diameter"

Analytical Procedures

Urease activity was measured by SUMNER AND GRAHAM'S method[6] using direct nesslerization of an aliquot. Catalase activity was followed by FEINSTEIN'S perborate method[7]. Total protein content was found by digestion with 5 *N* sulfuric acid and H_2O_2, followed by direct nesslerization**. The enzyme activities and nitrogen content of the original material, each foam fraction ("froth"), and the residue ("frothate") were measured.

Results

"Enzyme units" in a fraction were obtained by multiplying its volume (ml) by its activity (per ml). The "% enzyme in froth" was the ratio, enzyme units in froth/enzyme units in original

* The following gas dispersers were used:
a. "Corning" sintered glass discs and cylinders.
"Extra coarse" (160 μ average pore size)
"Coarse" (40 μ)
"Medium" (14 μ)
b. "Scientific Glass" 1-inch stainless steel dispersion tube D (65 μ).
c. Jetalloy spinnerette, five 0.05 mm diameter holes.

** Most froth fractions contained denatured protein (insoluble). This denatured protein was usually included in the determination of total nitrogen, although it could have been removed by centrifugation, thus raising purifications.

References p. 120.

material. The ratio, specific activity of fraction/specific activity of original material, was denoted by "purification". The "average purification" was defined as follows:

$$\text{average purification} = \frac{\sum_{i=1}^{n} (\text{purification})_i \, (\text{enzyme units})_i}{\sum_{i=1}^{n} (\text{enzyme units})_i}$$

where i = 1st, 2nd ... nth froth fractions (in certain cases, particularly with catalase, the frothate was included with several froth fractions). This average gives an indication of the purification of the average enzyme molecule in the froth (or froth — frothate) independent of concentration and volume of the fractions taken. It provides an index of the sharpness of the fractionation.

In presenting data on graphs, it will be seen that only one valid point can be had for each curve in the frothate, and each point was linked to the corresponding froth curve by a smooth line. The justification for this is that foaming is arbitrarily stopped and the frothate point is obtained. If foaming were continued, as was done in several cases, curves obtained followed the pattern shown in the graph, and the curves into the frothate section indicate trends.

RESULTS AND DISCUSSION

General observations

When gas is led into the liquid, a time lapse is observed before foaming starts. As the bubbles rise in the column, the smaller bubbles coalesce (Fig. 2B). Simultaneously the bubble walls become thinner; the excess liquid probably drains off through the voids created by the coalescence of the smaller bubbles and the breakage of the larger ones, which are generally less stable.

As the bubbles reach the top of the column, they grow and breakage increases*. A part of their liquid is trapped by the "surviving" bubbles, while the rest returns to the bulk liquid. In general, breakage increases loss by inactivations** and tends to increase purification[8], apparently due to increased drainage. Therefore, breakage is desirable to a point where a maximum purification can be obtained through allowing maximum drainage. There is also some evidence that excess drainage will lower recoveries and purifications due to the longer foaming required***.

As the foaming proceeds, bubbles grow and breakage increases even at column levels where, at the beginning of the foaming, the bubbles are small. This may be due to the reduction in protein concentration of the bulk liquid towards the end of the foaming. As a result, it is rarely advantageous to foam to exhaustion.

For the preparations used, urease as well as total protein concentrate in the central froth fractions (Fig. 3), while catalase concentrates in the final froth fractions and retains a relatively high concentration in the frothate (Fig. 4). These differences may be due to competition for the available surface between the different components. As a foaming proceeds, it is necessary gradually to increase the minimal gas pressure in order to be able to form foam. This is probably due to the differences between the surface tensions of the protein solutions.

* In one run, the foam column was bent so that a small portion of the tube was horizontal, and a capillary drain was attached to the horizontal portion. It was found that drainage constituted nearly 5/6 of the total foam volume, and that the enzyme activity in the drainage liquid was about 1/2 that of the foam proper (which came off the top of the column).

** Several experiments performed have shown that, in general, the making and breaking of foam, as in the case of shaking or foaming solutions of low stability, are destructive toward enzymes. Creating a stable foam by introducing a gas causes little destruction.

