A STUDY OF BENCE-JONES PROTEIN, AND OF THE METABOLISM IN THREE CASES OF BENCE-JONES PROTEINURIA.

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CONTENTS.

SECTION I. DESCRIPTION OF THE CASES STUDIED.

THE condition known as Bence-Jones proteinuria should not fail to interest the physiologist as well as the pathologist. The appearance, in the course of a disordered metabolism, of protein material which escapes breakdown, and passes the kidneys in such amount that the nitrogen contained in it may amount to a third or more of the total nitrogen excreted; the peculiar characters of the protein, and the fact that, though a highly complex substance forming colloid solutions, it may be passed in large quantities and for long periods by kidneys which remain impervious to plasma proteins, and which are—to all appearance at least—histologically intact: these are aspects of the condition of significance for all concerned with the processes of metabolism and excretion. A proper understanding of the disturbances involved could hardly fail to throw light on normal protein metabolism. The physico-chemical properties of the excreted product deserve, too, the attention of all concerned with the study of colloid solution.

Considerably more than a hundred cases of the condition have now been recorded, and there is every reason to suppose that it is much

commoner than was formerly supposed. It has hitherto been found (whenever reliable evidence has been forthcoming) always in association with disease of the bone marrow; in the great majority of cases with myelomatosis (multiple myeloma); more rarely with lymphoid hyperplasia of the marrow (A. V. Descatello). It is possible that it may be associated with true osteomalacia (Jochmann and Schumm; Gascard).

It is not necessary for the purpose of this communication that we should attempt here any general survey of the literature of the subject[1]. After the first description of the condition by Bence Jones[2] in 1848, only seven cases were recorded during the fifty years which followed; but, when at the end of that period the publications of Kuhne[3], Kahler and Huppert[4], A. Ellinger[5], and, in this country, of T. R. Bradshaw[6] had recalled general attention to the subject, the record of cases grew apace. Of the more complete studies, which have yielded results related to our own, we may mention particularly those of Magnus Levy[7], of Abderhalden and Rostoski[8], and of Allard and Weber[9]. Other references will be given later as occasion arises. Magnus Levy's work was of great importance in giving to the excreted substance its right place in the classification of proteins, and in indicating that its peculiar behaviour when urine containing it is heated is due to the influence of other urinary constituents. Abderhalden and Rostoski for the first time submitted the material to modern methods of analysis and upon these lines confirmed Magnus Levy's view that it is of the nature of an intact protein and not a product of partial hydrolytic cleavage. They added the interesting fact that the urinary protein yields a precipitin active with human serum, and must therefore represent assimilated material and not an exogenous product diverted directly from intestinal processes. Allard and Weber followed its excretion

[1] Among the more recent summaries may be mentioned those of Grutterink and de Graaff, *Zeits. physiol. Chem.* XXXIV. p. 393. 1902; Parkes-Weber, *Trans. Roy. Med. and Chir. Soc.* LXXXVI. 1903; C. W. P. Moffatt, *Lancet*, Jan. 28, 1905. For the histological side: Parkes-Weber and Ledingham (cited in text). See also the Articles by Mohr in V. Noordon's *Hdb. d. Path. d. Stoffw.* II. p. 864; and A. Ellinger in Oppenheimer's *Hdb. d. Bioch.* II. (2), p. 657.

[2] Bence Jones. *Phil. Trans. Roy. Soc.* I. 1848.

[3] Kuhne. *Zeitsch. Biol.* XIX. p. 209. 1883.

[4] Kahler and Huppert. *Prager Med. Wochensch.* XIV. p. 35. 1889.

[5] A. Ellinger. *Arch. klin. Med.* LXII. p. 255. 1899.

[6] T. R. Bradshaw. *Trans. Med. Chir. Soc.* LXXXI. and LXXXII.

[7] Magnus Levy. *Zeit. physiol. Chem.* XXX. p. 200. 1900.

[8] Abderhalden and Rostoski. *Ib.* XLVI. p. 125. 1905.

[9] Allard and S. Weber. *Deutsch. med. Wochensch.* p. 1251. 1906.

quantitatively for a more prolonged period than others had done, the most important result of their work being the proof that the amount of its excretion was largely independent of the amount and kind of protein ingested.

The present paper deals, more fully than any previously published work, with three aspects of the subject; the physical chemistry of the protein, particularly as regards the influence of electrolytes in determining its peculiar behaviour on heating; the pure chemistry of the substance, and especially its content of various amino-acids; lastly the metabolism of individuals excreting it, mainly on the lines of a comparison between the amount of protein and of the total nitrogen excreted.

Our observations have been made upon the urine of three persons, a woman and two men. The general properties of the protein were studied with material from all three cases. A complete analysis of it was made in two; so that a comparison became possible. The metabolism was followed for a long period in the case of the woman; briefer observations being made in the other cases. In the woman necropsy established the existence of extensive myelomatosis. No *post mortem* examination could be made in the other cases.

Case A. Female, aet. 65. The case was met with in the private practice of one of us (H. S.). The patient was first seen in Nov. 1904, when she complained of severe lumbar pain. The condition of the urine was first recognised in Dec. 1904, though it might have existed earlier. For nearly three years after this the woman's general health was sufficiently good to allow her to carry out household duties, though she was anæmic, and subject to much pain. The pain spread to the sternum and long bones and continued with occasional exacerbations till the end. Kyphosis of the lower dorsal spine developed. A few months before death there was fracture of the surgical neck of the right humerus as the result of a very slight accident, but good bony union followed. Late in 1908 the patient was in the Bedford County Hospital under the care of Mr Gifford Nash, and she died there on Jan. 25th, 1909. The necropsy was made in the presence of one of us (F. G. H.) by Dr F. Parkes-Weber. A careful study of the marrow growth found was made by Parkes-Weber and Ledingham, a circumstance which add greatly to the value of our own observations, making the description of this case perhaps the most complete in the literature. Weber and Ledingham's observations have been published in full[1], the results need, therefore, only brief reference here. The sternum, ribs, clavicles,

[1] *Proc. Roy. Soc. Med.* p. 193. 1909.

humerus, and femur were all found transformed into mere shells of very hard brittle compact bone filled with a dark red opaque jelly-like substance which replaced the normal red marrow. If it be permissible to speak of this substance as bone marrow at all (we quote Parkes-Weber and Ledingham) the total amount of bone marrow in the body must have been immensely in excess of the normal amount. The bodies of the vertebrae at the site of the kyphotic bend consisted of hard bony shells containing only a remnant of the normal cancellous tissue which was replaced by a jelly-like growth, similar to that found in other bones. The lungs were pneumonic. The liver and pancreas showed no obvious disease. Microscopic examination of the kidneys showed nothing abnormal beyond scattered spots of a chronic interstitial fibrotic change of very slight degree (this observation was confirmed by us). Weber and Ledingham found that the cells with which the marrow was abundantly infiltrated showed all the morphological characters of plasma-cells. Little evidence of active mitosis was seen; but the mitotic figures, when present, were of an abnormal type. Degeneration of the cells was more in evidence than active proliferation. Outside the dense infiltrates all varieties of the ordinary marrow elements were present, together with plasma-cells which pervaded the whole marrow. The giant cells were not increased in number; eosinophil myelocytes were very numerous; and small erythroblastic foci were observed.

Case B. Male (A. B. W.) aet. 60. In Sept. 1906 there was severe lumbago. Urine free from protein. In Dec. patient had severe attack of influenza. In March 1907 the Bence-Jones protein was identified in the urine by Dr Gilbert Kempe of Salisbury. *March* 28: Hæmoglobin 48 per cent.; red cells 3,366,000; protein 24 parts per mille by Esbach. Treatment with Atoxyl begun, 2–4 grains being injected. During April increasing kyphosis was observed and there was much pain in the ribs. *May* 3: Protein by Esbach 22 parts per mille, Atoxyl discontinued. *May* 26: Protein 20 parts. Hæmoglobin 48 per cent. Count of red cells could not be made on account of rapidity with which blood coagulated. *June* 1: The anterior thoracic wall has fallen in so as to show a marked saucer-shaped cavity; much pain on movement of arms or thorax. *June* 4: Protein by Esbach 16 per mille. Increasing asthenia. *June* 8: Collapse of thoracic wall greatly increased; kyphosis more marked; patient computed to have lost six or seven inches in height; ribs and sternum soft and bending on pressure. The general condition suggests an increasing toxæmia. *June* 10, 1909: Patient died comatose. In health, patient had been a heavy man, weighing at

least 15 stone; but there was much wasting during the illness. As the protein was absent when the urine was examined on one occasion nine months before death, the condition would seem to have had a much shorter duration than in cases *A* and *C*; the disease being in all respects more acute. There was no necropsy. We are greatly indebted to Dr Gilbert Kempe for sending us carefully collected 24-hour samples of the urine, and for the above account of the case which is abbreviated from his notes.

Case C. Male (F. L.) aet. 47. At the time of our first examination of the urine, near the end of the year 1909, the patient was at the National Hospital, Queen's Square, London, under the care of Dr James Taylor. Up to 1900 he had been exceptionally healthy and an athlete. In that year he met with a spinal accident which led to paraplegia. He was later able to walk, but showed a spinal curvature which afterwards continued to increase. When examined in 1909 there was no conclusive evidence for the presence of myeloma. The existing pain was then, as always, largely confined to the knee-joint, which towards the end of life showed increasing stiffness. There was, at no time throughout the history of the case, any bending or fracture of the long bones, ribs, or sternum. The evidence from the spinal curvature was complicated by the fact that this began with the injury. Though there is no sufficient reason to doubt the existence of bone-marrow disease, it would seem to have been less extensive than in the other two cases. It is noteworthy that the occurrence of "albumen" in the urine was observed so far back as 1901, and a urinary protein was continuously present till Bence-Jones protein was identified in 1909. As the patient never showed any symptoms of nephritis, and as no trace of blood proteins was ever found after the Bence-Jones substance was once identified, it is extremely probable that the latter substance was present (and was the only protein present) from the earlier date. If so, the condition existed for a period of nearly ten years. The patient's average weight during health was 10 st. 2 lbs.; at the time of our analyses it was 7 st. 3 lbs. During 1910 he developed phthisis, and died on Dec. 4 of that year. No necropsy was made.

We have to thank Dr C. M. Hinds Howell for bringing this case to our knowledge, and Dr A. J. Edge of Maidenhead for information concerning the patient.

The behaviour of the protein in the urine was precisely the same in all three cases, and agreed with the descriptions published in connection

with other typical instances of Bence-Jones proteinuria. In all the specimens of urine which we examined the protein coagulated at temperatures under 55° C. The coagulation point varied from 45° to 55° in accordance with variations in the acidity and saline content of the urine. When, as occasionally happened, the urine was alkaline the addition of acetic acid was necessary to ensure complete coagulation, but such occasions were rare. On boiling, the coagulum almost always completely dissolved, leaving the urine perfectly clear. The rare exceptions to this occurred in case *C*; and the few specimens from this case which remained cloudy on boiling always cleared completely upon the addition of a soluble lime salt in small amount (cf. Section II).

Saturation with sodium chloride or magnesium sulphate in the cold gave no precipitate; ammonium sulphate precipitated the protein, the precipitation limits being between 45 and 55 per cent. saturation, though varying somewhat in different samples of urine. Ferrocyanide of potassium and acetic acid induced a characteristically slow precipitation. Strong mineral acids gave precipitates which redissolved on boiling, usually completely. The urine, even when highly diluted, exhibited a white ring of precipitate when poured upon strong HCl (Bradshaw's test). All these reactions characterise the typical protein. As the result of a few attempts only, on the lines of the method used by Grutterink and de Graaf, we have failed to obtain the protein in crystalline form. In no one of the cases did the urine at any time contain renal casts or blood proteins. In case *A* examinations for evidence of nephritis were made at intervals for a period of four years; always with negative results.

For the purpose of study the protein was thrown out of the urine by alcohol or by ammonium sulphate; an aqueous solution of the precipitate being afterwards thoroughly dialysed (for details of the treatment *vide infra*).

Section II. The special relations between Bence-Jones protein solutions and electrolytes.

Introductory. The most familiar characteristic of the protein—its solubility upon rise of temperature after preliminary heat coagulation at lower temperatures—is displayed only when certain other substances, and, especially—as we hope to show—electrolytes, are associated with it in solution. The importance of associated substances was first clearly indicated by the observations of Magnus Levy. For reasons to be

mentioned later, this observer gave, however, chief attention to the effects of urea, and studied but slightly the influence of electrolytes. He found, indeed, that of the few salts employed by him in this connection—ammonium-chloride, sodium chloride, magnesium chloride, and mixtures of neutral and acid potassium phosphate—only the first (AmCl) increased the solubility of the protein at 100° C. This limitation to the action of salts was certainly not to be observed with our material, nor do we believe it to exist in connection with typical Bence-Jones protein. On the contrary we believe that the substance yields a very interesting case of relation between a particular colloid solution and electrolytes in general. Further reference to Magnus Levy's[1] results can be more conveniently left to the sequel.

No later workers have concerned themselves in detail with the effect of salts upon the heat changes. Moitessier[2] remarks in connection with a case studied by him, that, after the urine had been dialysed, the heat coagulum obtained was "less soluble at 100 degrees, but recovered its solubility after the addition of NaCl."

Ville and Derrien[3] made a similar observation in another case. The original urine behaved typically. After dialysis it gave a thermostable coagulum, but solubility at 100° was restored by adding sodium chloride in amount equal to that originally present. Grimbert[4] reports an observation of precisely similar nature.

We have considerably extended these preliminary observations on the action of salts, and our experiments indicate a relation which appears to be interesting and suggestive. We have made no attempt, however, to study completely the equilibrium changes in the colloid-electrolyte-water system, which, in such a case as this, must, of necessity, be especially complex. Lacking the completeness necessary for a full statement of relation our results are here dealt with mainly from a descriptive standpoint, in the hope that they may suggest a direction for more complete studies when opportunities arise. At the same time we have arrived at a point of view concerning the action of salts with which the facts, so far as our observations have brought them to light, are in harmony. This we shall present to serve as a preliminary statement of relation. In the development of the theory of colloid solution

[1] Magnus Levy. *Loc. cit.*

[2] Moitessier. *Soc. Biol.* LVII. p. 498. 1905.

[3] Ville and Derrien. *Soc. Biol.* LXII. p. 679. 1907.

[4] Grimbert. *Journ. Pharm. Chim.* [6], XXVII. p. 97; also *Compt. Rend. Soc. Biol.* LXIV. p. 14.

every case with special characters must be included, and we believe that our results are sufficient to strengthen the view that the solutions of this protein present such a case.

Our significant experiments bear, almost exclusively, upon the solution of the heat coagulum at 100°; but, before describing these, it will be well to call attention to certain properties of Bence-Jones protein solutions, which, while of a less special character, play a part in determining the influence of salts upon the reversible heat changes.

The protein, as obtained from either of our cases, exhibits itself, when pure and in aqueous solution, as a typical "hydrophile" colloid. Its solution is of the albumin type, yielding no precipitate on dialysis, and exhibiting none of that sensitiveness to electrolytes in low concentration which is characteristic of a suspension colloid. When a neutral, salt-free solution, or, to state the case with more certainty, when a solution, approximately free from associated salts, and of minimal grade of acidity, is heated, it coagulates at a temperature of about 65° and the coagulum is thermostable, remaining undissolved at 100°. With somewhat higher grades of acidity in the solution, some slight tendency to reversibility in the temperature changes may be observed, even when salts have been removed as completely as is possible by dialysis—the coagulum diminishing slightly at 100° and returning to its original amount upon cooling. With yet greater concentrations of (acetic) acid, coagulation fails, in salt free solutions, at all temperatures. For the typical phenomenon of reversible heat change to be complete definite concentrations of salts are necessary.

As Kühne first observed, solutions of the protein, after treatment with sufficient amounts of acid or alkali yield precipitates upon neutralisation. This indication of metaprotein (albuminate) formation was emphasised by Magnus Levy as proving that the protein is not an albumose. After such treatment, or, if boiled in the presence of even very dilute acid or alkali, its salt-free solutions also come to show, as do salt-free albumen solutions similarly treated, the stigmata of a "suspension" colloid solution. Precipitation is then induced by electrolytes in very low concentration, and occurs in accordance with the fundamental law of Hardy. In acid solution the negative ion is prepotent as a precipitant, and in alkaline solution the kation.

In this connection an observation involving a comparison with ox-serum may be described. A solution of the protein, after prolonged dialysis, was boiled with alkali (N/800 NaHO) and acid (N/100 HCl) respectively. Ox-serum, fully dialysed, and diluted till of exactly the same concentration (1·32 per cent. protein) was similarly treated.

