ON THE SEPARATION OF A PURE ALBUMIN FROM EGG-WHITE. BY F. GOWLAND HOPKINS, M.A., M.B.,

B.SC., *University Lecturer in Chemical Physiology.*

(*From the Physiological Laboratory, Cambridge.*)

I. INTRODUCTORY.

THE chemical study of animal proteids made one of its greatest advances when Hofmeister showed that under special conditions egg-albumins could be crystallised. When to this discovery was added the observation of Gürber showing that Hofmeister's method of crystallization could be extended to certain serum-albumins, there became available for research representative proteid material obtainable in practicable quantities, and possessing some guarantee of purity.

The work of the decade which has elapsed since these crystalline albumins became available for study cannot be said however to have defined the conditions necessary for obtaining a product with a just claim to actual chemical individuality.

It is quite certain that more than one proteid in egg-white remains unprecipitated after half-saturation with ammonium sulphate; and it is at least highly probable that on continued evaporation of the ammonium-sulphate-albumin mixture, in accordance with the original description of Hofmeister's process, more than one crystalline albumin eventually separates.

The well-known research of Bondzynski and Zoja[1] published in 1894 offered strong grounds for believing this to be the case; these observers finding wide differences in the specific rotatory powers of different crystalline fractions—an indication of great significance where proteids are concerned.

[1] *Zeitsch. f. physiol. Chem.* XIX. p. 1. 1894.

It will be convenient to give here certain of the data from Bondzynski and Zoja's experiments; the figures in the following table relating to successive fractions from the same preparation of egg-white. The fractions are in the order of increasing solubility.

No. of fraction	Specific rotatory power	Carbon	Hydrogen	Nitrogen	Sulphur
1	26° 0′	52·33 %	7·13	15·47	1·614
2	29° 16′	—	—	—	—
3	34° 18′	—	—	—	—
4	42° 54′	52·07	6·98	15·29	1·693

Professor Hofmeister, it is true, has not himself observed the occurrence in egg-white of crystallizable albumins of varying solubility, and is of opinion that Bondzynski and Zoja's fractions were admixed to a greater or less degree with uncrystallized proteid[1].

In support of this contention he adduces the fact that crystalline material analysed by himself (and by F. N. Schulz) gave a lower percentage of sulphur (a mean of 1·18)[1]. But even in the case of their most soluble fraction, that showing the very high rotatory power of 42° 54′, Bondzynski and Zoja observed that the product was wholly crystalline[2]; while as to the sulphur content, I venture to think that there are grave doubts if the chief crystallizable albumen of egg-white has a sulphur content so low as that found by Hofmeister (cf. *infra*). Moreover T. B. Osborne[3] has recently brought forward weighty evidence for the existence of more than one crystalline fraction; and I can myself speak to the extreme difficulty of obtaining with repeated recrystallizations by Hofmeister's process (at least in its original form) a product of constant rotatory power.

There remains therefore, as I have said, some considerable doubt as to the real chemical individuality of the egg-albumin hitherto employed for various lines of study; for when, as in several recent researches, crystalline products have formed the material employed, no guarantee of purity has been sought other than that of the crystallization itself.

It will be admitted moreover that at the present time great uncertainty exists as to the real percentage of sulphur in egg-albumins.

In the present paper a procedure is described by means of which an albumin is easily obtained from egg-white, in proportionately large amount, which offers what I think is satisfactory evidence of chemical

[1] *Zeitsch f. physiol. Chem.* XXIV. p. 166. 1898.
[2] *loc. cit.* p. 6.
[3] *Journ. Amer. Chem. Soc.* XXI. p. 477. 1899.

individuality. Special attention has been given to the rotatory power of the product, and to the percentage of sulphur contained in it.

Nearly two years ago I described, in conjunction with S. N. Pinkus[1], a method of crystallizing albumins which, while involving no more than a slight modification of Hofmeister's process, replaced a very lengthy and somewhat uncertain procedure by one which is extraordinarily easy and rapid.

This modification consisted in adding to the albumin solution when half-saturated with ammonium sulphate and filtered from the precipitated globulins, etc., sufficient dilute acetic acid to produce a slight permanent precipitate. With perfectly fresh eggs the process of crystallization begins at once, and a large yield of crystalline albumin is obtained in a few hours; the separation is accelerated by occasional gentle disturbance of the mixture, but no concentration is necessary.

When we described this method of crystallization we had given most attention to egg-albumin; but it was remarked in our paper that the addition of the acid to serum albumin, at the same stage in the process greatly accelerated crystal formation; and we showed that it induced the process in dogs' serum which had not before been crystallized. At the same time however we believed that crystallization could not be obtained by the acetic acid treatment so rapidly in serum as in egg-white, a view that I have since had occasion to modify.

More than six months after our paper dealing with these facts had appeared in this *Journal*, H. T. Krieger[2] described the use of sulphuric acid in the crystallization of serum-albumin, showing that this acid used in a similar manner greatly increased the efficacy of Gürber's method. F. N. Schulz[3] states that in the crystallization of egg-albumin the use of sulphuric acid as employed by Krieger is to be preferred to acetic acid as recommended by us, though he does not say in what respect it is superior. Pinkus and I did not discover any such superiority at the time our original observations were made; and on carefully comparing the action of the two acids at the present time I am quite unable to discover wherein such superiority lies. Very various acids may be used.

In place of acetic or sulphuric one may certainly employ—so far at least as the acquirement of a crystalline product is concerned—

[1] Hopkins and Pinkus. This *Journal*, XXIII. p. 130. 1898.

[2] Krieger. Inaugural Dissertation, Strassburg, 1899.

[3] *Zeitsch. f. physiol. Chem.* XXIX. p. 87. 1900.

either hydrochloric or nitric acid; and phosphoric acid gives equally good results.