*** Purification and recovery are not independent variables, since lower recovery implies less activity per unit protein, while the protein quantity is not altered by denaturation.

References p. 120.

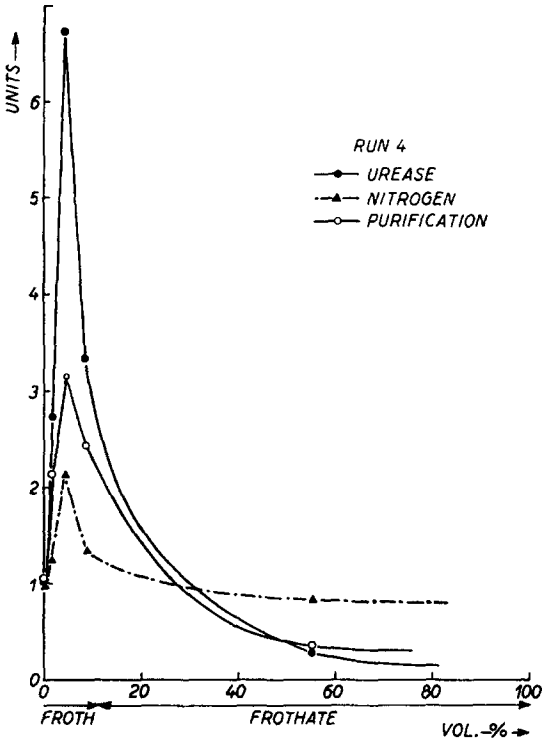

Fig. 3. Typical Urease Distribution. "Units" refer to the ratio of the urease activity of the fraction and the urease activity of the original material. The same holds for nitrogen content.

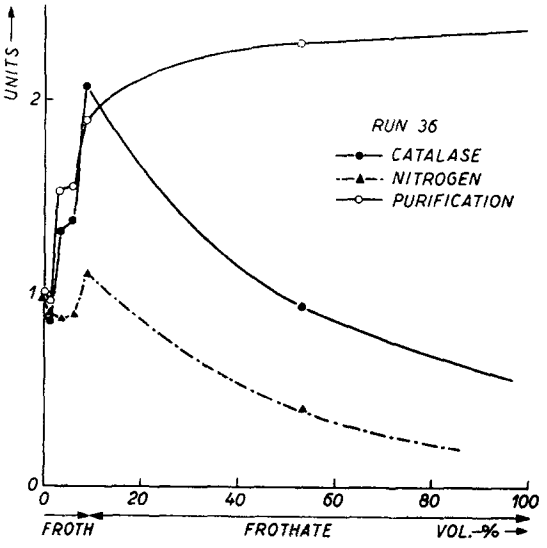

Fig. 4. Typical Catalase Distribution. "Units" refer to the ratio of the catalase activity of the fraction and the catalase activity of the original material. The same holds for nitrogen content.

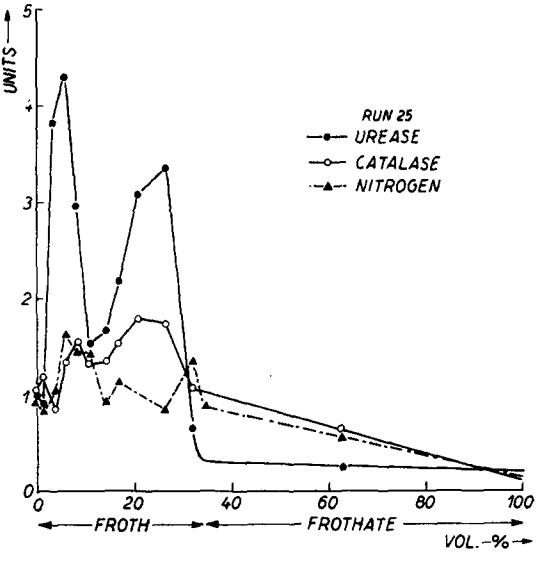

Fig. 5. Typical Distribution of a Mixture of Urease and Catalase. "Units" refer to the ratio of the urease activity of the fraction and the urease activity of the original material. The same holds for catalase activity and nitrogen content.