When salts were added to these solutions their orientation as precipitants was precisely the same in each case, and was in accordance with the familiar generalisation of Hardy; but there were quantitative differences. In the alkaline condition the Bence-Jones protein was less readily precipitated by positive ions than were the serum proteins, higher concentration of the salt, or higher temperatures, being necessary. Anions did not precipitate. On the other hand, in the acid state the Bence-Jones protein was more readily precipitated than the blood proteins by all negative ions (at lower concentration of salt, or lower temperatures for any given concentration). After boiling with N/100 HCl, as described, the Bence-Jones protein (but not the serum) was also readily precipitated even by such salts as $MgCl_2$, $CaCl_2$, $BaCl_2$, though of course at higher concentrations, or higher temperatures, than by salts with polyvalent anions. In the presence of higher concentrations of acid salts of the type of chlorides no longer precipitated. These results might be interpreted by assuming that the colloid particle in a Bence-Jones protein solution takes a negative charge more readily, and a positive charge less readily, than the particles of the serum proteins. The property will be found to be of significance in what follows. It makes it possible to study the influence of electrolytes in promoting coagulation and re-solution with a larger variety of salts when the solution is acid.

The effect of electrolytes upon the heat changes in solution: preliminary discussion. It is necessary, when our experimental results are under consideration, to bear in mind the general relations briefly set out in the foregoing paragraphs. In particular it should be remembered that the preliminary change which occurs when a solution of the native protein is heated—a change which, according to circumstances, may, or may not, be associated with actual coagulation—involves, in any case, such a change in its relation to electrolytes that it now approximates more nearly to one of the "suspension" type. When, it may be well to repeat, coagulation occurs in the absence of salts, as may happen in neutral solutions, or in solutions of very low grade acidity, no re-solution takes place on boiling. The coagulum is thermostable when electrolytes are absent[1]. In solutions definitely alkaline no preliminary coagulation occurs except when the protein content is high or when suitable electrolytes are present. When, under any circumstances, coagulation has occurred, the phenomenon of re-solution of the coagulum at 100° may, in all cases, be shown to have definite relation to the nature of the salts present in solution.

The description of our observations concerning salt action will be clearer if we give in advance some indication of their bearing. Experiment shows at once that the precise influence of any given electrolyte as a solvent is markedly affected by the presence of quite minimal concentrations of acid or alkali. In the acid condition the solubility of the coagulum in salts, upon rise of temperature, is favoured by

[1] Or unless weak bases such as urea are present; the effect of these is referred to later.

increasing valency of the positive ion; that is of the ion carrying a charge similar to that on the colloid particle, and opposite to the charge of the ion which (at lower temperatures) promotes coagulation. Other things being equal increasing valency of the latter ion diminishes the apparent solvent efficiency of a salt. In the alkaline condition, on the other hand, the negative ion is apparently prepotent. The solvent power of a salt at 100° increases with the valency of this ion and appears to diminish with an increase in that of positive ion. At first sight therefore the solution of the coagulum appears to be a function of the ions. But in passing from one of these opposing conditions to the other the colloid-salt-water system exhibits at one point a "neutral" phase, in which diverse salts tend to converge in respect of their solvent power, and in which ions of opposite charge appear to have equal potency. In this condition of the system the solvent power still increases with valency, but a salt, *e.g.* with monovalent basic ions and a divalent acidic ion exhibits the same potency as one with a divalent basic ion and monovalent acid ions. Thus, considering—for the moment—only salts of these two types, we have, as regards their influence in promoting solution of the coagulum, the relations:

acid:	$CaCl_2$ etc. > K_2SO_4 etc.
neutral:	$CaCl_2$ etc. = K_2SO_4 etc.
alkaline:	$CaCl_2$ etc. < K_2SO_4 etc.

In systems of neutral reaction the colloid particles are presumably electrically neutral or nearly so, and the phenomena of solution at 100° would appear to be always independent of charge. We may take therefore the standpoint that in acid or alkaline systems two distinct influences of any given salt may be opposed. Its solvent influence (however exerted) upon the molecules of protein may be opposed by the electric influence of one of its ions upon the colloid particles of the protein. In this case the salt will exhibit an (apparent) low solvent power. But when the electrical relations are such as not to oppose dispersion of the particles, the solvent power of such a salt is uncloaked, and appears to be relatively high. The true solvent power of the salt as a whole is, as already suggested, independent of electrical relations in the stricter sense; and is to be measured in uncomplicated relations only when the colloid particles are uncharged, that is when the reaction of the solution is "neutral."

The colloid-salt equilibrium is so sensitive to the very lowest concentrations of acid or alkali that the exact condition of neutrality, as

above defined in relation to salt action, is not easily demonstrated by the use of coloured indicators. The term "neutral point" is justified however, since it is a point which can always be approached from either direction during the process of neutralization. On either side of this point ionic charge intrudes as a prominent factor in influencing the apparent solvent power of a salt at 100°, and the ionic effects suffer a reversal in sign as the point is passed. When the point is just reached the complicating influence of ionic charge disappears, and the solvent power of any given salt is found to depend upon the qualities of the salt as a whole. Our results appear to show, indeed, that the coagulum dissolves because on rise of temperature the protein enters into molecular association with the salt. The special characters of the equilibrium in the case of the Bence-Jones protein (as compared, for instance, with globulins) are due to the fact that the association compound is stable only at higher temperatures. Further discussion may now await the description of our experiments.

Experimental. The solutions used for the experiments to be described were prepared by precipitating the protein from the urine either by saturation with ammonium sulphate, or, more usually, by the addition of alcohol; the precipitate being redissolved in water and the solution submitted to prolonged dialysis. The use of alcohol gave a product which was more free from pigment than that obtained by the use of ammonium sulphate. Precipitation by alcohol is, in most colloid systems, dependent upon the presence of electrolytes. In the case of proteins the rate at which they are rendered insoluble by alcohol is also greatly affected by the presence of salts. The character of the urine may therefore cause variations in the latter process. With material from cases A and B, for instance, it was comparatively easy to get, without special precautions, re-solution of the protein after precipitation by spirit. With that from case C it was usually necessary to precipitate ice-cold, and to filter or centrifuge as quickly as possible. As all the evidence in this paper points to identity in the proteins, we believe this difference to be due to variations in other constituents of the urine[1]. Repeated reprecipitation with spirit was in any case not

[1] It may explain certain contradictory statements in the literature if we note here the following observation. In our cases when the protein was separated from the urine by heat coagulation at 60° it was still (as would be expected from the facts given in this section) soluble in salts at 100°. If the coagulum was first thoroughly washed with water it could be subsequently kept for months under alcohol without losing this solubility; but if associated salts were not removed, alcohol, in their presence, slowly rendered the protein wholly insoluble.

possible, as with diminishing concentration of salts alcohol ceases to throw out the protein. As a rule, in our experiments, the first alcohol precipitate was centrifuged off, redissolved, thrown out for a second time with spirit, again centrifuged, and transferred, as a paste, to the dialysing tube. During the first few hours of dialysis the protein always went fully into solution. We usually dialysed for a week, the dialysis-tube being kept in motion by means of a water motor. The distilled water in the outer vessel was large in volume and was frequently changed. Chloroform was usually added to the outer liquid, but, as other observers have noted, the protein is remarkably resistant to bacterial decomposition. After such dialysis the water outside the tube would finally be found wholly salt-free, though the protein itself was never obtained completely free from ash by this method; about 1 per cent. ash being present in the dialysed product. The solution in the dialyser was usually very faintly alkaline to litmus, even when the protein had been precipitated from acid or amphoteric urine, and the dialysis indefinitely prolonged. On the other hand if acetic acid was previously added in excess, prolonged dialysis always failed to remove an acid reaction from the solution.

In comparing the solvent powers of different salts at 100° we adopted the simple plan of heating protein-salt-water mixtures in test tubes: observing that concentration of salt which was just sufficient to dissolve completely, after brief but brisk boiling, the coagulum which had first formed at some lower temperature. Equal quantities of a dialysed protein solution were measured into each of a series of tubes, and standard salt solutions in varying amounts added from small finely graduated burettes: water being added, also from a burette, in such quantity as to make the total volume of fluid in each tube the same. The tubes were first heated slowly until coagulation was apparently complete, and then as quickly as possible to boiling. With fresh preparations of the protein quite satisfactory end-points could be obtained; the transition from a mixture which remained opalescent on boiling to one which became crystal-clear, being quite sharp. Each

Exp. I. Protein from case A, precipitated from the urine with alcohol, and its solution dialysed for eight days. The solution as taken from the dialysis tube was very faintly alkaline to litmus, and faintly acid to phenolphthalein; it contained 1 per cent. protein, and was used, without treatment, for the observations described under I (c) (alkaline phase). The addition of 2·2 c.c. of 1 p.c. acetic acid to 400 c.c. produced the neutral solution, used for I (b); and the further addition of one drop of glacial acid to 200 c.c. of the neutral fluid yielded the solution used for I (a). The slight alterations in concentration of protein brought about by the above additions of acid were disregarded.

EXP. I (a). *Acid* condition. The solution heated alone, without salts, gave a thermostable coagulum not dissolving at 100°. Salts were added in M/2 solution.

Nature of salt added	Protein solution c.c.	Salt solution (half molecular) c.c.	Water c.c.	At 60° ±	At 100°[1]
NaCl	5	1·0	4·0	Heavy coagulum	Precipitate.
	5	2·0	3·0	,,	Slight precipitate.
	5	5·0	0·0	,,	Marked opacity.
	2	*8·0*	0·0	,,	Faint opacity.
$CaCl_2$	5	1·0	4·0	Coagulum	Opacity.
	5	1·2	3·8	,,	Slight opacity.
	5	1·35	3·65	,,	Very slight opacity.
	5	*1·40*	3·60	,,	Crystal clear.
$BaCl_2$	5	1·0	4·0	Coagulum	Op.
	5	1·2	3·8	,,	Sl. op.
	5	1·5	3·5	,,	V. sl. op.
	5	*1·6*	3·4	,,	Crystal clear.
$SrCl_2$	5	1·0	4·0	Coagulum	Op.
	5	1·5	3·5	,,	Sl. op.
	5	1·6	3·4	,,	V. sl. op.
	5	*1·65*	3·35	,,	Crystal clear.
$MgCl_2$	5	1·0	4·0	Coagulum	Op.
	5	1·5	3·5	,,	Sl. op.
	5	1·6	3·4	,,	V. sl. op.
	5	*1·65*	3·35	,,	Crystal clear.
K_2SO_4	5	1·0	4·0	Heavy coagulum	Precipitate.
Na_2SO_4	5	2·0	3·0	,,	,,
	5	3·0	2·0	,,	Op.
	5	5·0	0·0	Some coagulum	Sl. op.
	4	*6·0*	0·0	,,	Clear[2].
$MgSO_4$	5	1·0	4·0	Heavy coagulum	Sl. precip.
	5	2·0	3·0	,,	,,
	5	4·0	1·0	,,	Op.
	5	4·5	0·5	Coagulum	Nearly clear.
	5	*4·8*	0·2	,,	Clear.
$Na_3\overline{Cit}$	5	1·0	4·0	Heavy coagulum	Heavy precipitate.
	5	5·0	0·0	,,	Precip.
	3	7·0	0·0	,,	,,
	2	*8·0*	0·0	,,	,,

[1] In all cases, in this and other experiments (unless otherwise specified), the process was reversible; the coagulum after solution at 100° reappeared when the solution was cooled.

[2] In certain cases the mixture at 100° was so completely homogeneous as to show no trace of the Tyndall effect when held in a beam; in others, though at the final stage the solution was clear when viewed directly, some dispersion was visible in a beam, increase in the salt not affecting this. In the former case the expression "crystal clear" is used; in the latter "clear."

observation was made by means of a separate admixture. If the concentration of a salt is gradually increased in the same tube and the mixture boiled after each addition, good end-points are not obtained. The inertia of the solid phase tends to increase with successive heatings. To work with protein previously coagulated and washed, whether employed dry or in suspension, was also found disadvantageous when compared with the procedure described.

When the above simple method is used for comparing one type of salt with another the influence of the reaction of the fluid upon their

Exp. I (b): *Neutral* condition. The solution heated alone gave at 50° a thermostable coagulum, showing no tendency to dissolve at 100°.

Nature of salt added	Protein solution c.c.	Salt solution $\frac{M}{2}$ c.c.	Water c.c.	At 60° ±	At 100°
NaCl	5	2·0	3·0	Coagulum	Opacity.
KCl	5	3·0	2·0	,,	,,
LiCl	5	4·0	1·0	,,	V. sl. op.
(identical)	5	*4·2*	0·8	,,	Clear.
$CaCl_2$	5	1·0	4·0	Coagulum	Opacity.
	5	1·2	3·8	,,	Sl. op.
	5	1·3	3·7	,,	,,
	5	1·35	3·65	,,	V. sl. op.
	5	*1·40*	3·6	,,	Crystal clear.
$BaCl_2$	5	1·0	4·0	Coagulum	Opacity.
$SrCl_2$	5	1·30	3·7	,,	Sl. op.
$MgCl_2$	5	1·35	3·65	,,	V. sl. op.
(identical)	5	*1·40*	3·6	,,	Crystal clear.
$NaNO_3$	5	1·0	4·0	Coagulum	Marked opacity.
KNO_3	5	2·0	3·0	,,	Op.
	5	3·5	1·5	,,	Sl. op.
	5	*3·8*	1·2	,,	Crystal clear.
K_2SO_4	5	1·0	4·0	Coagulum	Opacity.
Na_2SO_4	5	1·2	3·8	,,	Sl. op.
	5	1·45	3·55	,,	V. sl. op.
	5	*1·50*	3·50	,,	Crystal clear.
$MgSO_4$	5	1·0	4·0	Coagulum	Opacity.
	5	1·5	3·5	,,	Sl. op.
	5	1·60	3·4	,,	V. sl. op.
	5	*1·65*	3·35	,,	Crystal clear.
$Na_3\overline{Cit}\ \frac{M}{50}$	5	1·0 $\frac{M}{50}$	4·0	Coagulum	Sl. op.
(freshly	5	1·2	3·8	,,	V. sl. op.
prepared	5	1·25	3·75	,,	Clear.
solution)	5	*1·30*	3·7	Sl. coag.	Crystal clear.

Exp. I (c). *Alkaline* condition. The solution without salts did not coagulate on heating.

Nature of salt added	Protein solution c.c.	Salt solution M/2 c.c.	Water c.c.	At 60° ±	At 100°
NaCl, KCl,	5	1·0	4·0	No coagulum	—
LiCl	5	5·0	0·0		
$CaCl_2$	5	1·0	4·0	Heavy coagulum	Precipitate.
	5	2·0	3·0	,,	Marked opacity.
	5	3·0	2·0	,,	Slight op.
	5	3·2	1·8	,,	V. sl. op.
	5	3·5	1·5	,,	Do. (not diminishing with increase of salt).
$BaCl_2$	5	1·0	4·0	Heavy coagulum	Marked opacity.
	5	2·0	3·0	,,	,, ,,
	5	3·0	2·0	,,	Sl. op.
	5	3·2	1·8	,,	Clear.
$MgCl_2$	5	1·0	4·0	Heavy coagulum	Opacity.
	5	2·0	3·0	,,	,,
	5	2·55	2·45	Coagulum	Slight op.
	5	3·0	2·0	,,	Clear.
K_2SO_4	5	1·0	4·0	No coagulum	—
Na_2SO_4	5	5·0	0·0		
$MgSO_4$	5	1·0	4·0	Coagulum	Sl. opacity.
	5	1·2	3·8	,,	V. sl. op.
	5	1·45	3·55	Slight coagulum	,,
	5	1·50	3·5	,,	Clear.
$Na_3\bar{C}i\bar{t}$	5	1·0	4·0	No coagulum	—
	5	5·0	0·0		

solvent properties must be studied at very low grades of acidity or alkalinity for reasons which will be clear in the sequel.

The "neutral" condition of solution, in which, for reasons already indicated, it is especially important to study the influence of electrolytes, could not (as already pointed out) be with certainty defined in advance by the use of indicators. After some preliminary knowledge of the relations had been gained our practice was to obtain the neutral point by comparing, at intervals during the process of careful neutralisation, the solvent powers of two such salts as $CaCl_2$ and K_2SO_4. When these become sensibly equal the "neutral" point is arrived at, and the influence of other salts can be studied. In this state the solution was always approximately neutral to sensitive litmus paper, but not always exactly so; the relative concentration of protein in solution being a factor of importance in determining the exactness of this relation. In

our experiments the "alkaline" solution was frequently the final contents of the dialyser, without added alkali. Only rarely did such a solution exhibit—in relation to salts—the properties of the neutral or acid condition. A portion of such alkaline preparations was reserved for experiment and to the rest very dilute acetic acid (N/100, or less) was gradually added in order to obtain solutions in the neutral or acid conditions respectively. Sometimes, on the other hand, the contents of the dialyser, after removal of salts, were made definitely acid with acetic acid, dialysis being then continued till acid ceased to pass the membrane. The final contents of the dialyser then served for observations relating to the acid condition, while neutral and alkaline preparations were made by addition to it of exceedingly dilute ammonium hydrate.