If the egg-white used be from perfectly fresh eggs, and if the acid added be not too strong, and yet of such strength that the ammonium sulphate is not over-diluted (about *normal* strength is in most cases the best), and if at the same time it is added in each case to a point where a definite precipitate is first produced, the mixture being afterwards occasionally gently shaken or stirred—crystallization will then prove about equally rapid with all the acids mentioned, and the ultimate yield of the product will be but little different. But it seems desirable—as it seemed originally to Pinkus and myself—in dealing with proteid material, to choose an organic rather than a mineral acid. Be this as it may, those who have had occasion to compare the exceedingly difficult task of preparing a first crop of crystals by evaporation of the alkaline mixture of Hofmeister's original method, with the extreme ease of obtaining crystals in the acid condition, will admit the importance of the modification first introduced by us. No better proof of the difficulties attending the original process could be adduced, than the fact that for many years after it was described the crystalline proteid was scarcely employed as material for research. In 1898, Hofmeister showed (*loc. cit.*) that a product can generally be obtained with rapidity if some crystals derived from a previous preparation be added to the mixture; then, for the first time, the crystalline albumin became really available material. But for the majority of workers this still leaves the necessity of preparing a preliminary supply of crystals, with the original difficulties attendant upon the task. By the simple device of adding acid, these difficulties are removed; while at the same time the yield of the product is greatly improved.

I shall now describe an application of the acetic acid method which I believe enables one to obtain quite easily a large yield of a well-characterised pure albumin.

II. Preparation of the pure albumin.

One of the facts which first came to light on further acquaintance with the process as previously described was that there was no need to avoid the formation of even bulky precipitates on the addition of acid to the mixture of proteid and ammonium sulphate. Such precipitates,

though apparently amorphous at first, change with remarkable rapidity into a crystalline form, and that without going through any intermediate stage of the globules or spherules which are so familiar as precursors to the crystals in Hofmeister's process. The crystals which form under these circumstances remain, it is true, of small size, and are, in the case of egg-albumin, acicular in form, so that they are seldom suitable for crystallographic studies; but their absolute purity and freedom from amorphous admixture is easily demonstrated under the microscope ($\frac{1}{8}$th obj. or its equivalent).

Taking advantage of the fact that the formation of amorphous precipitates is no hindrance to crystal formation[1] makes it easier to employ a definite excess of acid, and so to define better the conditions for separating a definite fraction while obtaining the maximum yield.

The egg-white is beaten, as usual, to a froth[2] with exactly its own bulk of saturated ammonium sulphate solution. The mixture, having stood over-night, or, at least, for a few hours, is filtered from precipitated proteid. The filtrate is now measured. Ten per cent. acetic acid (glacial acid diluted to ten times its bulk) is then very gradually added from a burette until a well-marked permanent precipitate first forms—a precipitate sufficient to make the mixture actually milky in appearance; not a mere opalescence, for which liberated gas-bubbles might be mistaken. The actual amount of acid required to produce such a precipitate will vary (chiefly because of the varying loss of ammonia which occurs when the mixture has previously stood in open vessels). The point corresponds roughly to an incipient acidity of the solution towards litmus; but the formation of the permanent precipitate forms itself the best indicator. This stage being reached, a measured quantity of the acid is now added, over and above that required to produce the first precipitate; one cubic centimetre extra being added for each hundred cubic centimetres of the filtered mixture as originally measured. The whole contains therefore approximately one part per mille. of free acid. In carrying out this stage of the process when large quantities of material are to be dealt with, it is

[1] Amorphous precipitates, that is, produced at the stage of adding acid; a precipitate produced by excess of ammonium sulphate before acidification may remain obstinately amorphous.

[2] I find that failures in the hands of some who first attempt the process are due merely to inefficient beating or whisking at this stage. It is absolutely necessary, as Hofmeister showed, to break up thoroughly the membranes contained in the egg-white.

best to note the amount of acid necessary for a measured half-litre of the mixture, and then to add a proportionate quantity to the whole.

The bulky precipitate thus produced is at first amorphous, but microscopical examination with a high power will show commencing crystallization within an hour or so; and, if the mixture be occasionally shaken, the amorphous precipitate will entirely give place to crystals within four or five hours. To obtain the full yield however the material should stand for 24 hours, the change in the original amorphous precipitate being complete some time before the whole product has crystallised from the solution.

It is essential that the eggs employed should be as fresh as possible; not more than about a week old in summer, nor more than a fortnight in winter.

The yield of the crystalline product should at this stage be at least 60 grammes per litre; with perfectly fresh eggs it is frequently more.

To arrive at a product which, as I shall show, offers satisfactory evidence of being chemically pure, this first precipitate needs to be once, and once only, recrystallized. Its solutions may show some slight pigmentation; and its rotatory power, though agreeing closely with that of the pure product, is apt to vary within small limits.

It is filtered off, and washed twice or thrice with half-saturated ammonium sulphate solution containing 1 per mille. acetic acid. Redissolved in a minimal quantity of water (solutions containing from 10 to 12 per cent. of proteid may be easily obtained), it is reprecipitated by the addition of a suitable quantity of ammonium sulphate solution. This should be added little by little, the mixture being meanwhile gently stirred or shaken, until a definite precipitate (not a mere cloud) remains; and then about 2 c.c. per litre are added in excess of this. The precipitate, as before, becomes rapidly crystalline and separation is complete in about 24 hours. At this stage slight mechanical disturbance from time to time is of especial use in accelerating the separation. I have frequently observed, in cases in which the process has appeared slower than usual, that slight shaking of the flask has been quickly followed by complete separation. On one occasion a solution of the product at this stage, to which ammonium sulphate had been added in quantity too small to produce any definite precipitate, remained, while undisturbed, practically clear for no less than three weeks. On then shaking the flask a bulky precipitate wholly composed of crystals

separated within an hour. The mixture behaved as a supersaturated solution. It should not be forgotten however that solutions of the crystalline albumin are especially prone to undergo mechanical coagulation, and should not be shaken with any violence.

This second crystalline product is, as I have said, the material which I believe to be homogeneous and to consist of a pure proteid. If the amount of water used to dissolve the first precipitate has not been excessive, the yield will be 80 per cent. of the latter; that is, in general, about 50 grammes per litre of egg-white. Not infrequently it considerably exceeds this figure.

On microscopical examination it is found to be quite unmixed with amorphous material, consisting as a rule of tyrosin-like aggregates of fine needles. Filtered off it is snow-white in colour, and its solutions are quite colourless. On fractionation by repeated recrystallizations, which may be carried out by adding ammonium sulphate to successive fresh solutions of the product, in a manner exactly similar to that just described, the rotatory power remains absolutely constant, and the content of sulphur in successive fractions, together with the other analytical figures, remain unchanged.