When a combined preparation of urease and catalase is foamed, a good separation between urease and catalase is effected, with most of the urease concentrating in the froth and most of the catalase in the frothate. However, the purifications of both catalase and urease (with regard to total protein) are slightly reduced (Fig. 5).

pH and concentration

It was found that both purifications and recoveries varied significantly with total

References p. 120.

protein concentration, attaining a distinct optimum at a concentration of about 0.16% (Fig. 6). Apparently protein does not concentrate enough in dilute solutions to form stable bubbles that can rise in the column. Excess protein makes a bubble too stable to allow for effective drainage.

Confirming the data of SCHUETZ[8], it was found that both urease and catalase showed the highest purification and recovery near their respective iso-electric points (Table I and Fig. 7)*.

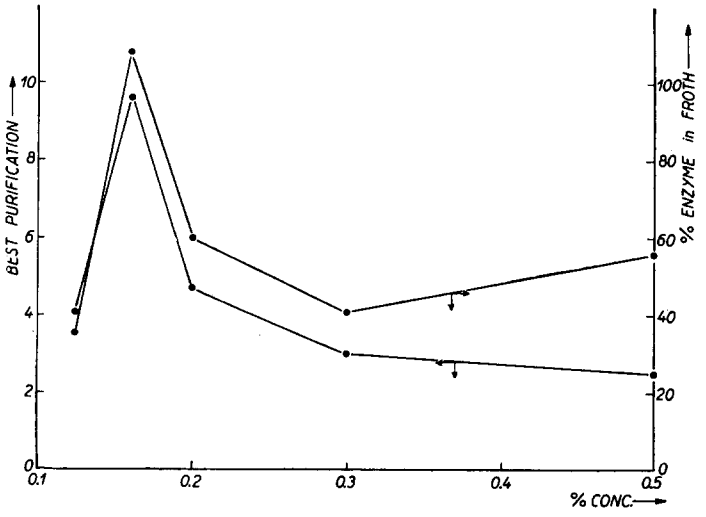

Fig. 6. Effect of Protein Concentration of Medium on the Foaming Characteristics of Urease. Several points are from the data of LONDON, COHEN AND HUDSON[4].

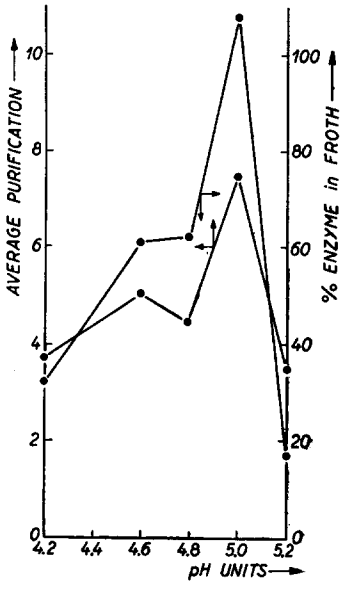

Fig. 7. Effect of pH of Medium on the Foaming Characteristics of Urease. Several points are from the data of LONDON, COHEN AND HUDSON[4].

TABLE I

THE EFFECT OF THE pH OF THE FOAMING MEDIUM ON THE FOAMING CHARACTERISTICS OF CATALASE*

Run	*pH*	*Purification (frothate)*	*% Enzyme in frothate*	*% Total recovery*
38	4.8	2.01	64	79
37	5.0	1.99	69	84
36	5.6	2.30	83	98
34**	6.8	0.97	61	89

* 100 ml of 0.03% catalase in 0.2 *M* acetate buffer were foamed in the apparatus of Fig. 1 (coarse disc). The fractions obtained were centrifuged prior to analysis.