It will be easily understood that the relations in alkaline solution are the more difficult to demonstrate by the method used. The high solvent power of hydroxyl ions adds a complication. If the original solution does not coagulate when heated alone coagulation may also fail in the absence of a potent coagulating ion (polyvalent-kation) and the phenomenon of re-solution cannot of course be observed. But if the protein solution be a concentrated one and its grade of alkalinity

EXP. I (cc). Protein from Case A precipitated and dialysed as above. The solution from the dialyser was just faintly alkaline to delicate litmus paper, and was used without treatment. It contained 4·2 p.c. protein and coagulated, though not completely, when heated alone. The coagulum did not dissolve at 100°.

Nature of salt added	Protein solution c.c.	Salt solution *M*/2 c.c.	Water c.c.	At 60°	At 100°
NaCl	5	1·0	4·0	Coagulum	Precipitate.
	5	2·0	3·0	,,	Opacity.
	5	2·5	2·5	,,	Sl. opacity.
	5	3·0	2·0	,,	Do. (not diminished at higher concentrations of salt).
$CaCl_2$	5	1·0	4·0	Heavy coagulum	Opacity.
	5	1·5	3·5	,,	,,
	5	1·7	3·3	,,	Sl. opacity.
	5	*1·8*	3·2	,,	Clear.
K_2SO_4 / Na_2SO_4	5	1·0	4·0	No coag. at 60°, coag. at 70°+	Opaque.
	5	1·2	3·8	,,	V. sl. opacity.
	5	1·3	3·7	,,	,, ,,
	5	*1·4*	3·6	,,	Crystal clear.
$MgSO_4$	5	1·0	4·0	Coagulum	Opacity.
	5	1·5	3·5	,,	Sl. opacity.
	5	*1·6*	3·4	,,	Crystal clear.

exceedingly low the solvent power even of salts which lack a polyvalent positive ion may be at least orientated as in Exp. I (cc). The solution being so near the neutral point the differentiation between such salts as $CaCl_2$ and K_2SO_4 is but slight; but the relation found in the acid condition is nevertheless seen to be reversed.

The above experiments were carried out with solutions prepared from the protein of Case A. The following will show that similar relations held when the proteins of Cases B and C were employed.

EXP. II. Protein of Case B thrown out with alcohol, and its aqueous solution dialysed. Acetic acid was added in small amount and dialysis continued. Finally one third was reserved for observation, (II (a)), one third was neutralised with ammonia, (II (b)), and the remainder further alkalised until approximately ·001 normal (II (c)). The acid and neutral portions were so diluted as to contain the same concentration of protein as the alkaline preparation (1·8 %). Each salt was carefully tested in increasing concentration as in Exp. I, but only the beginning and end of each set of observations appears in the tables.

EXP. II (a). *Acid* condition. The solution coagulated completely when heated alone and the coagulum was thermostable.

Nature of salt added	Protein solution c.c.	Salt solution M/2 c.c.	Water c.c.	At 60° ±	At 100°
—	5	0·0	5·0	Heavy coagulum	Unaltered.
NaCl	5	1·0	4·0	Heavy coagulum	Precipitate.
	4	*6·0*	0·0	,,	Clear.
$CaCl_2$	5	4·0	4·0	Coagulum	Opacity.
	5	1·35	3·65	,,	V. sl. op.
	5	*1·40*	3·60	,,	Crystal clear.
$BaCl_2$	5	1·0	4·0	Coagulum	Opacity.
	5	1·45	3·55	,,	V. sl. op.
	5	*1·50*	3·50	,,	Crystal clear.
$MgCl_2$	5	1·0	4·0	Coagulum	Opacity.
	5	1·5	3·5	,,	Sl. op.
	5	*1·55*	3·45	,,	Crystal clear.
K_2SO_4	5	1·0	4·0	Heavy coagulum	Precipitate.
Na_2SO_4	5	4·5	0·5	,,	Sl. op.
	5	*5·0*	0·0	,,	Clear.
$MgSO_4$	5	2·0	3·0	Coagulum	Opacity.
	5	3·8	1·2	,,	Sl. op.
	5	*4·0*	1·0	,,	Clear.
Na_3Cit M/2	5	5·0	0·0	Heavy coagulum	Precipitate.
	2	*8·0*	0·0	,,	Do. (not clear at higher concentrations).

EXP. II (b). *Neutral* condition. The protein solution heated without salts gave a thermostable coagulum.

Nature of salt added	Protein solution c.c.	Salt solution M/2 c.c.	Water c.c.	At 60° ±	At 100°
—	5	0·0	5·0	Complete coagulation	Unaltered.
NaCl, KCl,	5	2·0	3·0	Coagulum	Opacity.
LiCl	5	4·0	1·0	,,	Nearly clear.
(identical)	5	*4·1*	0·9	,,	Clear.
$CaCl_2$, $SrCl_2$,	5	1·00	4·0	Coagulum	Opacity.
$MgCl_2$, $BaCl_2$	5	1·35	3·65	,,	V. sl. op.
(identical)	5	*1·40*	3·6	,,	Crystal clear.
$NaNO_3$	5	1·0	4·0	Coagulum	Marked opacity.
KNO_3	5	3·5	1·5	,,	Sl. op.
(identical)	5	*3·7*	1·3	,,	Crystal clear.
K_2SO_4	5	1·0	4·0	Coagulum	Opacity.
Na_2SO_4	5	1·35	3·65	,,	V. sl. op.
(identical)	5	*1·40*	3·6	,,	Crystal clear.
$MgSO_4$	5	1·0	4·0	Coagulum	Opacity.
	5	1·65	3·35	,,	Sl. op.
	5	*1·70*	3·30	,,	Crystal clear.

EXP. II (c). *Alkaline* condition. The solution heated without salts gave a well marked coagulum which did not wholly dissolve at 100°.

Nature of salt added	Protein solution c.c.	Salt solution M/2 c.c.	Water c.c.	At 60° ±	At 100°
NaCl	5	1·0	4·0	Coagulum	Opacity.
	5	2·0	3·0	,,	Sl. op.
	5	*2·2*	2·8	,,	V. sl. op. (not diminishing on further addition of salt).
AmCl	5	1·0	4·0	Coagulum	Marked opacity.
	5	2·0	3·0	,,	Opacity.
	5	3·0	2·0	,,	Sl. op.
	5	*4·0*	1·0	,,	V. sl. op. (not diminishing on further addition).
$CaCl_2$	5	1·0	4·0	Heavy coagulum	V. sl. opacity.
	5	1·5	3·5	,,	,, ,,
	5	*2·0*	3·0	,,	Clear.
K_2SO_4	5	0·5	4·5	Coagulum	Sl. op.
	5	0·7	4·3	,,	Nearly clear.
	5	*0·9*	4·1	Coag. at 80° ±	Crystal clear.
KNO_3	5	1·0	4·0	Coagulum	Opacity.
	5	1·5	3·5	,,	V. sl. op.
	5	1·8	3·2	,,	,, ,,
	5	2·0	3·0	,,	Crystal clear.

Exp. III. Protein of Case C precipitated with alcohol, dissolved in water and thoroughly dialysed. The solution was nearly "neutral" in the sense defined, though slightly acid to phenolphthalein. The addition of a minute trace of acetic acid produced the solution used in III (b). For III (a) one drop of glacial acetic acid was added to 200 c.c.

Exp. III (a). *Acid* condition. Thermostable coagulum in the absence of salts.

Nature of salt	Protein solution, c.c.	Salt solution M/2, c.c.	Water, c.c.	At 60° ±	At 100°
—	5	0·0	5·0	Heavy coagulum	Unaltered.
NaCl	5	1·0	4·0	Heavy coagulum	Coagulum.
KCl	4	*6·0*	0·0	,,	V. sl. opalescence, not clearing on further addition.
$CaCl_2$	5	1·0	4·0	Coagulum	Marked opacity.
	5	1·36	3·64	,,	Sl. opacity.
	5	*1·39*	3·61	,,	Crystal clear.
$BaCl_2$	5	1·0	4·0	Coagulum	Opacity.
	5	1·39	3·61	,,	Nearly clear.
	5	*1·41*	3·59	,,	Crystal clear.
K_2SO_4	5	1·0	4·0	Heavy coagulum	Coagulum.
Na_2SO_4	5	4·5	0·5	,,	Opacity.
	5	*4·9*	0·1	,,	Clear.
$MgSO_4$	5	1·0	4·0	Heavy coagulum	Coagulum.
	5	3·9	1·1	,,	Sl. opacity.
	5	*4·0*	1·0	,,	Crystal clear.
		M/100			
Cerium	5	1·0	4·0	Slight coagulum	Nearly clear.
chloride	5	1·2	3·8	V. sl. coag.	V. sl. opalescence.
M/100	5	*1·22*	3·78	,,	Crystal clear.

Exp. III (b). *Neutral* condition. Thermostable coagulum in the absence of salts.

Nature of salt	Protein solution, c.c.	Salt solution M/2, c.c.	Water, c.c.	At 60° ±	At 100°
—	5	0·0	5·0	Heavy coagulum	Unaltered.
NaCl	5	1·0	4·0	Heavy coagulum	Heavy coagulum.
KCl	5	2·0	3·0	Coagulum	Sl. coagulum.
	5	3·5	1·5	,,	Sl. opacity.
	5	3·7	1·3	,,	Clear.
$CaCl_2$	5	1·0	4·0	Coagulum	Opacity.
$BaCl_2$	5	1·35	3·65	,,	Sl. opacity.
	5	1·39	3·61	,,	Crystal clear.
K_2SO_4	5	1·0	4·0	Coagulum	Opacity.
Na_2SO_4	5	1·39	3·61	,,	V. sl. opacity.
	5	1·40	3·60	,,	Clear.
$MgSO_4$	5	1·0	4·0	Coagulum	Opacity.
	5	1·4	3·6	,,	,,
	5	1·6	3·4	,,	V. sl. opacity.
	5	1·62	3·38	,,	Crystal clear.
		M/100			
Cerium	5	1·0	4·0	Slight coagulum	Opalescence.
chloride	5	1·15	3·85	V. sl. coag.	V. sl. opacity.
M/100	5	1·2	3·8	,,	Crystal clear.

Salts with ions of high valency. In this as in kindred investigations it was difficult to extend the enquiry to the influence of salts containing ions of more than diad valency, because of the tendency to hydrolytic dissociation displayed by such salts. This is especially the case when the acid constituent has high valency. Salts of the higher members of the aluminium, or earthy, group of metals yield, as is well known, aqueous solutions which are neutral, and therefore suitable for our purpose. Observations made with some of these, though carried out on the same lines as before, are described separately here, because the effects of the high potency of the kation make it necessary to tabulate the results somewhat differently.

The triad ion exerts of course a very potent precipitating influence upon a (negatively) charged particle, but salts of these triad metals have also, in the case of Bence-Jones protein, an exceedingly high solvent power which increases rapidly with temperature. In the acid or neutral condition re-solution of the primary heat coagulum may occur at temperatures considerably below 100°, and even a comparatively low concentration of the salt may prevent coagulation altogether. In alkaline conditions of solution these salts in low concentration greatly promote and accelerate coagulation, or lower the coagulative temperature; but while still in comparatively low concentration, they dissolve the coagulum freely when the temperature rises. If the concentration of salt be sufficient, the solvent property cloaks the coagulant effect altogether, and there may be no coagulation at any temperature, even of the negatively charged protein. The solvent power increases more rapidly than the coagulative power with increase either of temperature or of salt-concentration. To define completely such relations extensive quantitative work would be necessary; but our simpler experiments establish what has been claimed. We append one set of observations only, in which salts of lanthanum and cerium were used. It should be noted that they were added in M/100 solution, instead of M/2 as employed for salts of lower valency. In acid or neutral solution a salt of a triad metal as will be seen from the tables dissolves the protein at 100° with a concentration less than one-fiftieth of that required in the case of a salt with a diad metal. It happens, in the example given, that solution occurs in the alkaline medium at a concentration very similar to that found for the acid or neutral state. It should be remembered that the cases are different, since, when alkaline, the solution without the salt did not coagulate at all on heating. With very low concentrations of the salt the expected powerful coagulant effect is observed, but with increasing concentration the solvent effect

so rapidly replaces the coagulative that a change in the reaction of the medium (which influences in particular the ionic coagulant action) produces less differentiation than when salts containing constituents of lower valency are concerned. With rise of valency in the constituent base the solvent action of salts would appear to increase more rapidly than the precipitating power.

EXP. IV. Protein from Case C thrown out with alcohol and very thoroughly dialysed. Concentration 1·5 %.

(A) Made very faintly acid to delicate litmus paper: the solution gave a stable coagulum when free from salts.

Nature of salt	Protein sol. c.c.	Salt sol. M/100 c.c.	Water c.c.	Before heating	On heating	At 100°	On cooling
Lanthanum nitrate,	5	0·5	4·5	No ppt.	Heavy coag.	Coagulum	Heavy coag.
	5	0·75	4·25	,,	Coagulum	V. sl. coag.	Ppt.
Cerium chloride (identical)	5	1·0	4·0	,,	Sl. coagulum	Nearly clear	Sl. ppt.
	5	*1·25*	3·75	,,	V. sl. coag.	Crystal clear	Sl. ppt. after long standing
	5	2·0	3·0	,,	No coag. at any temp.	,,	No ppt.

(B) Just alkaline to delicate litmus. Solution did not coagulate without salts.

Ib.	5	0·5	4·5	Ppt.*	Ppt. increased to a heavy coag.	Coagulum	Heavy coag.
	5	1·0	4·0	Rather more ppt.	,,	,,	,,
	5	1·1	3·9	Less ppt.	Increase less, and re-solution began at 60°–70°	Sl. coag.	Ppt.
	5	1·2	3·8	Still less ppt.	Increase slight	Nearly clear	Sl. ppt.
	5	*1·3*	3·7	,,	Increase doubtful	Wholly clear	V. sl. ppt.
	5	1·5	3·5	,,	,,	,,	,,
	5	2·0	3·0	V. sl. ppt.	Disappears below 60°	,,	,,

(C) Made as nearly neutral as possible. The solution coagulated completely without salts, and the coagulum was thermostable.

Ib.	5	0·5	4·5	No ppt.	Heavy coag.	Coagulum	Coagulum
	5	0·75	4·25	,,	Slight coag.	Not clear	Ppt.
	5	1·0	4·0	,,	,,	Nearly clear	,,
	5	1·1	3·9	,,	,,	Slight opalescence	,,
	5	*1·25*	3·75	,,	V. sl. coag.	Crystal clear	Sl. ppt. after standing
	5	2·0	3·0	,,	No coag. at any temp.	,,	No ppt.

* That metallic ions of high valency precipitate, in very low concentration, the native unheated protein (we have found them to do the same with native egg- and serum-albumins) would seem to indicate that there is no sharp distinction between the hydrophile and suspension types of colloid solution.

In this as in other cases of relation between electrolytes and colloids the special effect of acids and alkalies is such that a complete study of the equilibrium changes calls for extensive experimentation. Not only does the solvent power of acid or alkali, in the absence of salts, require determination, but variations in the concentration of acid and alkali need to be plotted against salt-concentrations as regards their simultaneous effects upon both coagulation and solution. In connection with the present subject the shift of equilibrium under various conditions during rise of temperature, from below the coagulation point up to the point of solution, calls particularly for study. Such observations we have had no opportunity of making.

It may be claimed for our experiments, however, that they are sufficient to indicate clearly the more essential relations upon which the quite peculiar physico-chemical properties of the Bence-Jones protein depend. They are concerned only with solubilities at the neutral point, and in media of very low grades of acidity or alkalinity; a limitation which is quite necessary, with the method used, if diverse types of salt are to be properly orientated as regards their solvent power. An exact estimation of the relative solvent capacity of divergent types of salt at 100° is possible only when the original protein solution coagulates completely, or almost completely, in the absence of salt; or, otherwise, when the coagulative intensity of the salts under comparison is of the same order. When the concentration of acid or alkali is sufficient to interfere with the primary heat coagulation, or to cause appreciable solution of the coagulum in salt-free solutions, the relations, upon adding salts, become, of necessity, complex. If (say) enough of either acid or alkali is present to prevent coagulation in a salt-free solution—and very little free alkali is sufficient for this—then, owing to the ionic-charge relations, the addition of one type of salt will produce at some temperature complete, or almost complete coagulation; whereas another type will produce little coagulum, or none at all. Obviously therefore the changes of equilibrium at 100° cannot be directly compared. It is of course quite easy, within a range which is considerable, to show the effect of increasing acidity in depressing the solvent power of any salt which is coagulative for the acid condition (*i.e.* those with polyvalent anions), and, *mutatis mutandis*, to show the effect of the increasing charge on the particle when alkali is increased. The typical behaviour, too, of the protein—the solubility of its coagulum at 100° and its reappearance on cooling—can be shown with higher grades of acidity, and even of alkalinity, provided only that *suitable* electrolytes are present. But divergent types of salt (*e.g.* $B''A_2 - B_2A''$) can only be quantitatively compared in one and the same medium when the reaction of this is near the neutral point.