III. Analytical methods, etc.

The albumin from four different supplies of eggs was crystallized by the process just described, and then once recrystallized. Starting in each case from this second crystalline product, which was itself examined and analysed, four or five successive fractions were prepared; the rotatory power of each being determined and a portion of each reserved and prepared for analysis. The white of a hundred eggs was employed on each occasion, and the yield at the second stage of crystallization was from 105 to 115 grammes of proteid. To secure efficient fractionation each successive product was filtered off and dealt with as soon as it became fully crystalline, without waiting for the whole to separate from solution. So treated, the first product examined, after being thrice recrystallized, was reduced to 20 or 30 per cent. of its original quantity; otherwise 90 per cent. or more may separate afresh at each stage.

Rotatory power. Preliminary experiments having shown that varying proportions of ammonium sulphate in solution had not the smallest effect upon the rotatory power of the proteid, this constant was determined in the solution obtained by dissolving the crystals

direct from the filter, without subsequent dialysis or other treatment. Such solutions may be slightly opalescent from mechanical coagulation, but can be filtered crystal-clear. In filtering solutions of the pure proteid at any stage it is necessary however that the neck of the funnel should nearly reach the surface of the filtrate, else mechanical coagula are apt to form.

The estimations were made with a large Laurent's polarimeter, reading to hundredths of a degree. All the observations were taken at temperatures of from 15° to 17° C. The solutions mostly contained as much as from 8 to 10 per cent. proteid, and being perfectly clear and pigment-free, permitted the use of a 4 decimetre tube, so that large rotations were observed.

To estimate the percentage of proteid in solution I employed Devoto's method slightly modified. I am convinced that this when applied to the estimation of the pure albumin is susceptible of very great accuracy. I used it as follows: A quantity of the solution containing from ·5 to 1·0 gramme proteid (in my experiments generally 5 to 10 c.c.) is measured from a standardised pipette into 100 c.c. of saturated ammonium sulphate solution and a few extra crystals of the salt added. The beaker containing the solution is covered with a clock glass and heated, well immersed in the steam from a water-bath, for 2 hours. The coagulum is allowed to cool completely and is filtered cold through a hardened filter-paper. Washed back into the beaker with distilled water, it is again heated for half-an-hour, again cooled, filtered through the same paper, and finally washed with cold water until considerable quantities of the washings after concentration show no trace of sulphate upon adding barium chloride. The filtrate and successive washings should be tested by Heller's test, which if the heating has been properly carried out will show no trace of proteid. The precipitate is always in a form which permits of easy washing from a hardened filter, and it is best to complete the determination by washing it into a weighed platinum dish, evaporating to dryness, drying at 110° and weighing. To attain to the ultimate accuracy of which the process is capable, it is not unimportant to use at the final stage an efficient drying apparatus such as a Lothar Meyer's air-bath. In an ordinary oven the last few milligrammes of water are lost with surprising slowness, and the drying involves an undesirably long exposure to the high temperature. I made duplicate determinations in every case, and these always agreed with great exactness[1].

[1] To the extreme accuracy of which I have found the process capable, washing with cold rather than hot water appears to contribute.

It may not be out of place to discuss here certain considerations which may explain the superior accuracy of Devoto's process as a method of estimation when compared with heat coagulation under other conditions. Haycraft and Duggen[1] first called attention to the fact that a dilute solution of egg-white when quickly heated may form no true coagulum, but may become merely opalescent or milky. The condition thus assumed by the proteid has been recently studied from the physical standpoint by W. B. Hardy[2], who shows that it is analogous to that assumed by silica when in so-called colloid aqueous solution—"the heat by chemically altering the dissolved proteid has by a process of desolution, to use Lord Rayleigh's word, produced a suspension of particles having an average diameter commensurable with the mean wave-length of light." These suspended or dispersed particles are aggregated by the addition of electrolytes, and a precipitate or coagulum may result. I have found that ammonium sulphate is very efficient in producing this aggregation when present to near saturation; and proteids in the condition just discussed yield a precipitate on saturation with this salt which is not again taken up in any form by water. Starke[3] has shown that the material present in the opalescent fluid yielded by boiled egg-white is nearly all in the form of alkali albuminate; but the pure albumin itself though wholly free from associated alkali undoubtedly tends to undergo at least to a slight degree some analogous transformation, leading to incomplete coagulation on heating. The presence of the ammonium sulphate in Devoto's process either ensures aggregation of any part of the proteid which may assume this form of suspension, or what is more likely it inhibits the phenomenon. That the latter is more probable is suggested by a fact which I have observed, namely, that while ordinary heat coagula show some indications of chemical change, in that the percentage of sulphur contained in them is inconstant, a coagulum prepared as in Devoto's process yields exactly the same amount of sulphur as do alcohol coagula.

Believing that really accurate estimations of proteid in solution are to be made by Devoto's process, and having regard to the fact that the ammonium sulphate which adheres to the moist crystals has certainly no influence upon the rotatory power, I am of opinion that the most accurate determination of the latter is to be made in the manner I have adopted. To get rid of the salt completely by dialysis, with the

[1] *Brit. Med. Journ.* p. 167. 1890.

[2] This *Journal*, XXIV. 182. 1899, and *ibid.* p. 288.

[3] *Ref. in Jahrb. f. Thierchemie*, XXVII. p. 19. 1897.

aim of determining the percentage of proteid by simple evaporation, is by no means an easy task.

Preparation for analysis. The analyses detailed in the next section were all made upon alcohol coagula. A portion of the clear aqueous solution of each fraction was poured into four or five times its bulk of absolute alcohol, allowed to stand three or four days, and the coagulum then washed with cold water until entirely free from sulphate. This washing, as all observers have found, is a lengthy process. My own procedure generally occupied three days, and was as follows: The coagulum was ground up in a mortar with alcohol, and then transferred to a large bulk of distilled water, which was kept in motion with a mechanical stirrer for an hour. The proteid was then allowed to settle, the greater part of the water poured off, and the precipitate filtered through fine cambric, and squeezed dry. It was then transferred to a fresh quantity of water, and the process many times repeated. To keep the substance aseptic, freshly boiled water was used, and the coagulum after squeezing through the cambric filter was occasionally soaked with absolute alcohol. Two or three litres of the final washings were concentrated to small bulk and tested with barium chloride. The coagulum when free from sulphate was washed with alcohol and ether, air-dried, and finally dried at 110° C. Some analyses were made upon heat coagula and others upon mechanical coagula. These are briefly referred to in a later section.

After most of the analyses had been made, a method was contrived for washing the intact uncoagulated crystals free from sulphate; this is described below.