** 100 ml of 0.10% catalase in 0.05 *M* phosphate buffer were foamed in the apparatus of Fig. 1 (coarse disc).

* In a few runs recoveries above 100% were obtained. In order to see whether this was due to analytical error distinct from inhibition effects, large amounts of the ashed urease preparation as well as the whole boiled preparation (separate experiments) were added to enzyme solutions. In neither experiment were significant reductions of activity observed.

References p. 120.

Additions

It was expected that the addition of electrolytes[9] to the foaming medium would increase its foaming power*, since the spreading of protein films on surfaces is enhanced by adding salts to their solutions. However, it was found that the addition of Na_2SO_4 depressed purifications and recoveries. The effects of low temperature and ethanol were similar (Table II). In general, temperature variation does not significantly affect foaming characteristics, though the viscosity does change considerably. Comparison of 3 runs at 10, 20 and 30° C showed no significant differences.

TABLE II

THE EFFECT OF SURFACE PROPERTIES* ON THE FOAMING CHARACTERISTICS OF UREASE**

Run	*Addition*	*Interface behavior*	*Average purification*	*Total recovery*
2	None	—	3.1	84%
4	2.35% EtOH	EtOH concentrates in interface; lower protein-bubble stability	2.7	72
3	5% Na_2SO_4	Directs protein to interface	1.7	63
5	Foam at 1–4° C	Increase surface tension	1.5	47

* The additions in this table change several properties, *e.g.* viscosity and surface tension.
** 100 ml of 0.20% urease in 0.2 *M* acetate buffer pH 5.0 were foamed in the apparatus of Fig. 1. The "additions" were added to this solution before foaming.

It was observed that the non-ionic detergent "Tween 80" very markedly increased recoveries (Table III). In one experiment** a frothate which had shown virtually no residual activity (purification 0.08) was refoamed with the aid of a few drops of Tween 80 and yielded further froth fractions with purifications as high as 1.6. This represents a purification of 20 on the basis of the frothate as original material. These recoveries are due, at least in part, to the formation of stabler bubbles.

TABLE III

THE EFFECT OF TWEEN 80 ON FOAMING CHARACTERISTICS

Enzyme preparation	*Tween not added*			*Tween added*		
	Run	*Total recovery*	*Purification*	*Run*	*Total recovery*	*Purification*
Urease*	2	84%	3.1	8	100%	3.2
Catalase**	37	84%	1.99	44	111%	1.78

* 100 ml of 0.20% urease in 0.2 *M* acetate buffer were foamed in the apparatus of Fig. 1 (coarse disc). 4 drops of Tween 80 from a 1 ml pipette were added to the solution in run 8. "Purifications" referred to are average purifications of the froth.
** 97 ml of 0.03% catalase in 0.2 *M* acetate buffer pH 5.0 were foamed in the apparatus of Fig. 1 (coarse disc). 2 drops of Tween 80 from a 1 ml pipette were added to the solution in run 44. "Purifications" referred to are frothate purifications.

* "Foaming power" and similar expressions are vaguely defined in the literature[10,11] as the ability to produce foam in a solution. The vagueness is due to the difficulty in separating the physical measurements of foaming power and foam stability (usually defined as "foam life").
** 97 ml of 0.125% urease in 0.2 *M* acetate buffer pH 5.0 were foamed at room temperature in the apparatus of Fig. 1 (250 ml gas washing bottle). Two fractions with a combined volume of 5 ml and an average purification of 4.0 were foamed off. The frothate was exhausted and foamed only after the addition of Tween 80 (3 drops from a 1 ml pipette). The next fraction which came off had a purification of 1.6; the purifications dropped in the following fractions.

References p. 120.

Bubble size

SCHUETZ'[8] claim that foaming characteristics are generally improved by smaller gas-in-liquid bubble size was partially confirmed[4] (Table IV, runs 23, 20, 24). It is clear that the column diameter affects the results. A reversal of the effect of gas disperser porosity on foaming characteristics seems to occur at a column diameter of about 4 cm under the conditions of these experiments.