In the experiments we have described the departure from neutrality was, on either side, so small that the above considerations intrude but little. Yet the low grade of acidity or alkalinity present was enough to impose a positive or negative charge, respectively, upon the colloid particles, and to bring to light the important effect of this upon the capacity of different salts to dissolve the coagulum at 100°. In the acid solutions the results are fairly quantitative, and, in the alkaline, good orientation is obtained.

In neutral systems our observations yielded results showing remarkable consistency in a quantitative sense; not only in duplicate observations with the same protein, but also when the protein of one case was compared with that of another.

The experimental results just presented (and many others were obtained of precisely similar significance) will, we think, support the standpoint which was suggested in an earlier part of this section as a convenient one from which to view the facts.

When the protein-salt-water system departs from neutrality, what is observed on heating a primary heat coagulum to 100° is the resultant

of two independent actions of the salt, which may be opposed: an ionic (electrical) effect exerted upon the colloid *particles* of the protein, and a solvent influence of the salt in (what may be presumed to be) *molecular* relations with the protein. Simpler relations obtain in neutral solutions alone. We have, as regards *solvent power*, the series:

Acid $CaCl_2 = BaCl_2$ etc. $> MgSO_4 > K_2SO_4$ etc. $> KCl = NaCl$ etc.
Neutral $CeCl_3 > CaCl_2 = BaCl_2$ etc. $= K_2SO_4$ etc. = (nearly) $MgSO_4 > KNO_3$ etc. $> NaCl$ etc.
Alkaline K_2SO_4 etc. $> MgSO_4 > CaCl_2$ etc. $> NaCl$ etc.

When the system is acid the real solvent power of a salt may be masked, or its quantitative value apparently lowered, by the coagulating effect of its anion, which, as is well known, rises with valency. In alkaline solutions the kation of a salt may be responsible for a corresponding effect. When the solution is neutral, and the colloid particle therefore uncharged (Hardy), the solvent action is exhibited as an uncomplicated phenomenon, and a convergence in the solvent power of certain salts is observed. Here, too, valency relations obtain, but the significance of ionic sign disappears. Salts *e.g.* of the type $B''A_2$ become equal in solvent power to those of the type B_2A''. In general, the solvent power in neutral solution increases with the valency of either constituent radicle of the salt. It is a property of the salt as a whole.

Comparison with globulins. If we attempt to find analogies for the action of salts upon Bence-Jones protein it is natural to recall their influence upon the solubility of globulins, notwithstanding the obviously different temperature relationships in the case. Reference to published observations upon the globulins, especially those of Hardy[1], but also those of Osborne and Harris[2] will indicate that marked resemblances do, as a matter of fact, exist. In globulin systems the solvent power of salts is exerted, of course, upon native proteins, while in the case of the urinary protein they influence the solubility of a heat-altered product—the primary heat-coagulum. The latter however is characterised, no less than the globulin, by insolubility in neutral water, and by displaying the properties of a suspension colloid when dissolved in dilute acid or alkali. These characters they both share with ordinary metaproteins (albuminates) derived *e.g.* from albumins. But the heat-

[1] Hardy. *Journ. Physiol.* xxxiii. 1905.
[2] Osborne and Harris. *Amer. Journ. Physiol.* xiv. p. 151. 1905.

altered Bence-Jones protein also agrees with a globulin, while both differ from metaproteins, in being soluble in neutral salts. Between the two former the one essential difference is that the temperature range within which their association compounds (*infra*) with electrolytes exhibit stability, lies much higher in the case of the excreted protein.

Hardy found that in dissolving neutral globulin at room temperature salts exhibit increase of solvent power with increase in the valency of either their acid or basic constituent. His numerical data indicate indeed that the relative solvent powers of various types of salt are almost precisely those found by us for the solution of Bence-Jones protein in neutral condition at 100°[1]. The results obtained by Osborne and Harris respecting the solubility of the vegetable globulin, edestin, give similar indications. Hardy and also Osborne came to the conclusion that the solution of globulins by electrolytes is due to the formation of molecular association compounds, the phenomenon being in Hardy's view analogous with the formation of double salts. The amphoteric character of the protein molecule lends probability to such a view, and we believe that the hypothesis explains better than any other the process of solution of Bence-Jones protein at 100°. If we accept, for globulins, Hardy's conception of an analogy with double-salt formation, we may be tempted to remember in connection with the special behaviour of the urinary protein that certain known types of double salts are stable only above a definite transition temperature, and dissociate into the constituent salts at temperatures below it. But, doubtless, this analogy must not be pushed too far. The phenomenon of association on endothermic lines is, in the case of these salts, observed more particularly in the solid state, or usually in the presence of only such quantities of water as may arise from the water of crystallisation which separates on their formation. A dilute solution of such double salts has, in general, the properties of a mixed

[1] The effect of valency has so potent an influence in determining the solvent power of a salt for the urinary protein that it certainly overshadows other factors if these exert an influence. Since the above was written Schryver (*Proc. Roy. Soc.* LXXXIII. (B) p. 96 (1910)) has suggested that the surface tension of a saline solution plays a part in determining its disaggregating power for globulins. The method used by us is hardly delicate enough to determine minor differences between salts of the same order; but the sodium series—chloride, bromide and nitrate, iodide—seem to show an increasing solvent power (in neutral solution) in the order given. This was found for globulins by Schryver, and, as he indicates, it is in the order of decreasing surface tension.

solution of the constituent salts. Nevertheless it must be remembered that, under conditions, their association can persist in solution, and, moreover, that, in the case of the protein, the association with salt is also highly unstable unless a large excess of the latter be present (*infra*). If a neutral solution in salt at 100° be diluted with boiling water the Bence-Jones protein separates, just as does a globulin when its solution in salt is diluted at ordinary temperatures.

Analogies apart, the facts seem abundantly to justify the statement that, when the primary heat coagulum of the Bence-Jones protein dissolves on heating to temperatures near 100°, it is because some type of association compound is then formed with any salt present in the solution. This compound dissociates at lower temperatures, and so, on cooling the solution, a precipitate of the insoluble protein reappears. Only when the reaction of the fluid departs from neutrality do the quite independent effects of ionic charge upon the colloid particles complicate these otherwise simple relations. In the case of globulins, when associated with acid or alkali the solvent properties of salts are probably no less balanced against their ionic precipitating power as the published observations suggest[1]. But there is a difference in the case of the Bence-Jones protein, in that the temperature relationships are such as to isolate more completely these two actions of a salt, and to bring them into clear relief as distinct factors in equilibrium changes. We have here a case in which rise of temperature rapidly increases the tendency to molecular association. Now the coagulative effect of an ion upon a particle increases more slowly with temperature—presumably at the rate of increase in ionic velocities. Hence above a certain temperature the Bence-Jones protein may be dissolved even by salts which contain what is, for the given conditions, a potent precipitating ion. The conception that one action of a salt is upon the substance *qua* colloid (*i.e.* upon particles), while another is molecular, is, we believe, justified, and may be of importance to the understanding of equilibrium changes in other salt-colloid-water systems.

As a result of his own experimental work J. Mellanby[2] has come to a conclusion concerning the solution of globulin by salts which differs somewhat from the above. In his view the dissolved globulin is in equilibrium with the ions of the salt, each ion exerting a solvent influence increasing with its valency (charge), though independent of the sign of its charge. This view is, in the main, based upon the following results: (1) A very exact relationship between the solvent effects of a salt and the sum of the valencies of its con-

[1] Hardy. *loc. cit.* pp. 317–319.

[2] J. Mellanby. *Journ. Physiol.* XXXIII. p. 338 (1905).

stituent ions. Mellanby found that a divalent ion has four times the efficiency of a monovalent ion, and, in general, that the solvent power is proportional to the square of the valency value. This exactness of relation we did not find in the case of the Bence-Jones protein. When a solution had been very carefully neutralised, the solvent power of, say, NaCl was almost exactly one-third that of K_2SO_4 or $CaCl_2$, in accordance with Mellanby's rule; but that of $MgSO_4$, for instance, was very nearly equal to that of $CaCl_2$ or K_2SO_4. Our experiments, as we have said, orientated salts almost exactly as did those of Hardy for globulins[1]. (2) Mellanby found that the solubility of the globulin in salts increased only slowly with rise of temperature, being proportionate to the increase in ionic velocity. In this connection it is clear that the relations are quite different when the Bence-Jones protein is concerned; the temperature phenomena, in fact, seem altogether to prevent the application of Mellanby's hypothesis in explanation of the solution of this substance. We may consider once again the action of (say), a sulphate in an acid solution of the protein. The salt, in virtue of its anion, exerts a coagulative action, lowering the primary coagulation point of the system, or, within a certain temperature range, increasing the velocity of separation at any given temperature. Above a certain temperature however the same salt promotes solution of the coagulum. It is difficult to conceive of such a reversal in the influence of ions; the establishment of some new relationship with the salt at higher temperatures seems more probable. (3) When in Mellanby's experiments a given quantity of a solvent salt was added to excess of globulin in suspension, the amount of globulin dissolved increased with the concentration of its original suspension. This led Mellanby to postulate a "sphere of action" for the solvent ions. Similar relations were observed by Hardy, though he explained them differently[2]. The facts also hold in the solution of Bence-Jones protein at 100°.

It will be seen that in all our experiments hitherto described, the concentration in neutral solutions of, say, K_2SO_4, or $CaCl_2$, necessary just to re-dissolve the coagulum was about the same (*circa* 0·07 M), although the concentration of the protein solutions employed varied somewhat widely. The same thing is seen in the following direct comparison, carried out on lines similar to those of all the previous experiments. The protein was from Case A in 1·2 % neutral solution.

Protein solution in c.c.	Amount of salt solution required to give clear solution at 100°		
	$CaCl_2$	NaCl M.	Water
2	1·40	2·10	Water was added in each case to make
4	1·45	2·20	the total volume 10 c.c.
6	1·50	2·40	N.B. The NaCl was employed in molar
8	1·65	—	solution, the $CaCl_2$ in half molar.

It is seen that the amount of salt necessary for complete solution increases very slowly with increase in the concentration of the protein. This statement represents the facts of Mellanby's observations on globulin viewed from another aspect. Such relations do not seem difficult to explain on the assumption that a molecular compound is formed. In any molecular association between a protein and a salt the relative amount of the latter must be very small. What we measure in determining the solvent power of a salt is not a stoichiometric relation alone, but the total concentration of salt required to overcome the dissociative tendencies of the molecular compound formed. This latter calls for so large an amount of salt in proportion to that actually combined, that the amount of protein concerned, within a wide range, hardly affects the effective salt concentration as observed.

[1] Osborne and Harris also found, for Edestin, $MgSO_4 = CaCl_2 = Na_2SO_4$.

[2] Hardy. *loc. cit.* p. 310 (1905).

Effect of Weak Bases. Magnus Levy called special attention to the influence of urea in inducing the solubility of the protein at 100°. We have confirmed his observations with our material, but we find that the concentration of urea required is very high compared with effective concentrations of electrolytes. In the following experiment a 24% solution of urea was employed (M × 4). The protein (from Case C) was in 2% neutral solution, which, when heated alone, gave a completely stable coagulum, not dissolving at 100°.

Protein solution in c.c.	M×4 Urea solution in c.c.	Water c.c.	At 60°	At 100°
5	1	4	coagulum	precipitate
5	2	3	,,	marked opacity
5	3	2	,,	,,
5	4	1	,,	slight opacity
5	4·2	0·8	,,	very slight opacity
5	4·4	0·6	,,	Crystal clear. The coagulum reappeared on cooling
4	6·0	0	no coagulum	Crystal clear. No precipitate on cooling

Thus, while calcium chloride or potassium sulphate dissolved at a concentration of about 0·07 M, in the case of urea a concentration of 1·56 M is required (10·56%). When the protein dissolves in the original urine a complex equilibrium must of course be established; but it seems clear from our experiments that the urinary salts play a more important part in the solvent action than does urea.

Globulins at ordinary temperatures also dissolve in urea-solutions of sufficient concentration, and the fact that Bence-Jones protein so dissolves at 100° supplies yet another resemblance between the behaviour of the latter at high temperatures and that of the former at room temperatures.

The Bence-Jones coagulum will furthermore dissolve at 100° in solutions of such a base as aniline. In all these cases there can be little doubt that we have to deal with associative molecular compounds which in the case of the Bence-Jones protein are (except those with salts of highest valency) stable only at temperatures near the boiling point. On cooling the solutions, unless the concentration of the salt or of the base is considerably in excess of the minimum required for solution, the protein separates anew.

Salting out. The special effect upon colloids of electrolytes when in high concentration which is denoted by the term salting out is also exerted upon Bence-Jones protein when in solution at 100°. Such

behaviour might be well expected, but it is of interest to note it as completing the analogy with globulin solutions.

The salting out of the original native protein has been studied by others and has been referred to in the introductory section. The heat-altered product when dissolved in electrolytes at 100° can also be salted out at that temperature, though the quantitative relations to precipitating salts are somewhat different. We have not studied the matter fully, but the following statements can be made. If, using the technique of our previous experiments, magnesium sulphate be added to a neutral solution of the protein, the concentration of the salt may be increased till considerably in excess of the minimum required for solution, without any obvious reversal in its effects. But when it is present to approximately one-half saturation, it is seen that the heat coagulum induced at 50° no longer completely dissolves at 100°. When about two-thirds saturation is reached the coagulum does not dissolve at all. With ammonium sulphate this point is reached somewhat short of half saturation. Again, if to a solution of the protein, kept at 100°, a hot saturated solution of $MgSO_4$ be added, precipitation is seen to be complete when the amount added is equal to about twice the bulk of the original solution. A hot solution of Am_2SO_4 precipitates fully when about an equal bulk has been added.

It is clear that taking all the facts together the resemblance between the heat-altered urinary protein and a typical blood-globulin is, as regards solubility factors, exceedingly close. The fundamental difference between them is the difference of a temperature factor involved in their equilibrium with salts, etc. What is special in this temperature relation is the basis of what is special in the physical chemistry of Bence-Jones protein.

Addendum. It is necessary before closing this section to make some further reference to Magnus Levy's results. In the case studied by him it appeared that the only salt effective in promoting solution of the protein at 100° was ammonium chloride. As regards our own material it will have been observed that all the effects of salts as described were quantitatively, as well as qualitatively, identical in all three of the cases studied. It is difficult to reconcile Magnus Levy's results with ours save on the assumption that his material was different. This was almost certainly the case, though the difference did not necessarily concern the native protein in the original urine. The urine of Magnus Levy's case was not typical, in that the heat coagulum produced at 50° was not completely dissolved at 100°. We believe (as he did) that this was due to the nature of the urine rather than to that of the protein; but the circumstance led him to submit the latter to special treatment before its solutions were used for studying the effect of salts. He coagulated the protein under boiling alcohol, and then, since it was no longer soluble in water, dissolved it in weak ammonium hydrate. Such a solution when neutralised with HCl

(containing therefore AmCl) showed the typical heat phenomena. The protein coagulated at about 50°, dissolved on boiling, and reappeared on cooling.

Magnus Levy states that sodium chloride and magnesium chloride had no effect in promoting solution at 100°; but it is not quite clear under what conditions they were tested. If they were added to the neutralised solution, in which ammonium chloride was already present the conditions would be complicated; if to the ammoniacal solution, then, from the facts given in this section, it is clear that the conditions would be such as to make $MgCl_2$ of low potency as solvent, and this is the case with NaCl under any circumstances. Magnus Levy does not appear to have tried salts of the type of sulphates. He got negative results with phosphates, which we have not ourselves tested. In any case the preliminary treatment suffered by his material prevents it from being strictly comparable with ours. Other observers (quoted in the opening paragraphs of this section) have demonstrated the solvent influence of sodium chloride at 100°, since they have shown that its addition to dialysed urine restores the typical characters lost upon dialysis (*supra*).

Section III. Chemistry of the protein.

(A) *Elementary Analyses.*

	In per cent. of ash-free protein				
	C	H	N	O	S
Case A. (*a*)	51·64	6·66	16·19	24·33	1·17 1·18
(*b*)	51·71	6·80	—	—	—
Case B.	51·74	6·98	16·13	23·97	1·18
Case C. (*a*)	51·46	7·14	—	—	—
(*b*)	51·76 51·70	6·97 7·09	16·30	—	1·18
Means: A	51·67	6·73	16·19	24·24	1·17
B	51·74	6·98	16·13	23·97	1·18
C	51·64	6·71	16·30	24·17	1·18
Mean of all three cases	51·68	6·81	16·21	24·12	1·18

Amide-Nitrogen per cent.

A	1·30
B	1·30
C	1·29

Analytical details.