Estimation of sulphur. This was carried out by the Asboth-Düring[1] method. The materials used were carefully tested and proved free from sulphates. Quantities equal to those actually employed in the analyses were dissolved in hydrochloric acid, and the solution digested for some hours on the water-bath, after the addition of barium chloride. Only after some trouble did I obtain sodium peroxide which yielded no trace of precipitate under these circumstances. The fusions were carried out in nickel basins with a spirit-lamp burning pure alcohol and capable of giving sufficient heat to produce fluidity of the mixture. In some cases the estimations were checked by controls with Carius' method, and good agreement obtained. It is necessary however to heat for some hours at tempera-

[1] Düring. *Zeitsch. f. physiol. Chem.* XXII. p. 281.

tures over 300°; the proteid is refractory towards nitric acid, and many hours' heating at about 240°—250° may yield little more than half the sulphur as sulphate.

Nitrogen determinations were made by Dumas' method.

IV. Results of analyses.

The analytical data from which the following results were calculated are given in protocols at the end of the paper. All the fractions examined were entirely crystalline.

Preparation A. The original crystalline precipitate (A^1) produced by the addition of acetic acid contained 125 grammes proteid (from 100 eggs). It gave a rotatory power of 31·10° and contained 1·61 per cent. sulphur. The amount of proteid in A^2 was 110 grammes.

No. of fraction	Proportion of A^2 contained in later fractions	Specific rotatory power, αD	Carbon	Hydrogen	Nitrogen	Sulphur	Ash
A^2	100[1]	30·72°	52·63	7·13	15·43	1·58	—
A^3	65	30·71	—	—	15·41	1·58	—
A^4	42	30·68	—	—	—	—	0·060
A^5	26	30·73	52·82	7·21	15·38	1·56	0·054

Preparation B. The original precipitate B^1 contains 128 grammes proteid, and gave a rotatory power of 30·98°. B^2 contained 108 grammes.

No. of fraction	Percentage of B^2 contained in later fractions	Specific rotatory power, αD	Carbon	Hydrogen	Nitrogen	Sulphur	Ash
B^2	100	30·76°	52·81	7·16	—	1·59	—
B^3	72	30·70	—	—	15·51	1·56	—
B^4	52	30·73	52·69	7·09	—	1·58	—
B^5	35	30·68	—	—	15·45	1·54	0·060

Preparation C. The first precipitate C^1 was not analysed.

No. of fraction	Percentage of C^2 contained in later fractions	Specific rotatory power, αD	Carbon	Hydrogen	Nitrogen	Sulphur	Ash
C^2	100	30·67°	—	—	15·33	1·62	—
C^3	75	30·76	—	—	—	—	—
C^4	65	30·64	—	—	—	1·58	0·080
C^6	30	30·57	52·63	7·13	15·53	1·55	0·068

[1] In calculating these percentages allowance has of course been made for the amount of each fraction employed for analysis.

Preparation D. The rotatory power of the first precipitate D^1 was 31.45°. The amount from 100 eggs was 130 grammes. D^2 was 115 grammes.

No. of fraction	Percentage of D^2 contained in later fractions	Specific rotatory power, αD	Carbon	Hydrogen	Nitrogen	Sulphur	Ash
D^2	100	30·75°	52·82	7·01	15·51	1·57	—
D^4	52	30·71	—	—	—	—	—
D^5	38	30·75	52·66	7·12	15·41	1·51	0·053

Taking together the results from all four preparations the following mean values are obtained:

Rotatory power	30·70°.	Nitrogen	15·43.
Carbon	52·75.	Sulphur	1·57.
Hydrogen	7·12.	Ash	0·063.

It will be seen that throughout the series very great constancy in the rotatory power was obtained, the agreement being very exact in the different preparations, and no less so in the successive fractions from each preparation. As I have said, the rotations could be observed with great accuracy owing to the clear colourless nature of the solutions, and there is no question that Devoto's method, used as described, for determining the proteid in solution, gives very reliable results. The very striking constancy in this physical property observed after fractionation, offers strong evidence that the product is homogeneous. Apart from the estimations just given I have in the course of preparing the proteid for other purposes, made estimations of the rotatory power in product from eleven different supplies of eggs. In these fractionation was carried generally to the second stage, sometimes to the third. The extreme variations were from 30·51° to 30·75°—the mean was 30·61°.

T. B. Osborne[1] has prepared and analysed crystalline products from egg-albumin obtained by the acid process described by Pinkus and myself. For one preparation he employed acetic acid and for another hydrochloric acid. He found that the crystalline product separated more rapidly with the mineral acid, though the ultimate yield was somewhat larger with acetic (80 grammes from 1500 c.c. of egg-white). After separating the product produced by the original addition of the acid Osborne obtained three other fractions from the filtrates. The first of these in each case agreed very closely in properties with the original precipitate, the later ones showed higher rotatory power and a greater percentage of sulphur. The latest fractions (the fourth) were not crystalline however.

[1] *loc. cit.*

The first two fractions obtained by the use of acetic acid agree in all respects, save that of their content of ash, very closely with the product I have described; as will be seen on comparing Osborne's figures with those given above:

	Acetic acid I. (Osborne)	Acetic acid II. (Osborne)
Rotatory power	29·17°	29·23°
Carbon	52·60	52·61
Hydrogen	7·02	6·94
Nitrogen	15·54	15·76
Sulphur	1·61	1·612
Ash	0·87	

Osborne did not recrystallize these products before analysis, and this, as I have shown, is necessary to obtain a fully homogeneous preparation. Moreover the difference (which is not great) between his determinations of rotatory power and my own, is I think chiefly due to the different method adopted for determining the percentage of proteid in solution. Osborne evaporated the solution to dryness, weighed the residue, and deducted ash. This is doubtless preferable to any coagulation method if ammonium sulphate can be first completely got rid of; but to remove this efficiently takes so long that there is some danger of the proteid decomposing. Coagulation, with the precautions described above, yields I believe more reliable results. Moreover, I have myself on more than one occasion obtained a rotatory power of under 30° for products produced at the first stage of acidification; though one recrystallization of these has always yielded material giving a rotation agreeing exactly with the figures of the above series.

Taking these considerations into account the agreement between Osborne's figures and my own is very satisfactory. Our analytical results differ however in a remarkable way from those published by Hofmeister and F. N. Schulz.