TABLE IV

EFFECTS OF COLUMN DIAMETER AND GAS DISPERSER POROSITY ON THE FOAMING CHARACTERISTICS OF UREASE*

Porosity	*2.54 cm internal diameter*			*3.50 cm internal diameter*			*6.04 cm internal diameter***		
	Run	*Average purification*	*Total recovery*	*Run*	*Average purification*	*Total recovery*	*Run*	*Average purification*	*Total recovery*
Extra-coarse	80	2.96	87%	81	3.1	81.3%	23	2.6	47%
Coarse	79	2.01	64%	82	3.01	104%	20	2.9	61%
Medium		—			—		24	6.1	100%

* 265 ml of 0.16% urease in 0.2 *M* acetate buffer pH 5.0 were foamed in the apparatus of Fig. 2B-a. Liquid height 10 cm, foam height 50 cm. The durations of the foamings varied between 25 and 32 minutes. Gas rates varied between 1.1 and 1.4 ml CO_2/sec, but were increased towards the end of each run. For the coarse porosity, Corning cylinders (40 μ) were employed; for the extra coarse porosity, the stainless steel disc (65 μ) was used.

** 300 ml of 0.16% urease in 0.2 *M* acetate buffer pH 5.0 were foamed in apparatus similar to that of Fig. 1; however, the gas-washing bottle was replaced with a 1000 ml cylinder. Liquid height 10 cm, foam height 33 cm. Foamings were carried out till exhaustion. Gas rates were not controlled. Corning discs were used.

A large bubble size is conducive to better drainage and sharper separation. When the column diameter exceeds a critical size which depends on the conditions of the foaming, the bubbles produced by larger pores can no longer be supported by the column walls and by each other. Excessive breakage, denaturation and inability to climb the column (hence premature cessation of foaming) ensue, reducing purifications and recoveries.

Column characteristics

An increase in column diameter was shown to improve both purifications and recoveries (Table V). When column diameters were increased beyond about 6 cm, the foam column became (for extra-coarse porosity, see Table IV) structurally unstable, because there was less column surface per bubble for support.

The foam height was also shown to affect the separation (Table VI). An optimal height was found to exist for each column diameter. This is due to the opposing effects of increasing drainage, which improves separation, and increasing denaturation with increasing foam height. The optimum tends to move towards lower foam heights with increasing diameters, due to excessive breakage in tall, wide columns.

The "liquid height" was found to affect significantly the foaming characteristics of urease, particularly the percentage of activity remaining in the frothate (Table VII). This seems to indicate that a considerable amount of inactivation takes place at the interface between the rising gas bubble and the bulk liquid phase. As a result, some of the runs were carried out in columns of varying cross-sections. Larger diameters were used for the liquid column than for the foam column (see Fig. 2B-a).

References p. 120.

TABLE V

EFFECT OF COLUMN DIAMETER ON THE FOAMING CHARACTERISTICS OF UREASE*

Run	*Internal diam. (cm)*	*Average purification*	*% Enzyme in frothate*
73**	1.28	1.4	28.3
75	1.92	1.6	18.6
79	2.54	2.0	28
82	3.50	3.0	36.3

* 265 ml of 0.16% urease in 0.2 *M* acetate buffer pH 5.0 were foamed in the apparatus of Fig. 2B-a. Liquid height 10 cm, foam height 50 cm. Durations of the foaming varied between 24 and 32 minutes. Rates: 1.1–1.5 ml CO_2/sec. "Coarse" discs.

** As above, but foam height 89 cm, duration of foaming 35 minutes.

TABLE VI

EFFECT OF FOAM HEIGHT ON THE FOAMING CHARACTERISTICS OF UREASE

Foam height (cm)	*Internal diam. 1.3 cm**				*Internal diam. 1.3 cm***				*Internal diam. 1.9 cm***				*Internal diam. 2.5 cm***			
	Run	*Total recovery*	*Best purifn.*	*Average purifn.*	*Run*	*Total recovery*	*Best purifn.*	*Average purifn.*	*Run*	*Total recovery*	*Best purifn.*	*Average purifn.*	*Run*	*Total recovery*	*Best purifn.*	*Average purifn.*
28	63	76%	1.49	1.36	—	—	—	—	—	—	—	—	—	—	—	—
51	—	—	—	—	71	99%	2.4	1.97	75	74%	2.60	1.61	79	64%	2.13	2.01
89	60	65%	1.88	1.90	73	89%	2.1	1.27	74	74%	2.14	2.01	77	67%	2.08	1.89
128	52	55%	1.64	1.51	—	—	—	—	—	—	—	—	—	—	—	—