A. (*a*) 0·1695 grm. subst. gave 0·3210 CO_2 and 0·1016 H_2O; 0·2330 gave 31·0 c.c. N at 15° and 775 mm.; 1·1155 grm. gave 0·0950 $BaSO_4$.

(*b*) 0·1500 gave 0·2844 CO_2 and 0·0918 H_2O.

B. 0·1550 gave 0·2941 CO_2, and 0·0974 H_2O; 0·1710 gave 23·2 c.c. N at 16° and 760 mm.; 1·50 grm. gave 0·1770 $BaSO_4$.

C. (*a*) 0·2155 subst. gave 0·4067 CO_2 and 0·1385 H_2O.

(*b*) (1) 0·1503 gave 0·2853 CO_2 and 0·0943 H_2O; (2) 0·2573 gave 0·4875 CO_2 and 0·1645 H_2O; 0·2185 gave 29·2 c.c. N at 15° and 772 mm.; 1·5 grm. gave 0·1771 $BaSO_4$.

The protein employed for the above analyses, and also for the purpose of hydrolysis, was obtained by heating the diluted urine, after acidification with acetic acid, to temperatures not exceeding 60°. The coagulum was filtered off at the pump and washed very thoroughly with faintly acidulated water. It was then washed many times with alcohol (some of the material stood, at this stage, for long periods under alcohol), then again with water and finally with alcohol and ether. It was dried at 110° till of constant weight, and was practically ash-free. We found that prolonged washing with water was necessary to remove an associated substance which somewhat lowered the carbon-content.

(B) *The Amino-Acids.*

A study of our material has confirmed the belief that the Bence-Jones protein yields all those amino-acids which are to be obtained from typical proteins, and is therefore not a fractional product arising from the partial breakdown of protein in metabolism. We analysed side by side the material from two distinct cases, and our results appear to offer evidence of identity.

Proof that two different persons may excrete identical material does not of course carry the corollary that the urinary protein is a chemical individual. But if the protein were shown to be the same in all cases, or in certain groups of cases, the fact must be of significance when its origin is under consideration. It is of course true that with the available methods the separation of only a very few of the amino-acids is even approximately quantitative, and this remains true in spite of certain advances towards accuracy which have been made quite recently. Not claims on the part of those who have essayed to separate the constituent amino-bodies of proteins, but the current practice (in text-books and elsewhere) of publishing quantitative statements and comparisons, without reference to those shortcomings in methods which are frankly avowed in the original papers, makes it desirable at the present time to emphasise the existence of such deficiencies[1]. With respect however to certain amino-acids some claim for considerable accuracy in estimation can be made, and it is upon a satisfactory agreement among these, when the proteins from the two cases are compared, that our belief in their identity is based.

[1] For recent discussions concerning these see, *e.g.* Osborne and Breese-Jones, *Amer. Journ. Physiol.* xxvi. p. 212, 1910; *ib.* 305. Also Abderhalden, *Zeits. physiol. Chem.* lxxxvi. p. 477, 1910. We are ourselves convinced that analytical difficulties have not always the same incidence when different proteins are under treatment. This has struck us forcibly when comparing Bence-Jones protein with casein.

(1) *Amino-acids separated without preliminary esterification.*

Tyrosine. Fifty grammes of dry ash-free protein from each case were boiled for 20 hours with 500 c.c. 25 % sulphuric acid. The acid was quantitatively removed with baryta and the barium sulphate precipitate washed until the washings even after concentration gave no trace of colour with Millon's reagent. The filtrate in Case A was evaporated and three successive crystalline fractions obtained of which the last gave only a very slight tyrosine reaction. A fourth fraction gave no reaction. The three first fractions were united, dissolved in weak ammonium hydrate and the solution boiled with blood charcoal. Upon concentration and standing the tyrosine separated in white silky needles which after a further recrystallisation from ammonia gave good figures on analysis.

The protein of Case A yielded 2·07 grms. = 4·14 %. From that of Case C after almost precisely similar treatment 4·23 % was obtained.

A. 0·1560 subst. gave 0·3410 grm. CO_2 and 0·870 H_2O
C. 0·1681 „ „ 0·3665 „ „ „ 0·952 „
A. Carbon = 59·61. Hydrogen = 6·19.
C. „ = 59·46. „ = 6·29.
Calculated for $C_9H_{11}O_3N$, Carbon = 59·67 Hydrogen = 6·08.

Tryptophane and Cystine. These were separated from the protein of Case A only. A quantity equal to 263 grms. of the dry ash-free protein was digested with 1 gram of Pancreatin Rhenania. After ten days 5 % by volume of strong sulphuric acid was added and the tryptophane and cystine precipitated together by means of Hopkins and Cole's mercuric sulphate reagent. After decomposing the mercury precipitate and filtering off the mercuric sulphide the mixture was not further fractionated with the mercury reagent in the usual manner, but after removal of sulphuric acid the solution was at once concentrated. Cystine separated before there was any separation of tryptophane, and it proved quite easy to prepare the pure products by fractional crystallisation. A little charcoal was employed to decolorise the final product in each case. Of tryptophane 2·147 grms. were obtained, and of cystine 1·495 grm.—equal to 0·82 and 0·57 % respectively.

The somewhat low percentage of tryptophane obtained is far from showing that the Bence-Jones protein is relatively poor in this substance. The always rather serious loss in the mother liquors is relatively higher

when small amounts of material are dealt with. Thus, from casein, it is not difficult to get 1·5 % and more, when two or three kilos have been digested, but from 250 grms. the yield is not often more than 0·5 or 0·7 %. The yield of cystine was of course not quantitative, but the separation of the pure substance from a pancreatic digestion is of some interest in itself; the product had a normal rotatory power.

Tryptophane 0·1670 grms. gave 19·45 c.c. moist N at 18° and 75 mm. N = 13·67 %. Theory 13·72

Cystine. 1·063 grm. dissolved in 20 % HCl. Total weight of solution 35·068 grms. = 3·03 %. Rotation in 4 dcm. tube = 27°16. $[\alpha]_D^{20°} = 224$.

Glutamic Acid. The ease with which glutamic acid separates as hydrochloride from the products of HCl hydrolysis seems to vary with the protein under treatment. Whenever we hydrolysed the Bence-Jones protein we always found that, no matter at what concentration of the products the saturation with gaseous HCl was carried out, the glutamic hydrochloride separated with exceptional slowness and difficulty. For its estimation we hydrolysed fifty grms. of the dry protein (Case A) by boiling for 20 hours with 250 c.c. of strong HCl. The mixture was saturated ice-cold with gaseous HCl and was then allowed to stand for several weeks in the ice-chest. The crystals obtained were for some reason exceptionally small and they did not settle to the bottom of the fluid. Instead of filtering through linen or asbestos we found it necessary to centrifuge the material in corked vessels. This secured very complete separation, and the product obtained was, from the first, nearly colourless. The mother liquors were concentrated and again saturated with HCl when a second small fraction was obtained by centrifuging. The two fractions were combined, dissolved in a minimal quantity of water and the glutamic acid again separated by saturation with gaseous HCl. The product could now be filtered through linen. It was colourless and free from ammonium chloride. Dried *in vacuo* over sulphuric acid and caustic soda till constant in weight, it weighed (Case A) 4·70 grms., equal to 9·40 %, or 7·53 % glutamic acid. In Case C 25 grms. only of protein were hydrolised. After similar treatment it yielded 2·515 grms. of hydrochloride = 10·06 %, or 8·05 % of free acid.

Case A. 0·3214 grm. hydrochloride gave 0·2525 AgCl; Cl = 19·47 %.
„ C. 0·3300 „ „ „ 0·2590 „ „ = 19·39 „
Calculated for $C_5H_{10}NO_4Cl$ „ = 19·35 „

Aspartic Acid. Aspartic acid, when separated directly from the products of hydrolysis as its mercury compound, is, as one of us has found, nearly always to be obtained in better yield than when the ester fractions are dealt with. Fifty grams of the dry protein were boiled for eighteen hours with 25 % sulphuric acid (500 c.c.). The sulphuric acid was then quantitatively removed with baryta and the fluid concentrated to 250 c.c. and cooled. The tyrosine which separated was filtered off. A warm solution of mercuric acetate was then added to the filtrate until no further precipitate fell. Barium hydrate in hot solution was added till the fluid became alkaline, and then more mercuric acetate; the two reagents being added alternately till any precipitate produced by either was no longer white but yellow. The precipitate was then filtered off and washed at the pump with a cold saturated solution of mercuric acetate. It was suspended in water and the mercury precipitated with SH_2. The mercury sulphide being filtered off and well washed, the filtrate was concentrated to 150 c.c. and boiled up with copper carbonate in excess. After filtering, the fluid was further evaporated in vacuo over sulphuric acid when copper aspartate separated in characteristic crystals. From the mother liquor of these a further crop was obtained. Subsequent evaporation gave no further yield of crystals but only an amorphous residue of copper salts. The copper aspartate proved to be pure without further treatment.

Case A yielded 2·141 grms. air-dried copper salt = 4·282 % = 2·07 % aspartic acid.

Case C gave 2·24 grms. air-dried copper salt = 4·48 % = 2·17 % aspartic acid.

C. 0·3525 grm. air-dried Cu-salt gave 0·1010 CuO. Cu = 22·88 %. Calculated for $C_4H_5NO_4Cu$, $4\frac{1}{2}H_2O$ = 23·07.

Arginine, Histidine, and Lysine. The estimations of diamino acids were made according to the familiar method of Kossel and Kutscher, as modified by Kossel and Patten, but with a further slight modification in connection with the separation of arginine and histidine. An identical procedure was applied to the protein in each case. Fifty grms. of material were hydrolysed with 25 % sulphuric acid to serve for the estimations. After the first precipitate of arginine- and histidine-silver had been decomposed with sulphuric acid, the silver and sulphuric acid were removed, and the solution made slightly acid with nitric acid. Excess of silver nitrate was then added in the usual way. In the fractional precipitation of this solution with barium hydrate,

instead of using ammoniacal silver solution as a test for the removal of histidine[1], we added the baryta gradually, in small quantities at a time, and filtered off at the pump three successive fractions of the precipitate. The first fraction was that obtained when the solution had been just neutralised to litmus. Each fraction was washed, ground up with sulphuric acid, and the silver removed from solution as sulphide. The content of sulphuric acid was then, in each fraction, brought up to 5 %, and acid mercuric sulphate solution was added to precipitate any histidine present. The first fraction gave an abundant precipitate, the second very little, and the third none, even upon long standing. Histidine being absent from the last fraction, no further fraction was precipitated from the original solution, which now contained arginine alone. Mercury and sulphuric acid were removed from the solution of the third fraction and it was mixed with the original liquid containing the arginine. From the other two fractions the mercury-histidine compound was filtered off, and mercury removed from the combined filtrates, which were then evaporated to small bulk, and a determination of nitrogen made by Kjeldahl's method upon an aliquot part (*infra*). The histidine compound was suspended in water, decomposed with SH_2, the mercuric sulphide filtered off, and the sulphuric acid removed quantitatively with baryta. The filtrate from the barium sulphate was concentrated, treated with excess of hydrochloric acid, and evaporated to a syrup. The histidine hydrochloride crystallised almost immediately in the characteristic rhombic form, and was nearly free from colour. It was dried in a desiccator till of constant weight.

Case A gave 0·5775 grm. histidine dichloride = 1·155 %.
„ C „ 0·6200 „ „ „ = 1·240 „
Histidine (A) 0·843 %; (C) 0·780 %.

The original solution after removal of histidine was now saturated with baryta, and the arginine liberated from the silver precipitate in the usual manner. Its solution was exactly neutralised with nitric acid and taken down to complete dryness. The residue which proved to be pure arginine nitrate (*infra*) was weighed.

Case A yielded 3·792 grms. = 7·584 % = 5·370 arginine %.
„ C „ 3·841 „ = 7·682 „ = 5·433 „ „

[1] A preliminary experiment had shown that the relative amount of histidine was small, and that it was more than ordinarily difficult to define the stage of separation by the use of ammoniacal silver solution.

To these amounts must be added the small quantity of arginine determined by Kjeldahl in the filtrate from the histidine precipitate as described above. The N thus found was, for Case A, 0·21 % of the protein, and, for Case C, 0·20 % = 0·650 and 0·629 % arginine respectively.

Total Arginine: Case A, 6·020 %.
Case C, 6·062 %.

The arginine nitrate after being weighed was in each case dissolved in water, boiled with excess of copper carbonate, the solution filtered and evaporated nearly to dryness. On standing the residue set to a solid magma of well-formed uniform crystals. After drying in the air they were weighed.

The weights of arginine-copper-nitrate so obtained agreed almost exactly with the amounts calculated to correspond with the original weight of arginine nitrate, thus proving the purity of the latter.

(A) 3·792 grms. $C_6H_{14}N_4O_2 . HNO_3 + \frac{1}{2} H_2O$ gave 4·498 grms. copper compound.

(C) 3·841 grms. $C_6H_{14}N_4O_2 . HNO_3 + \frac{1}{2} H_2O$ gave 4·560 grms. copper compound.

Calculated for $(C_6H_{14}N_4O_2)_2 Cu(NO_3)_2 + 3H_2O$
4·542 grms. and 4·601 grms. respectively.

The copper, and the loss on drying at 110°, were determined for the copper compounds

(A) 4·498 grms. lost 0·445 grms. gave 0·6065 grms. CuO.
(C) 4·560 „ „ 0·439 grms. gave 0·6068 grms. „
(A) water 9·89 %, copper 10·77 %.
(C) „ 9·62 „ „ 10·63 „
Calculated, water 9·16 „ „ 10·77 „

The histidine estimations agreed fairly well, considering the small amount present, and the arginine estimations agreed very exactly. In the lysine separation however an unexplained difficulty presented itself, and we were unable to make a quantitative comparison. In the filtrate from the silver precipitate lysine was, as usual, thrown out with phosphotungstic acid, and the precipitate thoroughly washed. When this was decomposed in the orthodox manner, and the solution containing the lysine concentrated to small bulk, the addition of picric acid or sodium picrate failed in both cases to give a satisfactory crystalline

precipitate, even after long standing. We found it necessary to add alcohol to the solution before adding the picric acid, and to filtrate off a somewhat bulky amorphous precipitate produced by the alcohol. In the filtrate from this a typical picrate was obtained, but the experimental manipulations prevented us from obtaining more than a small part of the lysine from Case A. From Case C the yield was probably not far from quantitative. The picrates were weighed after recrystallising from hot water. From (A) 3·733 % of picrate were ultimately obtained; (C) yielded 9·425 %, equal to 3·67 % lysine.

(2) *Amino-acids separated after Esterification.*

One hundred grammes of the dry protein, from Cases A and C respectively, were boiled for eighteen hours with strong hydrochloric acid and the products esterified by Fischer's method. Glutaminic acid was not separated before esterification. For transferring the esters to ether we used the barium method of Levene[1] and found it highly convenient. The residue was esterified a second time, but the yield of esters was then so small that a third esterification was not thought necessary. Upon distillation the following fractions were obtained:

Fraction	Temp. of bath up to	Pressure	Weight	
			Case A	Case C
I	100°	30 mm.	10·20 gms.	11·10 gms.
II	70°	0·5	16·15	16·00
III	105°	0·5	6·75	6·20
IV	200°	0·5	25·25	24·10

The first fraction consisted largely of alcohol and ether and its weight is not significant. It will be seen that the two cases agree closely in their yield of the later fractions. All were saponified in the usual way.

Glycine. Fraction I was taken to dryness in the presence of excess of HCl, the weight of the residue being (in Case C) 3·9 grms. only[2]. It was, in each case, covered with 50 c.c. of absolute alcohol, and the liquid saturated with gaseous HCl, when all went into solution. It was then sown with a crystal of glycine-ester hydrochloride, but no separation of glycine could be obtained from either case, even after the solutions had stood for several weeks in the ice-chest. None was

[1] *Journ. Biol. Chem.* I. p. 4. 1905; *ib.* VI. p. 419. 1909.

[2] We omitted to weigh this residue in Case A.

obtained after concentration and further standing. The alcohol was therefore evaporated off, the residue dissolved in water and the solution well boiled. HCl was then removed with silver sulphate, and the sulphuric acid, quantitatively, with barium hydrate. The solution was then further boiled under a vertical condenser. The amino acids present were then separated by fractional crystallisation into three fractions, of which the most soluble weighed 1·2 grms. in Case A, and 1·35 grms. in Case C. In each case this fraction was dissolved in a small quantity of hot water, and, while hot, mixed with an equal volume of an alcoholic solution of picric acid. On cooling a crystalline picrate separated which was filtered off and recrystallised from hot water. It melted at 192° (uncorr.) in each case, and the melting point was not altered when glycine picrate, prepared from pure glycine (melting also at 192°) was mixed with the products. There can be no doubt that glycine was present in the protein of both cases, but the amount of picrate obtained (about 0·2 grm. in each case) is no measure of its quantity. While Magnus Levy, using Spiro's method, could find no glycine in the protein of his case, Abderhalden and Rostoski found, in their material, 1·7 %. As we were careful to ascertain that no loss of glycine occurred, either by separation from the alcohol during the original esterification, or during the removal of ether before fractional distillation, we believe that our material almost certainly contained less than the amount found by Abderhalden and Rostoski.