	Carbon	Hydrogen	Nitrogen	Sulphur
Hofmeister[1]	53·28	7·26	15·00	1·18
Schulz[2]	52·26	7·40	15·19	1·23

Hofmeister's determinations were made upon crystalline material obtained without the use of acid. Schulz employed sulphuric acid in preparing his product. The mean of 1·18 as a value for the sulphur was calculated by the first of these observers from four determinations

[1] *loc. cit.*

[2] *Zeitsch. f. physiol. Chem.* XXIX. p. 88. 1900.

(1·01, 1·18, 1·24, 1·27; the last two determinations being made by Schulz, using the Asboth-Düring process). The mean of 1·23 Schulz obtained from two estimations (1·29, 1·18) made by Hammarsten's method.

I am unable to explain in any way this remarkable difference in the percentage of sulphur. It should be noted that Osborne has obtained a percentage of 1·70 from a crystalline product prepared in accordance with Hofmeister's most recently described procedure (*i.e.* "sowing" the proteid and ammonium sulphate mixture with crystals previously obtained). By the same method I prepared a product in which I found 1·64 per cent. (without recrystallization) and 1·59 per cent. (after recrystallization).

Notwithstanding the fact that the alcohol coagula upon which the analyses of my pure product were made were washed with extreme care, and although the figures for sulphur agreed satisfactorily with those of Osborne, and with those which Bondzynski and Zoja obtained from their less soluble fractions, one felt that in view of Hofmeister's results (those of Schulz had not yet been published) a very rigorous proof was necessary that the higher results were not due to unremoved sulphate. Hofmeister's suggestion that the higher percentages might originate from admixture with amorphous proteid (for the mixed albumins present in egg-white yield a percentage of sulphur which is undoubtedly higher: Hammarsten found 1·67—1·93) is certainly not applicable to the products analysed by me. Feeling that the question of sulphur-content could be better settled if an electrolyte, or mixture of electrolytes, other than sulphates, could be found, under the influence of which the proteid would crystallize, I have—doubtless in common with other observers—spent much time in an endeavour to find such. I have tried many and various salts in various combinations with different acids; but hitherto quite without success. There appears to be something highly specific in the influence of the ammonium sulphate of Hofmeister's original discovery. But I eventually found a medium—one familiar enough to physiological chemists—by means of which the proteid crystals may be washed free from sulphate, while themselves remaining intact. This is saturated sodium chloride solution, containing acetic acid.

V. Data obtained after washing the intact and uncoagulated crystals free from sulphate.

The pure egg-albumin described in this paper is wholly precipitated from its solution in an amorphous form by saturation with common salt and the subsequent addition of acetic acid. (The saturation however must be very complete, and the acid must reach a strength of nearly 1 per cent. The solubility increases rapidly with even slight diminution in the amount of salt present.) The acid salt solution was therefore tried as a medium for washing the crystals free from sulphate, and with complete success. If distilled water be fully saturated with sodium chloride and contain 1 per cent. acid, the crystals remain quite unaltered when suspended in it, and retain their form perfectly during the somewhat prolonged treatment necessary for removing all the ammonium sulphate present.

That the form and doubly refractive power are fully retained is better demonstrated upon large crystals obtained by slow crystallization of the albumin[1]; and best of all upon the large well-formed prisms obtainable from horse-serum (for these are equally well conserved in the medium). But even with the small acicular crystals which the egg-proteid yields in the process described in this paper the conservation of form can be shown with high magnification; and the fine silky lustre of the crystalline precipitate which is seen when it is suspended in the medium and stirred, is still observable when the last trace of sulphate has been washed away.

After this treatment the crystals remain fully soluble in water, and I find the rotatory power of the proteid to be quite unaltered.

Solutions, however, containing only so much chloride as may be adherent to the crystals before they are dissolved, together with the corresponding trace of acetic acid, will grow opalescent upon standing and may even slowly precipitate to some degree, the precipitate being highly insoluble in water. I am unable to explain this phenomenon, but it is certainly not due to any chemical change occurring during the washing of the crystals. The solutions are at first perfectly clear, and the rotatory power, being then determined, is found, as I have

[1] Very large and well-formed crystals can frequently be obtained by the acid process as we originally described it, adding however only a minimal excess of acetic acid. The proportion of ammonium sulphate is for this purpose best increased to a little short of precipitation point before adding the acid. The mixture should then remain undisturbed in a closed flask.

said, to be absolutely unaltered. Moreover, if later on, when some precipitate has formed, the solution be filtered, the proteid still contained in the clear filtrate again shows no change in rotatory power, and the whole may be again crystallized by means of ammonium sulphate in a perfectly typical manner, possessing then all its original properties.

If therefore a chemical and not a mere physical change is involved in the phenomenon in question, it certainly occurs only after solution, and the product separates *pari passu* with its formation. It is the result apparently of some specific influence of the chloride, for if one adds to a solution of ordinary egg-albumin, which has undergone no preliminary treatment, common salt up to some 4 or 5 per cent., and then acid in an amount which under other circumstances has certainly no effect upon the proteid, this insoluble precipitate is slowly formed. If on the other hand the albumin be wholly thrown out of solution by saturation with the salt in the presence of 1 per cent. acid, the precipitate remains so far as I have observed indefinitely capable of being redissolved.

Whatever may be the nature of the change, its occurrence does not detract in the least from the value of the method for washing the crystals, which quite certainly remain wholly unaltered during the treatment.

The opportunity thus offered of washing the intact crystals perfectly free from adherent sulphates is of value from two points of view.

In the first place it yields an important method of checking the sulphur estimations made after washing coagula direct; as one may clearly be more certain of washing away the last traces of sulphate from a crystalline precipitate than from a coagulum. In the second place it offers the possibility of observing whether, when the adherent sulphate of the mother liquor of the crystals is fully removed, there remains any of the salt molecularly associated with the crystals, which, if present, we should expect to be dissociated from the proteid on solution. To this latter point I will return.

Two distinct preparations of the pure proteid were made; the one (M) being recrystallized twice; the second (N) recrystallized once; ammonium sulphate being employed in the usual way. The crystalline precipitates (each containing about 10 grammes proteid) were collected at the pump upon fine platinum gauze and when well drained were transferred into about a litre of the acid sodium chloride solution (carefully saturated with the salt). After gentle stirring the crystals

were allowed to settle for some hours, the supernatant fluid poured off, and the precipitate again collected on the gauze. This process was many times repeated; two or three washings being given after the stage when the liquid (diluted) ceased to show any trace of opalescence with barium chloride.