* Solutions of 0.16% urease in 0.2 *M* acetate buffer pH 5.0 were foamed in the apparatus of Fig. 2B-b (gas disperser spinnerette) with liquid heights of 30 cm (run 52: 73½ cm) at a pressure of ½ atm. gauge, at a rate of 0.64 ml CO_2/sec for 14 minutes.

** Solutions of 0.16% urease in 0.2 *M* acetate buffer pH 5.0 were foamed in the apparatus of Fig. 2B-a ("coarse" disc) with liquid heights of 10 cm (run 71: 28 cm) at a pressure of about ½ atm. gauge, at a rate of 0.6–1.4 ml CO_2/sec for 20–25 minutes.

TABLE VII

EFFECT OF LIQUID HEIGHT ON THE FOAMING CHARACTERISTICS OF UREASE*

Run	*Liq. height (cm)*	*Best purification*	*Average purification*	*Total recovery*	*% Enzyme in frothate*
56	19	1.7	1.7	56	42.5
57	31	2.2	1.7	87	51
53	36	3.4	3.4	85	56.5
51	81	1.08	1.08	39	30

* Solutions of 0.16% urease in 0.2 *M* acetate buffer pH 5.0 were foamed in the apparatus of Fig. 2B-b (gas disperser: spinnerette), with 1.3 cm internal diameter columns. Foam heights varied between 48 and 63 cm. This variation seems to be negligible considering the data of Table VI.

Gas flow rate

One of the major factors affecting foaming characteristics is the rate of flow of foam bubbles in the foam column and gas bubbles in the liquid column. Both could be controlled only by the gas flow rate (Table VIII).

A slower foaming probably improves purifications by improving drainage. The decrease in recovery with increasing flow rates may be due to having reached the point of exhaustion earlier in the run.

References p. 120.

TABLE VIII

THE EFFECT OF THE GAS FLOW RATE ON THE FOAMING CHARACTERISTICS OF UREASE*

Run	*Gas flow rate (ml CO_2/sec)*	*Average purification*	*Total recovery*
64	0.55	1.91	105%
57	0.65	1.67	87
66	0.78	1.23	74
65	0.89	1.03	59

* 40 ml of 0.16% urease in 0.2 *M* acetate buffer pH 5.0 were foamed in the apparatus of Fig. 2B-b (column diameter 1.3 cm, liquid height 31 cm, foam height 65 cm, spinnerette) at a pressure of ½ atm. gauge for 10 minutes.

CONCLUSIONS

The data show that the optimal concentration range of protein for foaming is between about 0.01 and 0.2% (Table IX). Within this range, enzymes or proteins of systems which tend to concentrate in the froth are best purified when a higher concentration of protein is present, while those which remain in the frothate are best purified if lower protein concentrations are employed.

TABLE IX

SELECTION OF OPTIMAL CONCENTRATION RANGES FOR FOAM FRACTIONATION OF PROTEINS*

Enzyme	*Where purified*	*High concentration*		*Best concentration*		*Low concentration*	
		% Protein	*Best purification*	*% Protein*	*Best purification*	*% Protein*	*Best purification*
Urease	Froth (initial)	0.15	3.0	0.080	9.7	0.062	4.0
Catalase	Froth (final) and Frothate	0.052	1.6	0.037	2.3	0.022	1.99
Acid phosphatase (prostate)**	Frothate	0.050	2.0	0.010	15	—	—

* Foaming medium at pH 5.0.
** M. LONDON AND P. B. HUDSON, *Arch. Biochem. and Biophys.*, 46 (1953) 141.