Alanine. Alanine could not, unfortunately, be obtained in a pure condition from either case. It is clear that the amino-acids of low molecular weight were present in relatively small amount, and the 100 grms. of protein hydrolised were not enough to permit of efficient separation. After the glycine had been precipitated as picrate, and picric acid removed from the filtrate, the whole material of Fraction I (Case C) was mixed, and then separated into four fractions by crystallisation from water. The larger and most soluble fraction contained 44·02 % C and 8·60 % H. It could not be further separated. The least soluble fraction gave 52·22 C and 9·72 H. Both leucine and valine were probably present in small amount. Similar indications were obtained in Case A. Since no glycine could be detected in the first mentioned fraction, its low carbon content suggests that alanine was present admixed with leucine or valine.

Proline. Fractions II and III after saponification were taken completely to dryness on the water bath, dried at 100°, and each thoroughly extracted with absolute alcohol. The alcohol was allowed

to evaporate at 30°, and the residues again taken up in absolute alcohol. This process was repeated until the material went up easily and completely into cold absolute alcohol, the slight insoluble residue at first obtained being returned to the main fractions. The alcoholic extracts were then mixed and evaporated under reduced pressure, the residue being finally dried and weighed

Case A yielded 2·674 grms.
„ C „ 2·714 „

A large proportion of the copper salts was in each case soluble in alcohol. Of the lævo-prolin copper (dried at 110°) from A, 1·5210 grm. gave 0·4115 CuO, and, from B, 1·2665 grms. gave 0·3405 CuO.

(A) Cu = 21·61 %; (C) 21·50 %.
Calculated for $C_{10}H_{16}O_4N_2Cu$: Cu = 21·81 %.

The inactive prolin copper was obtained crystalline from water.

Valine and Leucine. Fractions II and III after extraction of the proline were combined. They weighed together 14·49 grms. in Case A, and 14·14 grms. in Case C. A separation of valine from leucine by the method of Levene and Van Slyke[1] was carried out on the material from Case C. To judge from our experience this method gives excellent results.

The original mixture of amino acids contained 52·70 % carbon, indicating (on the assumption that only valine and leucine were present) the presence of about 40 % of leucine isomers

$$\left(\frac{52{\cdot}70 - 51{\cdot}24}{3{\cdot}68} \times 100 = 39{\cdot}70\right).$$

For the separation 12·75 grms. of the mixture were suspended in 90 c.c. of water, heated to boiling, and treated with 20 c.c. of concentrated aqueous ammonia. To the solution 24 c.c. of 1·1 M lead acetate were slowly added. The mixture was well shaken and cooled, and after one hour the precipitate was filtered off and washed with alcohol and ether. After drying *in vacuo* till constant it weighed 10·361 grms.

0·300 grm. yielded 0·1946 $PbSO_4$ = 0·1329 Pb. Pb = 44·30 %.
Calculated for Pb $(C_6H_{12}O_2N)_2$ 44·29 %.

[1] *Journ. Biol. Chem.* VI. p. 391. 1909.

The precipitate was therefore pure lead-leucine. The valine fraction was recovered from the filtrate after precipitating the lead as sulphide. The residue obtained on evaporation was treated with alcohol-ether mixture (3:1) and dried. It contained 51·89 % carbon, a figure indicating that it was still admixed with 17·7 % leucine. It was dissolved in ammonia and precipitated with a quantity of lead acetate calculated from the above datum. There were thus obtained 2·71 grms. (= 2·78 grms. from the whole fraction) of lead salt, yielding 44·09 % Pb, and consisting therefore of nearly pure lead-leucine. The valine fraction was again recovered from the filtrate. Its appearance and behaviour on heating indicated that it was not yet pure, but after recrystallising from water, which involved some loss, 4·931 grms. of pure valine were obtained, equal, after allowing for the material removed for the determination of carbon given above, to 5·054 grms. from the 12·75 grms. used in the separation. When rapidly heated it decomposed at 296°.

The lead-leucine obtained (10·361 grms. + 2·78 grms. = 13·141 grms.) corresponds to 5·82 grms. leucine. This calculated to the total amount of the residue from Fractions I and II $\left(5{\cdot}82 \times \frac{14{\cdot}14}{12{\cdot}75}\right)$ gives the leucine yield from the protein as 6·46 %. A similar calculation for the valine gives 5·60 %. We think there can be little doubt that, as Levene and Van Slyke urge, the use of the lead separation will show that the valine content of proteins is higher than has been previously suspected.

Case C: Analyses.

Leucine mixture.

Kjeldahl: 0·5 grm. yielded ammonia neutralising 15·2 c.c. $\frac{N}{4}$ acid N = 10·64 p.c.; $C_6H_{13}O_2N$ requires 10·68 p.c.

Rotation: 3·373 grms. dissolved in 20 % HCl; total weight of solution 40·9430 grms. Rotation in 4 dem. tube = 6°·26; $[\alpha]_D^{20°} = 19{\cdot}01$.

Valine.

Analysis: 0·1520 substance gave 0·2849 CO_2 and 0·1320 H_2O C = 51·11; H = 9·64. Calculated for $C_5H_{11}O_2N$ C = 51·24; H = 9·47.

The lead-leucine fractions were united and the lead removed as sulphide. A determination of the rotatory power of the free leucine isomers so obtained, when dissolved in 20 % HCl, gave $[\alpha]_D^{20°} = 19{\cdot}01$. This indicates that the mixture contained 15·64 % of isoleucine and 84·36 % l-leucine. The HCl was removed from the portion employed for the determination of rotatory power, and the leucines returned to

the original fraction. Of this, 5 grms. were now converted into copper salts, and the mixed salts extracted with methyl alcohol according to the method of F. Ehrlich. The copper salt soluble in this yielded, when decomposed, free amino acid closely corresponding to the expected yield of isoleucine (0·81 grm. from 5 grms.). Owing to an accident however the rotatory power of this could not be determined and the presence of isoleucine cannot be claimed as conclusively proved. The rotatory power of the leucine mixture indicates that the protein yielded 5·45 % of ordinary leucine and 1·01 % isoleucine.

Phenylalanine. Fraction IV before saponification was shaken in each case with an equal bulk of dry ether, and the ether extract subsequently washed thrice with its own bulk of water. The ether was evaporated off and the residue taken to dryness several times with strong HCl. The hydrochlorides, after standing in a vacuum desiccator over sulphuric acid and caustic soda, weighed 11·75 grms. (Case A) and 11·57 grms. (Case C) respectively. These residues were, however, far from being pure phenylalanine hydrochloride. They contained in each case a little leucine and some aspartic acid. As the ether extract was thoroughly washed we are unable to explain the presence of the latter. The hydrochlorides were recrystallised twice from strong HCl, that is to say they were twice dissolved in about 20 c.c. of water and thrown out by saturating the solution with gaseous HCl. By this means pure phenylalanine hydrochloride was obtained, weighing after drying over H_2SO_4 and NaHO) 5·98 grms. from Case A, and 6·02 from Case C; equal to 4·73 and 4·92 % respectively of phenylalanine.

After removing the HCl from the filtrates an attempt was made to identify the amino-acids which accompanied the phenylalanine when the esters of Fraction IV were extracted with ether. A small fraction was obtained having the characters of leucine, and aspartic acid was identified as a copper salt. In Case C the phenylalanine was liberated from its hydrochloride by evaporation with ammonia and subsequent washing with cold water. It melted with decomposition at 284°.

Analyses: Hydrochloride, Case A: 0·500 grm. gave 0·3561 AgCl; Cl = 17·62 %. 0·1473 grm. gave 8·8 c.c. moist N at 18° and 751 mm. N = 6·90 p.c.

Calculated for $C_6H_{11}O_2N$. HCl, Cl = 17·61, N = 6·94.

Phenylalanine, Case C. 0·1600 grm. gave 11·4 c.c. moist N at 11° and 741 mm. N = 8·40 %. Theory 8·49.

Aspartic and Glutaminic Acids. As both these acids were estimated by direct methods we did not follow up completely their isolation from the esters. After removal of the phenylalanine ester, Fraction IV was saponified with barium hydrate in the usual way. From the insoluble barium salt in Case A 0·766 grm. of aspartic acid was obtained. After removing the glutaminic acid as the hydrochloride and freeing the filtrate from HCl, a small amount of copper aspartate was obtained, weighing, air-dried, 0·270 grm., equal to 0·130 grm. aspartic acid. We separated therefore from the esters only 0·91 % of this acid, though the direct separation gave 2·17 % (*supra*)[1]. The glutaminic acid obtained from Case A amounted to 7·2 %–80 % of the quantity separated direct; the distillation residue was not dealt with however. In Case C aspartic and glutaminic acids were not determined quantitatively in the ester analysis. In both cases we endeavoured to identify serine after removing the above acids from Fraction IV. The quantity of material was however too small for success.

The following is a summary of our results, the figures representing material weighed pure.

	Case A per cent.	Case C per cent.
Glycine	present	present
Alanine	probably present	probably present
Valine + Leucine	[14·49]	[14·14]
Valine	—	5·60
Leucine Isomers	—	6·51
l-Leucine	—	5·45
Isoleucine	—	(?) 1·01
Aspartic Acid	2·07	2·17
Glutamic Acid	7·53	8·05
Proline	2·67	2·71
Phenylalanine	4·73	4·92
Tyrosine	4·14	4·23
Tryptophane	—	0·82 +
Cystine	—	0·57 +
Arginine	6·020	6·062
Histidine	0·843	0·780
Lysine	—	3·670

Our belief that the same material was excreted in Cases A and C is based especially upon the close agreement in the figures obtained for *e.g.* tyrosine, phenylalanine, proline, arginine and aspartic acid (separated

[1] Osborne and Breese-Jones recovered in the esters only 42·5 % of the aspartic acid present in an artificial mixture of amino-acids of known constitution. *Amer. Journ. Physiol.* XXVI. p. 325. 1910.

directly) these being just those amino-acids in our estimations of which we felt the greatest confidence. The figures for glutamic acid and histidine are of the same order in both cases, and, as regards the former, considering the difficulties experienced in its separation the agreement is not unsatisfactory. Further evidence in support of the identity is obtained from the close agreement in the weight of the original ester fractions, and in the weight of the crude valine-leucine mixture. Again we find satisfactory agreement among the elementary analyses, including the sulphur estimations. In these the proteins from all three cases showed agreement, as did their quantitative relations to salts as described in Section II. It should be understood as regards Cases A and C that the material used for analytical work was obtained from urine collected over long periods.

To judge from our results the Bence-Jones protein is characterised by a high content of the aromatic acids, the values for tyrosine and phenylalanine taken together being considerably higher than any yet described for a blood- or tissue-protein[1]. Judging, indeed, by our figures as a whole, the protein would seem to stand by itself in respect of its chemical composition as much as it does in respect of its physico-chemical properties. But data concerning blood and tissue proteins are as yet too scanty to allow of any satisfactory comparison. For other proteins from the human body we have no data at all.

Section IV. Metabolism.

The amount of protein excreted compared with the total nitrogen of the urine. The effects of variation in the diet. In Case A a large number of estimations of protein and of total nitrogen were made during the year which followed the diagnosis of the condition. In the middle of the following year a considerable number was made; but later the case passed out of our direct control, and our observations were few. They were sufficient however to indicate the progress of the case, and the interval between our first estimations and the last was nearly four years. The patient of Case B died shortly after we first received the urine for analysis, and we have the record of metabolism

[1] Gross and Allard on administering Bence-Jones protein to an alkaptonuric person obtained so large a proportionate rise in the homogentisic acid excreted that they were led to conclude that the protein must be rich in aromatic groupings (*Zeitsch. f. klin. Medicin.* LXIV. p. 359. 1907). We are indebted to Dr A. E. Garrod for calling our attention to these observations.

for six days only. From Case C only a few 24-hour samples were analysed.

Methods. The protein was estimated by weighing the coagulum obtained by heating the urine. Twenty c.c. were diluted with an equal bulk of water, acidulated with 2 c.c. of acetic acid, and heated for two hours in a water bath kept at 60° C. The beaker used was then cooled in ice, and the coagulum filtered at the pump, either through a weighed Gooch crucible or through a filter paper supported by a perforated plate. It was thoroughly washed with ice-cold water, and, when a filter paper had been used, was subsequently transferred to a weighed platinum dish. In all cases it was dried at 120° till of constant weight. When, as in our cases, no blood proteins are present, this simple method is eminently satisfactory. Determinations were always made in duplicate, and agreed with great exactness. The filtrate was always tested by Bradshaw's test (ring test with strong HCl), and if this was not entirely negative a fresh estimation was made. If a protein-free filtrate were mixed with one-tenth of a c.c. of the original urine the mixture would always show a positive result with Bradshaw's test. The heat-coagulation method therefore gave results with an accuracy equal to at least one part in two hundred. The wash waters were often evaporated and tested, but never contained protein. In a very few instances, and only when the urine was strongly alkaline the method failed of accuracy; complete coagulation not occurring, in spite of acidification. On such rare occasions 20 c.c. were neutralised, mixed with 60 c.c. of strong alcohol, and allowed to stand for a week. The coagulum was then suspended in water acidulated with acetic acid, heated, and treated as above. The total nitrogen of the urine was determined by Kjeldahl's method.

A comparison of the cases gains in interest from the fact that they show so wide a variation in the grade of total metabolism. The woman of Case A, when upon uncontrolled diet, excreted no more than some 8 grms. of total nitrogen daily. The nitrogen representing protein actually utilised in metabolism was upon the average less than 6 grms., often falling considerably below 5 grms. In other words when guided by her appetite alone she catabolised some 40 grms. of protein only, or 0·75 grms. per kilo of body weight; half the normal amount.

On the other hand the patient of Case B was, at the time of our analyses, excreting more than 22 grms. of nitrogen a day; 16·5 grms. of this representing metabolised protein. If therefore the excreted protein be entirely left out of account this individual showed an average

grade of nitrogenous metabolism, utilising some 1·5 grm. of protein per kilo per diem.

In Case C metabolism was apparently of intermediate grade. The man excreted daily about 12·5 grms. of total nitrogen; eight grms. representing protein breakdown or about one grm. of protein catabolised per kilo.

Roughly speaking the amount of Bence-Jones protein excreted was in our cases adjusted relatively to the general level of metabolism, so as to bear much the same ratio in each case to the protein broken down. In Case A, when the diet was uncontrolled, the ratio of the nitrogen excreted in the form of Bence-Jones protein to the total urinary nitrogen was of the order of one to four. Later it rose to one to three. (The low ratios in May and June 1906 are explained below.) In Case C it was also one to three; in Case B it was only a little less. These are average values, under conditions when appetite was the guide to consumption. As will be evident later, wide fluctuations in the amount of ingested protein greatly affected the ratio (Case A).