The crystals, which were quite obviously intact in each case, were then dissolved in water, the solution filtered and a portion of it while still perfectly clear poured into alcohol. In the remainder the rotatory power was observed, and the percentage of proteid determined (*vide* protocols at end of paper). The solutions became slightly cloudy after standing for four hours.

The alcohol-coagula were washed free from chlorides in exactly the same way as was employed to remove sulphates in the earlier experiments, and received similar subsequent treatment.

The following results were obtained from these two preparations:

	αD	Carbon	Hydrogen	Nitrogen	Sulphur
M gave	30·61°	52·66	7·00	—	1·57
N gave	30·67	52·71	6·99	15·41	1·55

VI. Is ammonium sulphate molecularly associated with the proteid in the crystals?

The alcohol used to coagulate the preparations described in the last section was concentrated nearly, but not quite to dryness; the copious aqueous washings of both coagula were concentrated to small bulk, and all the liquids were mixed. Finally the mixture was reduced by boiling down in a beaker to about ¼ litre and acidified with HCl. There was now a very faint opalescence produced on the addition of barium chloride. On digesting for some hours on the water-bath a minute precipitate of barium sulphate separated, which was found to weigh 4 milligrammes, representing 2·3 milligrammes of ammonium sulphate. The proteid contained in the two coagula taken together weighed 11·2 grammes; or there was roughly, 5000 of proteid to 1 of ammonium sulphate. As the molecular weight of the latter is 130, it seemed unlikely that the extremely small proportion found could be of significance in pointing to a molecular association with the proteid.

I prepared however a further quantity of crystals and washed them with the acid salt solution (using for the preparation of this, carefully purified sodium chloride) some six or eight times after sulphates had

ceased to be detectable in the washings. The intact crystals were dissolved in water, poured into alcohol, and the washings of the coagulum treated as before. No trace of sulphate could be detected although the dried proteid weighed over 12 grammes.

It may be that such results are not actually conclusive, and that associated ammonium sulphate might be removed from the crystal during treatment, without altering its more obvious optical characters. It is however very difficult to see what kind of evidence could be obtained with regard to this question other than the proof that washing may leave the form of the crystal intact and the proteid unaltered, and yet remove all sulphate. Such evidence appears to suggest very strongly that the proteid crystals are formed under the influence of the electrolyte, but not in association with it.

VII. The ash.

There remains for consideration the not unimportant question of ash, the percentage of which in my preparations was surprisingly low. I early observed that quantities of material such as used for carbon combustions left no residue which could be weighed with certainty. After I had seen some estimation of ash by other observers, more particularly those by Osborne, who found as much as 0·87 per cent. in a crystalline product prepared with the use of acetic acid, my own results became a matter for surprise. I therefore made estimations upon large quantities of material—3 to 4 grammes (cf. protocol at end)—in the case of certain of the fractions which had been analysed. I was unable to do this upon all, as much of the material of each fraction had not been coagulated for analysis, but was employed for the preparation of bromine derivatives which were under study at the time. The estimations, which were all made upon later fractions, gave a mean of no more than ·063 per cent.

With so low a proportion as this it seemed probable that any molecular inorganic matter was as a matter of fact absent from the crystals, and that the small amount found was due to contamination. I recently prepared a quantity of the proteid for the special purpose of making a very careful estimation of ash. All my preparations had been made with very pure ammonium sulphate; and on this occasion I used distilled water specially prepared, for washing the coagula. The original product was recrystallized twice.

Ten grammes were incinerated in a muffle furnace at low red heat;

the material being added to the platinum dish in small quantities at a time. When reduced to a small bulk the carbonaceous residue was transferred quantitatively to a weighed platinum boat, and burnt at a low red heat in a current of oxygen. The residue weighed 3·2 milligrammes only—a percentage of ·032.

One obtains therefore by the process described in this paper what is practically an ash-free product. The estimations were made of course upon coagula very thoroughly washed; but it seems exceedingly probable that the crystals themselves contain no molecular inorganic matter.

VIII. Analyses of heat- and mechanical-coagula.

All the analyses hitherto detailed were made, as was stated, upon alcohol-coagula. It is almost, if not quite, essential to coagulate the proteid for analysis after crystallization by means of ammonium sulphate. Most workers have employed alcohol-coagula. If any chemical change is involved in coagulation by cold alcohol it is almost certainly one of simple dehydration, while heat coagulation may certainly involve something more.

I have made however partial analyses of heat-coagula, and of the highly interesting mechanical-coagula studied by Ramsden, to the formation of which egg-albumin is especially prone. I do not propose however to burden this paper with further analytical details, because such results as I have obtained in this connection do not bear upon the proof of the purity of the proteid.

Of the heat coagula I need merely say that when the albumin in aqueous solution is coagulated at different temperatures, and even when a solution is poured directly into boiling water, the percentages of sulphur obtained do not apparently show the stable values shown by alcohol-coagula. In the case of a native solution of the proteid containing associated alkaline salts this would be expected. It is certain for instance that some albuminate is formed when dilute egg-white is boiled; and hydrogen sulphide is liberated when such a boiled solution is afterwards acidified: my experiments seem to indicate however that some loss of sulphur occurs on heating even when the proteid is quite free from associated alkali[1].

[1] This is apparently not the case however when the proteid is precipitated before heating. When for instance excess of ammonium sulphate is added before the temperature is raised, the heat coagulum which results appears to be of identical composition with an alcohol-coagulum.

It seemed of more particular interest to compare the composition of a coagulum produced mechanically, with one derived by such a process as treatment with alcohol; if agreement was not obtained any differences found could hardly fail to be instructive.

I obtained a highly satisfactory supply of mechanical-coagula by placing strong glass bottles containing the proteid solutions (whether they contained ammonium sulphate or not) in a 'railway truck' shaking apparatus, the yield being especially good when a few inches of play was allowed between the bottle and the padded ends of the truck. The sharp impacts which then occur during shaking seem greatly to favour coagulation. I have often obtained under these circumstances as much as 50 per cent. of the proteid in a wholly insoluble condition after four or five hours' shaking.