The optimal pH of the foaming medium seems to be in the range of pH 4.8 to 5.6, which coincides with the range of iso-electric and maximum stability points of most proteins. Normal variations of room temperature do not affect the fractionation significantly.

It was also found that, in selecting gas rates, gas disperser porosities, and column dimensions, both the drainage of the bubble and denaturation have to be considered. The best results are obtained under conditions which favour the formation of bubbles of maximal size in columns of maximal diameter, while still minimizing bubble breakage.

The liquid height should be kept low. This implies a larger cross-section for the liquid column than for the foam column (Fig. 2B-a). Variation in cross-section should be very gradual to avoid the formation of slow-moving pockets or trapped foam. Newly generated foam by-passes these pockets, causing undesirable turbulence in the foam column.

References p. 120.

The foam height should be kept as close as possible to the optima dictated by drainage and denaturation. These optima are lower for larger column diameters (in these experiments they varied from 10 to about 90 cm, with column diameters varying between 1 and 7 cm).

The gas dispersers should be chosen to generate the largest bubbles compatible with bubble stability. The disperser should be placed as high in the liquid as is compatible with satisfactory stirring of the liquid.

In certain cases, particularly in the purification of surface-sensitive, dilute solutions and poorly foaming proteins, Tween 80 and other non-ionic detergents may prove useful.

ACKNOWLEDGEMENT

We wish to thank Professor JOHN M. REINER for his timely discussions, encouragement, and suggestions during the course of these experiments.

SUMMARY

Variations of foam purification conditions for urease and catalase, separately, were studied. It was demonstrated that impure urease and catalase mixtures could be separated considerably from one another, and each could be separated from most other contaminating protein.

The effect of several variables on the foaming method of separating enzymes from protein was investigated, and some general rules were presented for the fractionation.

RÉSUMÉ

La purification par la méthode des mousses de l'uréase et celle de la catalase ont été étudiées sous diverses conditions. La catalase et l'uréase impures, en mélange, peuvent être séparées l'une de l'autre et des autres protéïnes contaminantes.

La purification des enzymes par la méthodes des mousses a été étudiée en fonction de différentes variables et quelques règles générales de fractionnement sont présentées.

ZUSAMMENFASSUNG

Verschiedene Bedingungen für die Schaumreinigung von sowohl Urease wie Katalase wurden untersucht. Es wurde gezeigt, dass unreine Urease- und Katalasemischungen beträchtlich von einander getrennt werden konnten. Jede konnte von den meisten anderen, sie verunreinigenden Proteinen getrennt werden.

Der Einfluss mehrerer Variablen auf die Schaumtrennungsmethode für Enzyme von Protein wurde untersucht und einige allgemeine Regeln für die Fraktionierung wurden vorgelegt.

REFERENCES

1 F. SCHUETZ, R. BADER AND M. STACEY, *Nature*, 154 (1944) 183.
2 A. DOGNON, *Surface Chemistry*, London (1949) p. 253.
3 M. LONDON AND P. B. HUDSON, *Arch. Biochem. and Biophys.*, 46 (1953) 141.
4 M. LONDON, M. COHEN AND P. B. HUDSON, *J. Am. Chem. Soc.*, 75 (1953) 1746.
5 S. HENNICHS, *Ber.*, 59 (1926) 218.
6 J. B. SUMNER AND H. GRAHAM, *Proc. Soc. Exptl. Biol. Med.*, 22 (1925) 504.
7 R. N. FEINSTEIN, *J. Biol. Chem.*, 180 (1949) 3.
8 F. SCHUETZ, *Trans. Faraday Soc.*, 42 (1946) 437; *ibid.*, 38 (1942) 85.
9 I. LANGMUIR AND V. J. SCHAEFER, *Chem. Revs.*, 24 (1939) 181.
10 H. KIMIZUKA AND T. SASAKI, *Bull. Chem. Soc. Japan*, 24 (1951) 230.
11 W. OSTWALD AND A. SIEHR, *Kolloid-Z.*, 76 (1936) 33.

Received July 14th, 1953