To judge from the results obtained from Case A the amount of the protein excreted may vary from time to time, and an alteration in its amount may occasionally be abrupt. On the other hand the excretion is apt, for long periods to become set at a certain level, and remain strikingly constant from day to day. When our record began in March 1905 the excretion of four days upon an uncontrolled diet showed an average of 14·22 grms. per diem; but it then fell to a somewhat lower figure, and was for several months remarkably constant. Thus, 20 determinations during April gave an average of 11·22 grms.; eight in June gave one of 11·44 grms. Six estimations in July showed a daily excretion of 10·78 grms., and three in November 10·92 grms. An inspection of the figures for this period will show that when no special circumstances were present the daily fluctuations were small. In 1906 a series of determinations was made in the middle of the year, the diet being controlled during a portion of the period for the purpose of a balance experiment. It is remarkable that the protein excreted at this time had fallen to an average of only eight grms. per diem. It remained very constant in spite of great variations in the diet, which during part of the time was of exceptionally high nitrogenous value, and later was wholly vegetarian with little nitrogen. This period fell some six months after the close of the earlier estimations which showed a higher value for the protein. Later observations in the year showed the excretion to be still constant at the level of some eight grms. per

TABLE A. *General Metabolism in Case A.*

Date 1905	Urine c.c.	Total N per diem	Protein per 100 c.c.	Protein per diem	Protein-N per diem	Non-protein-N per diem	Pr. N×100 / Tot. N	Diet, etc.
March 23	868	—	1·65	14·32	2·32	—	—	Uncontrolled diet
25	1092	8·66	1·22	13·32	2·15	6·51	26·9	,, ,,
26	1148	8·93	1·26	14·46	2·34	6·59	26·2	,, ,,
27	1145	6·62	1·05	12·02	1·95	4·67	30·0	Milk diet
Apr. 1	1092	7·69	1·13	12·34	2·00	5·69	26·0	Uncontrolled diet
3	1274	8·42	0·890	11·34	1·84	6·58	21·8	,, ,,
5	1232	6·63	0·850	10·47	1·70	4·93	25·6	Second day of a gelatine diet
May 4	1344	—	0·780	10·48	1·70	—	—	Uncontrolled diet
6	1064	6·34	1·00	10·64	1·72	4·62	27·1	,, ,,
8	896	7·47	1·32	11·83	1·92	5·55	25·7	Protein in diet intentionally increased
9	1120	9·98	1·06	11·87	1·92	8·06	19·3	
10	1125	8·70	1·16	13·05	2·11	6·59	24·2	
11	1008	6·49	1·16	11·69	1·89	4·60	29·0	Uncontrolled diet
12	952	5·80	0·96	10·30	1·67	4·13	28·8	Bone-marrow administered and continued for one week
13	1400	7·69	0·820	11·48	1·86	5·83	24·2	Uncontrolled diet
14	1680	9·64	0·670	11·29	1·83	5·81	24·0	
15	1400	8·11	0·610	8·54	1·38	6·73	17·0	
16	1512	6·39	0·640	9·67	1·57	4·82	24·5	
20	1624	—	0·995	16·16	2·62	—	—	Day following cessation of bone-marrow
22	1680	—	0·620	10·42	1·69	—	—	—
23	1624	8·31	0·757	12·09	1·96	6·35	23·5	Ordinary diet
25	1792	5·94	0·547	10·80	1·75	4·19	29·5	Second day of gelatine diet
26	1465	7·11	0·732	11·48	1·86	5·25	26·0	Ordinary diet
27	1008	7·08	1·10	11·05	1·79	5·29	25·2	,, ,,
28	672	6·52	1·79	12·03	1·95	4·57	30·0	,, ,,
29	728	7·28	1·74	12·66	2·04	5·24	28·0	,, ,,
30	728	—	1·52	11·06	—	—	—	,, ,,
31	1120	—	0·97	10·83	—	—	—	,, ,,
June 13	1120	7·34	0·850	9·52	1·54	5·80	20·9	—
15	1120	7·84	0·970	10·76	1·73	6·11	22·0	—
17	1400	9·50	0·822	11·50	1·86	7·64	19·5	1 lb. of beef per diem added to ordinary diet
18	1344	11·32	0·922	12·39	2·01	9·31	17·7	
19	1512	11·54	0·782	11·82	1·91	9·63	16·5	
20	1624	11·97	0·857	13·96	2·26	9·71	18·9	Uncontrolled diet
21	1064	7·97	1·125	11·97	1·94	6·03	24·3	,, ,,
22	1456	6·85	0·702	10·19	1·65	5·20	24·1	,, ,,
July 18	1176	6·50	0·763	8·97	1·45	5·05	22·3	,, ,,
21	1064	8·22	1·185	12·61	2·04	6·18	24·8	,, ,,

Date 1905		Urine c.c.	Total N per diem	Protein per 100 c.c.	Protein per diem	Protein N per diem	Non-protein-N per diem	Pr. N×100 / Tot. N	Diet, etc.
Aug.	12	1120	6·57	0·867	9·71	1·57	5·00	23·9	Uncontrolled diet
	15	1064	7·97	1·185	12·92	2·09	5·88	26·2	,, ,,
	18	1120	—	1·005	11·26	1·82	—	—	,, ,,
	22	1120	6·94	0·822	9·21	1·49	5·45	21·4	,, ,,
Nov.	3	952	—	1·11	10·57	1·71	—	—	,, ,,
	5	1120	9·12	1·06	11·87	1·92	7·20	21·0	15 grms. pancreatic digestion product of casein
	6	1120	7·55	0·922	10·33	1·67	5·88	22·4	Uncontrolled diet
1906									
May	29	740	10·06	1·100	8·14	1·32	8·74	13·0	Beginning of nine
	30	850	11·47	0·932	7·92	1·28	10·19	11·1	days period in which
	31	1090	11·23	0·741	8·08	1·31	9·92	11·7	the *N*-balance was
June	1	1030	13·00	0·814	8·39	1·36	7·03	10·4	estimated (*vide* text)
	2	1523	14·30	0·591	9·01	1·46	12·84	10·0	
	3	1072	12·72	0·744	7·98	1·29	11·43	10·1	
	4	902	9·00	0·797	7·19	1·16	7·84	12·9	
	5	846	10·18	0·942	7·97	1·29	8·89	12·6	Witte's peptone (20 grms.) formed part of the diet
	6	846	10·11	0·944	7·99	1·29	8·82	12·7	End of balance period
	25	896	—	0·975	8·73	1·41	—	—	Uncontrolled diet
	29	1512	—	0·565	8·54	1·38	—	—	4th and 5th days of a
	30	1344	—	0·600	8·06	1·30	—	—	rigid vegetarian diet
July	1	840	—	0·910	7·64	1·24	—	—	Ordinary diet
	6	336	—	2·800	9·50	1·54	—	—	After 3 days of bone-marrow
	7	392	—	2·050	8·04	1·30	—	—	Ordinary diet
Nov.	14	—	—	—	7·80*	—	—	—	,, ,,
	20	—	—	—	8·29*	—	—	—	,, ,,
Dec.	9	—	—	—	7·84*	—	—	—	,, ,,
1907									
Oct.	13	840	6·43	1·582	13·30	2·15	4·28	33·3	Uncontrolled diet
	14	928	7·08	2·147	15·65	2·53	4·55	35·7	,, ,,
	15	952	7·69	1·560	14·85	2·40	5·69	31·2	,, ,,
	18	672	6·12	1·800	12·11	1·96	4·16	32·0	,, ,,
1908									
Jan.	14	616	7·81	—	—	—	—	—	,, ,,
	15	560	6·80	2·740	15·34	2·48	4·32	36·5	,, ,,
	16	504	8·21	3·537	18·24	2·95	5·26	35·6	,, ,,
1909									
Jan.	24	420	5·54	0·802	3·37	0·546	5·00	10·0	Day immediately preceding death

* The experimental data for these three days are lost. The total quantity of protein was recorded however in a table drawn up at the time.

diem. Unless therefore it can be assumed that the progress of pathological change in the bone marrow is at times in abeyance, the amount of protein excreted would not seem to depend upon the actual amount of the abnormal tissues existing at any time. In 1907 only four determinations were made; in the month of October. The daily excretion then averaged 14 grms. a day. The protein therefore, though in excess of the amount excreted during the greater part of 1905, and nearly double that of the year 1906 was yet in no greater quantity than when our experiments began, two and a half years earlier. The increasing kyphosis at this time, and the general history of the case leave little doubt that the amount of myelomatous growth had meanwhile greatly increased. In the middle of January 1908 three samples of urine were examined and the amount of urinary protein was at this time increased, amounting on three consecutive days to 15·30, 15·34 and 18·24 grms. respectively. We had no opportunities of making analyses upon measured 24-hour samples during the last year of the patient's life; though individual specimens received from time to time showed that the excretion of the protein continued. Not until the day before death did we receive the whole urine of the preceding 24 hours. This measured only 420 c.c. and the protein contained in it amounted to no more than 3·37 grms. The only point of interest in connection with this last analysis is the circumstance that, the patient being *in extremis*, the protein excretion seemed to show a much greater proportionate reduction than did the total nitrogen.

Perhaps the most important matter for consideration here is the influence of protein ingestion upon the excretion of the Bence-Jones protein. We found it impossible to put the patient upon such extremes of diet as would determine this relation unequivocally. But our data indicate, we think beyond doubt, that the excretion was largely independent of protein intake; that, in other words, the protein is an essentially endogenous product. In this our results agree not only with those of Parkes-Weber and Bradshaw, whose observations were few, but with those of Allard and Weber who followed the excretion in their case for some weeks. They differ on the other hand from those of Voit and Salvendi[1] who found that the amount of protein eaten produced a large effect upon the excretion. These last observers give however no figures in their paper.

The independence of protein intake is not absolute, any more than it is in the case of some other excretives usually classed as endogenous.

[1] *Münch. med. Wochenschr.* No. 24. 1904.

In Case A, upon May 8th to 10th, inclusive, and upon June 18th and 19th (1905), the protein of the diet was, with intention, largely increased. On each occasion there was a definite increase in the excreted protein which, as should be observed, was delayed.

But if the fluctuations of total-N and protein-N throughout the period of observation be compared, it will be seen that they are to a large extent independent. In general, as inspection of the table will show, whenever the total-N rises the proportion which the protein-N bears to it falls; when the total-N falls this ratio rises. The protein output is the more stable. Thus, taking the excretion of non-protein nitrogen as a measure of metabolism, we find 24 days in the Table in which its value was over six grms., and 11 days in which it was under five grms. The average of the first gives the protein-N as amounting to 18 % of the total-N; the average of the second gives this proportion as high as 30 %.

That the excretion depends but little on the immediate ingestion of protein is shown by the results of gelatine feeding. On April 4th and 5th and also on May 24th and 25th (1905) the patient took a dietary almost free from protein, consisting of arrowroot biscuits and gelatine in the form of jellies. The excretion of the second day is in each case given in the table and the amount of protein will be found scarcely below the average level of the period. As the urinary protein is characteristically rich in aromatic groupings, it could hardly on these occasions have taken origin as an exogenous product by direct diversion from intestinal processes, since gelatine was—and had been on each occasion for 24 hours previously—the sole nitrogenous material undergoing digestion. This evidence would have been more conclusive had the gelatine feeding been longer extended, but we were unable to insist upon a longer consumption of an unwelcome dietary.

The observations made in 1906 would, we are convinced, have offered a marked illustration of the relatively small influence of diet had our determinations of total nitrogen been complete. The patient was, at first, for a period of nine days (balance experiment, *infra*), upon a flesh dietary which had a nitrogenous value much in excess of what accorded with her general habits. This led to abnormally low values for the ratio protein-N : total-N, such as would be expected on the assumption that the former is of endogenous origin. A fortnight later she took for five days a strict vegetarian diet. Unfortunately the total-N during the latter period was not determined though it was quite certainly very much below that of the preceding flesh period. The urinary protein showed however no decrease at all.

Considered as a whole our results indicate that the Bence-Jones protein has the essential stamp of an "endogenous" metabolite in the sense now generally attached to the word. That its amount is entirely unaffected by those activities of the tissues which are involved in digestion and assimilation is unlikely and against the weight of such evidence as is available.

As bearing upon this matter we give here the results of two experiments (Case A) in which the excretion of protein and total-N were followed in two-hour periods. No food was taken till 1 p.m., the hour of the chief meal. Another, smaller, meal was taken in the evening.

	Nov. 10		Nov. 19	
	Non-protein N per hour	Protein per hour	Non-protein N per hour	Protein per hour
7 a.m. to 1 p.m.	·19	·35	·26	·33
1 p.m. to 3 p.m.	·15	·34	·18	·34
3 p.m. to 5 p.m.	·29	·43	·30	·41
5 p.m. to 7 p.m.	·23	·21	·25	·24
7 p.m. to 7 a.m.	·23	·41	·17	·25

It is seen that an appreciable rise in the protein accompanies the postprandial rise in the urea. No conclusion as to the effect of sleep can be drawn from these two experiments, as, in one, the night period showed an excretion above the average for 24 hours; in the other it was somewhat below it.

TABLE B. *General Metabolism; Cases B and C.*

Date 1907	Urine c.c.	Total N per diem	Protein per 100 c.c.	Protein per diem	Protein N per diem	Non-protein-N per diem	Pr. N×100 / Tot. N	Diet, etc.
Case B.								
April 26	1755	23·30	2·24	39·30	6·40	16·90	27·5	Uncontrolled
27	1698	22·58	2·40	40·75	6·64	15·94	28·5	,,
28	1472	19·89	2·31	35·00	5·70	14·19	28·7	,,
29	1754	21·41	2·09	36·68	5·98	15·43	28·0	,,
30	2207	24·50	1·85	40·83	6·65	17·85	27·5	,,
May 1	1811	24·09	2·12	38·39	6·26	17·83	26·0	,,
Case C.								
1910								
Jan. 15–20 mixed sample	—	—	2·73	—	—	—	30·4	,,
Feb. 23	1460	12·26	1·67	24·38	3·97	8·29	32·4	Flesh
25	1600	12·64	1·47	23·52	3·83	8·81	30·3	Milk
April 16	1195	10·54	1·73	20·67	3·37	7·17	32·0	Milk and fish
17	1130	11·94	2·08	23·50	3·83	8·11	32·0	Flesh

In *Case B* the quantity of protein excreted was high, amounting on the average to 38 grms. daily. No experimental variations in the diet could be made; the patient, as already stated, died a short time after we began to examine the urine. The output of protein during the six days covered by our estimations was remarkably constant; but so also to a somewhat less degree was the nitrogenous metabolism as a whole. The nitrogen excreted in the form of the protein constituted 28 % of the total nitrogen.

In *Case C* the output of protein was found to be nearly 24 grms. daily; its nitrogen amounting to nearly a third of the whole nitrogen excreted.

It would have been satisfactory could we have made a prolonged study of metabolism, similar to that made in Case A, on one of these two cases with a high output of protein. But the data given are sufficient to indicate the varying grade of metabolism which the cases respectively present. That B and C differ widely in this respect from Case A makes more interesting such proof of identity in the excreted protein as is offered in Sections II and III.

Nitrogen-Balance Experiment. Case A. It seemed important to determine how far an individual suffering from so grave a disturbance of protein metabolism as this condition of proteinuria betokens, could be in nitrogenous equilibrium. The comparatively long duration of life found in Cases A and C, and some other published cases, after the establishment of the condition suggests that some conservative element is present. Whatever its origin, the protein, it would seem, cannot represent a loss to the tissues which is wholly unreplaceable. We have no continuous record of body weight in Case A, but the data we possess show that, for months together, the loss might be slow. When she first came under observation the woman weighed 50 kilos. In December 1905 she weighed exactly 49 kilos. Four months later the weight was the same. In August 1906 it was 45·5 kilos. In October of that year it was unaltered. In January 1909, the last record we possess, it had fallen to 43 kilos. This loss was of course more than sufficient to cover the protein excreted if reckoned as protein, but not enough to correspond to the loss of tissue which such protein loss would represent. That the cellular growth in the marrow was all the time increasing in bulk must of course be borne in mind.

A nine days' N-balance experiment was carried out in this case. It lasted from May 29th to June 6th, 1906, inclusive, and the protein excretion, with that of total-N, is scheduled in Table A. The comparison between income and outgo is given in the following Table.

Date 1906	N in Food grms.	N in Urine grms.	N in Fæces grms.	Total Excretion grms.	Balance
May 29	15·86	10·06	0·65	10·71	+5·15 grms.
30	13·32	11·47	,,	12·12	+1·20
31	13·67	11·23	,,	11·88	+1·79
June 1	14·39	13·00	,,	13·65	+0·74
2	13·76	14·30	,,	14·95	−1·19
3	12·88	12·72	,,	13·37	−0·49
4	9·45	9·00	,,	9·65	−0·20
5	13·77*	10·18	,,	10·83	+2·94
6	11·84	10·11	,,	10·76	+1·08
Total for 9 days	118·94	102·07	5·85	107·92	+11·02
Average per diem	13·21	11·34	0·65	11·99	+1·22

* Witte's peptone (19·7 grms., containing 3·33 grms. nitrogen) formed part of the total ingesta on this occasion.

The food chosen was such as to be easily manipulated quantitatively, and consisted of beef and mutton as free as possible from fat and connective-tissue: of eggs, milk, butter, bread, and biscuit. A little cocoa was drunk. The meals taken during the period were personally supervised by one of us (H. S.). The items of food were weighed out, and what was unconsumed carefully weighed back each day. Each item, including the successive supplies of milk for instance, was analysed. The fæces proper to the period were isolated by means of charcoal given with the first and last meals.

On the balance of nine days there was therefore retention of nitrogen to the extent of 11 grms.; or 1·22 grms. a day. The experiment as carried out involved, it is true, certain complications which were unintentional and unforeseen. It was impossible to secure the consumption of a uniform dietary each day, and therefore the plan of determining accurately the unconsumed residue was adopted. Either the formality of the proceedings, or the fact that the supply was more abundant than usual had a psychological effect which led the woman to consume very much more food than was her custom—a circumstance not fully realised by us till the analyses came to be made. The first day of the balance experiment was one, therefore, in which there was a sudden rise in the protein intake, associated with an exceptionally large retention of nitrogen. On the fifth day there was some disturbance to digestion, and the conditions were not quite normal. On the 7th day a failure of appetite considerably reduced the amount of food taken, and on the following day, when the higher consumption was resumed, there was again notable retention of nitrogen. Not only in connection with this

experiment, but on other occasions when the protein of the diet was intentionally increased, we noted a tendency to retain nitrogen which appeared to be more marked than is normal.