But on working with moderately large quantities of material, so that 8 to 10 grammes or more of coagulum are formed, the remarkable fact comes to light that sulphur is undoubtedly liberated, either as hydrogen sulphide or some other volatile sulphide, during the process of coagulation. If the vessel be allowed to stand until the froth has broken down, a lead paper suspended above the liquid containing the coagulum is immediately blackened, and the smell of sulphuretted hydrogen may be quite obvious. The result is of course not due to putrefactive changes. It may be obtained after a few hours' shaking of perfectly fresh material and in the presence of antiseptics[1].

The phenomenon is of no little interest as illustrating the instability of a sulphur combination in the molecule of egg-albumin; but I am at present quite unable to throw any light upon its relation to the process of mechanical coagulation. For, to my surprise, when determinations of sulphur came to be made upon the coagula, it was found to be practically identical with that of the alcohol-coagula, the lowest figure obtained from a mechanical-coagulum being 1·51 per cent., and the mean of four determinations was 1·54 per cent. It can scarcely be, therefore, that loss of sulphur is part of the coagulative process; but rather that a part of the proteid undergoes some secondary change, perhaps analogous to that occurring (under certain conditions) during heat coagulation. I have not determined the amount of sulphur given off in any case, nor have I been able to study the phenomenon any further. I originally prepared mechanical-coagula as material for analytical study which might supplement the data obtained from

[1] Dr Ramsden in a private communication tells me that he has himself observed this phenomenon.

alcohol-coagula, in proof of the composition of the proteid; but the occurrence of this liberation of sulphur and the obscurity of the process involved make the coagula unsuitable material for this purpose.

IX. Concluding remarks.

To those accustomed to work only with materials which can be crystallized from pure solvents such methods as have hitherto proved applicable to proteids must appear cumbersome. It is likely however that a special technique will always prove necessary in dealing with most members of the proteid group; and that the absence of this has been one of the chief reasons for the delay in their study upon purely chemical lines. The great importance of Hofmeister's discovery lies in the fact that it is probably destined to form a foundation for the elaboration of such a technique. The extraordinary ease with which crystals are obtained in the case of an egg-albumin and certain serum albumins, when the conditions are properly defined, offers a reasonable expectation that all members of the group may eventually be obtained pure.

For the proof of chemical individuality in a proteid it is clear that constancy of empirical composition (if we except the datum of sulphur-content) is of little moment. Proteids that are undoubtedly different may vary in this respect only within limits which are commensurate with errors of analysis. In selecting some physical character, with regard to which a proof of constancy may be held to offer good evidence of purity, one is undoubtedly justified in choosing rotatory power. Not only does this vary greatly in proteids of different origin, but it is markedly affected in any instance by treatment which involves any degree of decomposition.

In studying the product described in this paper it seemed desirable therefore to determine this factor upon each crystalline fraction; and the great constancy obtained in the numerous analyses thus made, offers evidence of purity and for the stability of the product during treatment, which cannot, I think, be ignored.

The temperature of heat coagulation I have not employed as a test of purity, finding it to vary largely with the amount of salt present. In products which have been prepared by the use of ammonium sulphate we have no method of removing the adherent salt (save by replacing it by another) except dialysis. I possessed no apparatus by which the sulphate could be thus efficiently removed, without long exposure under

conditions in which decomposition is to be feared. Moreover no evidence of value is to be obtained from an observation as to whether the proteid does or does not wholly separate from solution within a given range of temperature. This depends so entirely upon conditions, that a failure to separate completely—in the absence of a certain minimum of an electrolyte—is certainly no proof of admixture. The change of condition upon heating, which was discussed in an earlier section, will often account for part of the proteid not being aggregated by heat[1].

In determinations of rotatory power after crystallization with ammonium sulphate, it is of special convenience that the rotation can be observed, and the percentage of proteid determined, without removal of adherent salt, so that the constant can be determined for a crystalline product directly it is prepared.

The percentage of sulphur—apart from its importance in the calculation of minimal values for molecular weight—is certainly of value as a test of purity. This again is a factor which varies greatly in different proteids, even of the same class, and it is easily affected by destructive treatment.

It is greatly to be regretted that there exists such conflicting evidence with regard to its value in crystalline egg-albumin. In the numerous estimations made upon the product described in this paper which have led to the mean value of 1·57 per cent., the very greatest care was taken to ensure accuracy; and I think that the close agreement with this figure which was yielded by products from which all sulphate was removed prior to coagulation, gives important support to its correctness. The close agreement with Osborne's results (1·61 per cent.)—of which I was wholly ignorant when most of my determinations were made—obtained from a product which differs from mine only (I venture to think) in being a little less pure[2] is also confirmatory.

The observations I have myself made, in connection with the work of this paper, do not necessarily indicate that more than one albumin capable of crystallization is present in egg-white—probable as I believe this to be from the work of others. If to the filtrates from the acetic acid precipitate which has formed the starting-point for my preparations, further ammonium sulphate be added, additional quantities of proteid separate, with a rotatory power which most certainly increases with the quantity of salt necessary for precipitation. Under these conditions of preparation however the later fractions do not crystallize

[1] Upon the behaviour of the crystalline albumin on heating cf. Osborne, *loc. cit.*

[2] I ought to say that Osborne in the paper quoted (*loc. cit.* June 1899) has stated his intention of studying the fractionated product.

with the ease of the first product; and I have never obtained, even those which stand nearest to the acetic acid precipitate, absolutely free from some amorphous admixture. It is possible therefore that the rise of rotatory power is due to such admixture, and that the crystals present really belong to the proteid which is present nearly pure in the acetic acid precipitate itself. In this connection the large yield of the latter (under the conditions I have defined) must be remembered. It is certain that any crystalline material obtainable after its removal bears a very small proportion to the acetic acid precipitate itself. Whether the pure product has to be separated from other crystalline albumins, or only from amorphous proteid, the process described in this paper offers a means of attaining to purity with great ease, and of obtaining a highly satisfactory yield of product.

The serum of the horse yields crystalline products under treatment similar to that described for the egg-proteid, and with no less ease and rapidity. I was led to pursue the endeavour to separate a homogeneous product from the latter rather than from the former, because data obtained from a preliminary study suggested that the matter would prove easier. My experiments suggested that in the serum there are at least two crystallizable albumins (as found by Gürber himself), of which the solubilities lie nearer together than do those of the albumins of egg-white. I found it, at least, more difficult to arrive at a product with a rotatory power which was satisfactorily constant. It is certainly much more difficult to obtain products yielding solutions free from pigment.

I did not, in my experiments, discover any important differences in the influence of sulphuric and acetic acid, respectively, upon the rate of crystallization. I may quote the following observations bearing upon this point: Ten per cent. acetic acid added to the albumins of three different specimens of horse-serum half-saturated, as usual, with ammonium sulphate up to the point of commencing precipitation, induced crystal formation, in all three, within one hour. Normal sulphuric acid had precisely the same effect; whereas addition of the more dilute acid used by Krieger ($\frac{1}{5}$th normal), owing, apparently, to the greater dilution involved in bringing the mixture to the requisite acidity, produced no crystals until after three hours. After 24 hours all the preparations were equally crystalline; and the yield in each case was almost exactly the same. The crystals produced by the more dilute sulphuric acid were somewhat larger than those in the other preparations.

In preparations from oxalate plasma obtained from the same three

animals, normal sulphuric acid brought about crystallization within two hours; ten per cent. acetic not producing the result till the preparations had stood over-night. But when to the albumin mixture from the oxalate plasma, extra ammonium sulphate solution was first added, up to a point just short of precipitation, the acetic acid, no less than the sulphuric, yielded a crystalline product in less than one hour. There is no doubt that the rapidity depends greatly upon conditions, and varies *inter alia* with the relative proportions of the salt and acid added. By a close study of such conditions it is likely that crystallization may be obtained in more refractory sera.

One matter is worthy of notice here. In my experience the first product from serum, when acid (at least, acetic acid,) has been used, is never wholly crystalline, though upon recrystallization it always becomes so. But the slight amorphous admixture is not an albumin, but a phosphorus containing proteid (probably the nucleo-proteid) of the serum. It is left wholly undissolved upon solution of the crystalline albumin. I am at present studying this substance.

SUMMARY.

A procedure is described by means of which a crystalline albumin may be separated from egg-white, which upon repeated fractional crystallization shows absolute constancy of rotatory power (– 30·7°), and a constant proportion of sulphur. The product is obtained with great ease and rapidity, and the yield is 50 grammes and upwards per litre of egg-white.

The albumin has the following percentage composition

C 52·75, H 7·12, N 15·43, S 1·57, O 23·13.

It is obtained practically ash-free.

It is shown that albumin crystals, obtained by the use of ammonium sulphate, may be washed wholly free from sulphate by means of a saturated sodium chloride solution containing 1 per cent. acetic acid; the crystals meanwhile retaining their form and their solubility in water.

The crystals being thus freed from adherent sulphate and dissolved in water, coagulation of the dissolved proteid liberates no further trace of sulphate. It is unlikely therefore that ammonium sulphate enters into the formation of the original crystal.

In the last section some remarks will be found upon the crystallization of serum albumins.

Analytical Data.

Rotatory Power.

	Rotation observed in 4 decimetre tube	Percentage of proteid in solution		Rotation observed in 4 decimetre tube	Percentage of proteid in solution
A^2	11·06°	8·984	C^3	11·10°	9·020
A^3	13·12	10·648	C^4	10·98	8·960
A^4	9·88	8·050	C^6	13·72	11·220
A^5	11·47	9·323	D^2	8·19	6·660
B^2	8·22	6·680	D^4	13·02	10·600
B^3	8·51	6·936	D^5	8·22	6·668
B^4	6·65	5·408	M	6·60	5·390
B^5	8·44	6·877	N	5·98	4·874
C^2	13·04	10·630			

Combustions.

A^2 0·1916 grm. gave CO_2 ·3697 grm., H_2O ·1229 grm.
0·1970 ,, ,, 26·5 c.c. moist N at 22·5° and 770 mm.

A^3 0·2025 grm. gave 27·2 c.c. moist N at 26·5° and 765 mm.

A^5 0·2100 grm. gave CO_2 ·4067 grm., H_2O ·1363 grm.
0·2800 ,, ,, 38·0 c.c. moist N at 21° and 756 mm.

B^2 0·2000 grm. gave CO_2 ·3873 grm., H_2O ·1289 grm.

B^3 0·2100 grm. gave 28·6 c.c. moist N at 21° and 760 mm.

B^4 0·1985 grm. gave CO_2 ·3835 grm., H_2O ·1266 grm.

B^5 0·2020 grm. gave 27·3 c.c. at 22·5° and 768 mm.

C^2 0·2705 grm. gave 34·7 c.c. moist N at 13·5° and 770 mm.

C^6 0·1995 grm. gave CO_2 ·3850 grm., H_2O ·1280 grm.
0·2925 ,, ,, 40·15 c.c. moist N at 21° and 756 mm.

D^2 0·2210 grm. gave CO_2 ·4280 grm., H_2O ·1394 grm.
0·2490 ,, ,, 34·2 c.c. moist N at 21° and 756 mm.

D^5 0·2090 grm. gave CO_2 ·4035 grm., H_2O ·1339 grm.
0·2008 ,, ,, 27·0 c.c. moist N at 22° and 769 mm.

M 0·2221 grm. gave CO_2 ·4288 grm., H_2O ·1399 grm.

N 0·2145 grm. gave CO_2 ·4145 grm., H_2O ·1349 grm.
0·2200 ,, ,, 29·55 c.c. moist N at 22° and 769 mm.

Sulphur Estimations.

A^2	0·7165 grm. gave ·0826 $BaSO_4$		C^2	0·573 grm. gave ·0676 $BaSO_4$
A^3	0·6369 ,, ,, ·0733 ,,		C^4	0·9554 ,, ,, ·1102 ,,
A^5	1·100 ,, ,, ·1249 ,,		C^6	0·7075 ,, ,, ·0802 ,,
B^2	1·140 ,, ,, ·1326 ,,		D^2	1·00 ,, ,, ·1140 ,,
B^3	1·004 ,, ,, ·1140 ,,		D^5	1·00 ,, ,, ·1096 ,,
B^4	1·010 ,, ,, ·1162 ,,		M	0·800 ,, ,, ·0914 ,,
B^5	0·798 ,, ,, ·1120 ,,		N	1·001 ,, ,, ·1130 ,,

Estimation of Ash.

A^4	3·00 grms. gave 0·0018 ash		C^4	3·50 grms. gave 0·0028 ash
A^5	3·00 ,, ,, 0·0016 ,,		C^6	3·50 ,, ,, 0·0024 ,,
B^5	4·00 ,, ,, 0·0024 ,,		D^5	3·00 ,, ,, 0·0016 ,,