A positive result from a balance experiment would have been of more significance perhaps if the patient had consumed an amount of food more in accordance with her custom. The fact remains however, and is of interest, that, in spite of the grave disturbance to protein metabolism, a gain of nitrogen to the body is possible in this condition, at any rate for a considerable period.

The exceptionally high consumption of protein during the experiment accounted for the low ratio of *Protein-N : Total-N* in the urine (Table A). This constituted part of the evidence, already discussed, for the endogenous origin of the Bence-Jones substance.

Other Aspects of Metabolism. In Cases A and C evidence was sought as to whether in addition to the Bence-Jones body any simpler complex of amino acids was at the same time present. No trace of any polypeptide precipitable by ammonium sulphate, nor of any substance yielding a biuret reaction could at any time be found in the urine after careful removal of the protein by heat coagulation. In Case A, on three occasions, at widely separated intervals, 500 c.c. of the fresh urine (after removal of protein) were concentrated to 100 c.c. alkalised with sodium carbonate, and shaken for two hours with an etherial solution of naphthalin sulpho-chloride. Only minute traces of synthetic derivatives could be separated; no more than could be obtained from normal urine under similar circumstances. In Case C a similar observation was made with equally negative results. At an earlier stage of our work we believed, with Magnus Levy, that the protein of the urine took origin directly from the bowel. It seemed of no small interest therefore to test the effect of giving predigested protein by the mouth. We intended to replace entirely the protein in a day's dietary by hydrolised products, to test the possibility of demonstrating an intestinal synthesis. As a preliminary experiment, on November 5, 1905 (*vide* Table A) 15 grms. of the products obtained by digesting casein for one month with Pancreatin Rhenania were given. (It was intended to administer 30 grms., but the patient was unable to take more than half of this.) The effect upon the protein excretion was practically nil. The realisation of the fact that the urinary protein is essentially endogenous in origin robbed this type of experiment of the special significance which we supposed at the time to be attached to it; but the urine of the day in question was tested on the above lines with naphthaline sulphochloride and found

to be free from polypeptides and amino acids. The experiment showed therefore that there was no failure to metabolise either such amino acids as would be liberated by prolonged pancreatic digestion or the polypeptides associated with them. In Case C, on one occasion, 1 grm. of pure tyrosine was given by the mouth. Working with a large proportion of the whole day's excretion we were unable to demonstrate any trace of tyrosine in the urine.

When the diet was uncontrolled as was, perforce, the case with most of our observations, no great importance can be attached to the figures of a general analysis of the urine. We made therefore comparatively few determinations other than those of protein and total nitrogen. Certain results may be given here, however, the general tendency of which is to show that if the protein of the urine be wholly left out of account, the ratio $\frac{\text{urea N} \times 100}{\text{Total N}}$ remains normal. The indication is that, side by side with the exceptional line of metabolic activity which results in the production and excretion of the special protein, a normally balanced nitrogenous metabolism continues.

Case and date	Total Non-protein N	N as urea	Urea N in percentage of Non-protein N	Method of urea estimation
A. 1905				
April 3	6·58	4·80	73	Morner-Sjoquist
5	4·93	3·70	75	,,
May 28	4·57	3·12	70	,,
July 1–10 Mixed sample of 10 days	5·20	3·90	75	Folin
B. 1907				
April 30	17·85	17·07	90	Morner-Sjoquist
May 1	17·83	16·22	91	,,
C. 1910				
Feb. 23	8·29	6·68	80	,,
25	8·81	7·20	82	,,

Thus, the ratio falls with diminished total metabolism, and if the above figures be compared with those given by Folin[1], it will be seen that for similar levels of total metabolised nitrogen (Non-protein N in our cases) the relative proportion of urea is of the same order.

Of some significance is the fact that the excretion of *Creatin* appears to be characteristic of Bence-Jones proteinuria. Evidence was obtained with regard to this from Cases A and C only. Estimations of preformed creatinine, and of total creatinine after the dehyrolysis of creatin were

[1] *Amer. Journ. Physiol.* XIII. p. 70.

made by Folin's method. The conversion of the creatin into creatinine was secured by heating the urine for five hours in the water-bath after mixing it with twice its bulk of normal HCl.

Case and date	Preformed Creatinine p.d.	Total Creatinine	Creatin estimated as Creatinine
A. 1908			
Jan. 14	0·542	0·769	0·227
15	0·425	0·649	0·224
16	0·534	1·360	0·826
B. 1909			
April 26	1·690		
27	1·555		
28	1·270		
29	1·270		
30	1·440		
May 1	1·470		
C. 1910			
Feb. 23	0·847	1·460	0·613
25	0·840	1·451	0·611
April 16	0·968	1·220	0·252
17	1·096	1·412	0·316

This excretion of creatin was not associated with acidosis. At no time were acetone substances detected in the urine.

SECTION V. SOME EXPERIMENTS MADE WITH THE BONE-MARROW GROWTH AND THE ORGANS OF CASE A; ETC.

Absence of the protein from the growth and organs. We failed to extract either from the myelomatous tissue (of which we had a large supply, chiefly from the shafts of the long bones) or from the liver, kidneys, and intestinal mucosa of Case A any substance with the physico-chemical properties of the Bence-Jones protein. As at the time the examinations were made we were in possession of most of the facts of Section II, we are not likely to have failed to detect the protein had it been present. With some knowledge of the influence of salts upon its solubility the substance is less likely to be missed. It seemed possible that the protein might be an early product of the autolysis of the tissues. The above observations were made upon the fourth day after death; but, as the material was upon ice during a greater part of the period which had elapsed, autolysis could not have proceeded very far. We therefore placed portions of the above mentioned organs—ground up with sand and mixed with chloroform in closed flasks—in the incubator

at ·35°. The bone-marrow growth was similarly treated, and so also were mixtures of the growth and organs. In no case could any trace of the Bence-Jones substance be detected at any stage of the autolysis.

Injection of the tumour into animals. Each of three white rats received three successive injections of the bone-marrow growth. Each injection consisted of about 1 grm. of the growth suspended in normal saline. The rats lived for several months. Their urine at no time showed the presence of the characteristic protein, and the bone marrow was found *post mortem* to have normal characters.

Attempt to obtain a precipitin. We injected five rabbits, not with urine which was apparently used by Abderhalden and Rostoski, but with dialysed solutions of the separated protein. Three of the animals were treated with material from Case A, and two with that from Case C. Each injection consisted of 10 c.c. of an approximately 2 % solution, and each rabbit received five injections at intervals of a week. In no case did we obtain a precipitin reaction from the serum of the animals thus treated, whether it was added to a solution of the protein, or to human serum at any dilution. This negative result must not be held to weaken the positive evidence obtained by Abderhalden and Rostoski[1]. The amount of material injected by us was very small especially as the injections were intraperitoneal. Moreover we found that anti-human serum supplied by the firm of Meister Lucius and Co. gave a definite, if slight, reaction with solutions of the protein. But we think the above observations show that the pure Bence-Jones protein has less power as an antigen than have serum proteins.

Section VI. Discussion and summary of results.

Much of the evidence which has been supposed to indicate that the peculiar behaviour of Bence-Jones protein upon heating may be exhibited by various and quite diverse types of protein seems to us unsatisfactory. Voit and Salvendi[2] state that the urine of their case contained material both of the albumen and globulin type though the coagulum of the whole was soluble on boiling. As A. Ellinger[3] remarks, such differentiation cannot be considered as justified when based upon precipitation limits alone. Ville and Derrien[4] considered that the material examined by them was a histone, though it exhibited the

[1] *loc. cit.*
[2] *loc. cit.*
[3] Oppenheimer's Handbuch, *loc. cit.*
[4] *loc. cit.*

"thermolability" characteristic of Bence-Jones protein. Their view was grounded upon the fact that after dialysis and treatment with very dilute hydrochloric acid, the protein became insoluble in ammonia if sufficient ammonium chloride were present. We could repeat this observation with our material, though the protein, whether judged by its analysis, or otherwise, was certainly not a histone in the proper sense of the term. The behaviour described is really due to the fact that under the influence of very dilute acids the protein undergoes that change which T. B. Osborne[1] has called "protean" formation, a protean being a derivative, standing between the native protein and an acid albuminate (metaprotein), which exhibits certain of the properties of a histone.

More difficult to interpret are the results recently published by O. T. Williams[2]. This author considers the material studied by him to be mucinoid in nature, probably related to the osseomucoid of Hawke and Gies or to the tendomucoid of Loebisch, Chittenden and Gies. The case examined was one of undoubted multiple myeloma. The urine, which showed the typical heat phenomena, gave, on one out of three occasions mentioned, a precipitate with acetic acid in the cold, though not on the other occasions. "A specimen," writes the author, "of this precipitate was obtained from 500 c.c. of urine, purified, and found to contain a carbohydrate radicle and no phosphorus; it is therefore shown to be a mucine." It is not stated what proportion of the whole protein present was so precipitated. A slight precipitate on adding cold acetic acid has been observed by others[3], and by ourselves, though the precipitate had no mucinoid characters. It is also not stated how the carbohydrate radicle was identified, nor whether it was proportionately abundant, as in mucins and mucoids. The evidence as presented does not seem very strong in support of the presence of a mucoid. Our material gave always a well defined, but by no means intense Molisch reaction. It was comparable with that given by most typical proteins and much weaker than that obtained from mucins or egg-albumen. Because of a marked variation in the etherial sulphates in the urine (of two days) and because "the Bence-Jones body contains loosely combined sulphur" the conclusion was drawn that it might be related to the above mentioned connective tissue mucoids. The force

[1] *Zeit. physiol. Chem.* XXXIII. p. 225. 1901, *Journ. Amer. Chem. Soc.* XXIV. p. 28. 1902, *The Vegetable Proteins* (Longmans, London), p. 37.

[2] *Biochem. Journ.* V. p. 225. 1910.

[3] Cf. Abderhalden and Rostoski, *loc. cit.* p. 129.

of the evidence is elusive, since all typical proteins contain cystine and therefore loosely bound sulphur.

"On February 18th from 500 c.c. of urine 0·4 grams of the protein were obtained in a pure condition. It contained 10·5 % of sulphur. On March 2nd, 0·3 grams of protein were obtained, and it contained only 0·55 % of sulphur." These results show certainly an extraordinarily wide variation in the sulphur, and the higher figure is a most remarkable one, indicating a much greater proportion of this element than exists in any known protein or albuminoid, not excepting the keratines. The exceedingly small amount of material available however can hardly have permitted accuracy in analysis. It will be seen from the figures given in Section III that the sulphur determinations in all our cases were identical within the limits of experimental error, the results showing an average of 1·18 %. As regards possible daily variations we can offer the following negative evidence. We happened to possess the protein separated from two individual days' excretion in the case of the woman patient (Case A), and since the appearance of Owen's paper we have determined the sulphur in each of these samples, 1·5 grams of material being employed in each case. The protein of March 20th, 1905, gave 1·22 %, and that of April 16th, 1906, gave 1·18 %. It is of course possible that the body excreted by Owen's patient was different from that described by ourselves and others, but we must confess that the evidence given to award it a place among the mucoids does not seem very strong.

The general characters of the protein excreted in our cases agreed with the descriptions given of the substance as found in the majority of other cases. In its native condition it resembled an albumen more nearly than any other type of protein, though the special relations between its heat coagulum and salts stamp it as different from any known albumen.

We have given the evidence on which is based our belief that it was identical in all three cases. Strictly speaking it is, for the most part, evidence showing only that the average material excreted was the same; because perforce the protein had to be accumulated for analysis from the urine of long periods. But we could find no evidence of any variation in one period as compared to another, and, considering that the material was collected under conditions and for periods controlled entirely by chance, the very fact that the accumulated excretion of three diverse individuals was similar is against the likelihood of its varying from day to day in any one. As the individuals concerned differed in

sex, in the acuteness of their disorder, in the grade of their metabolism, and in other particulars, the cases could not well be more diverse, and we believe that the same protein will prove to be characteristic of, at any rate, a large group of the cases which exhibit the condition. But Abderhalden and Rostoski obtained from the protein analysed by them yields of the various amino-acids so strikingly different from ours that there can be little doubt that they were handling different material. They worked it is true under the disadvantage of a very limited supply of material, the whole analysis being made upon 75 grams of protein[1]. But neither this fact, nor the differences in the methods used, could account for the variation in the results. They found for instance only 1·5% phenylalanine and 1·7% tyrosine, while we obtained (as an average from two cases) 4·82% and 4·18% respectively. It is not, we believe, unimportant to remember that Abderhalden and Rostoski's case was one exhibiting parenchymatous nephritis. Abundant epithelial, granular, and hyaline casts had been present in the urine, and, *post mortem*, chronic nephritis was revealed. Casts and blood proteins were always absent from the urine of our cases, and the necropsy of Case A, the only one examined, showed no nephritis. Though, as the above authors point out, the material analysed by them was wholly soluble on boiling, we think that cases in which nephritis is present should, in studies of the excretion, be distinguished from those in which it is not.

We are still unfortunately without any real information as to the source of the protein, and there are no facts yet available to explain the association of its excretion with myelomatosis. Any discussion of the matter can only be based upon hypothesis.

Magnus Levy's view that it arises from perverted or incomplete intestinal synthesis was entirely plausible until evidence had accumulated to show that it was essentially an endogenous product. If we are right in supposing that the protein is, in typical cases, a uniform substance (not necessarily, be it understood, a chemical individual), identical in all such cases, the view is suggested (though not established) that it arises in the body from some individual tissue. Its exceptional characters and special associations suggest further that it takes origin from an abnormal tissue—the neoplasm in the bone marrow. This view, which has been more than once advanced, receives perhaps some support from the observations mentioned in Section IV, which suggest that the production of the protein goes on as it were in

[1] As a matter of fact the authors claim only that they were able to demonstrate (nachweisen) the presence of the various amino acids concerned.

a water-tight compartment in metabolism, accompanied by a general nitrogenous metabolism which is normal. But the special physico-chemical characters of the substance might depend upon a comparatively slight and superficial modification in the molecule of a normal tissue-protein, and the metabolic relations just mentioned may receive other interpretations. Its origin from the myeloma would be more probable if it could be demonstrated to be uniformly present in the diseased marrow after death. In Case A, as stated in Section V, we were quite unable to discover it either in the growth or elsewhere in the body. Some probability for its origin from the growth would exist could it be shown that the balance of amino-acids in its molecule was quite different from that of any normal tissue protein. But although our own determinations indicate that it differs in this respect from any protein yet analysed the available data are not sufficient to decide the matter.

There are certainly grave difficulties associated with viewing it as a mere metabolite of the growth[1]. Magnus Levy pointed out that the large amounts excreted would call for an extraordinarily intense protein metabolism on the part of the cellular constituents of the neoplasm. This consideration does not perhaps apply with entire cogency to the circumstances of Case A of this paper, in which the amount of diseased tissue was, at death, proportionately very great. But even in this case it must be remembered that the tissue was largely fatty, and the actual mass of its protoplasmic elements would be but a small fraction of that of the normal tissues of the body, though the protein nitrogen amounted to a third of the whole nitrogenous excretion. Moreover, as was shown in Section IV, the amount of urinary protein found was as high early in the history of the case as it was much later, when, to judge from the greatly increased bone deformation, the amount of growth must have greatly and actively increased. If we might judge from our three cases alone the amount of protein would seem to be far more nearly proportionate to the grade of general metabolism than to the amount of growth present. The view that it arises from the growth has been brought forward by more than one writer, and therefore we have discussed it. But if it once

[1] It is not altogether fanciful to suppose that the protein might itself be related to precipitin formation. Conceivably a foreign protein produced by the growth might act as an antigen and call forth precipitin formation in the tissues. The special features in the case would then reside in the fact that the conjugate formed passes the kidneys instead of remaining in the body.

becomes fully established that an identical substance may be excreted in conditions from which myelomatous growth is absent, in Osteomalacea for instance, any possibility of its being a specific metabolite of a specific pathological tissue will of course disappear.

Fr. N. Schulz[1] long ago suggested that the Bence-Jones protein might prove to be related to globin, and the importance of the bone marrow in hæmoglobin formation commends such a view to the attention. We know of course nothing about the quantitative constitution of the protein of human blood-pigment. If we compare the analysis of the globin of horse-hæmoglobin made by Fischer and Abderhalden[2] with ours of the urinary protein, we find some resemblances; but any close relation is at once negatived by the high content of histidin in the former (10·96 %). Owen's suggestion that the protein is derived from bone and tendon is interesting, but seems to us, as we have said, quite insufficiently supported by the evidence offered by him. If the protein really arises from any individual tissue, the large amount which may be excreted suggests the muscles as its source; and the fact that our cases excreted creatin, gives (if we accept current views) a certain amount of support to this suggestion.

An assumption which has much to justify it, and which has stimulated productive research in connection with the congenital anomalies of metabolism, is that which looks upon any unusual excretive as being, not a product wholly foreign to the body, but rather a product due to an arrest in the normal processes. If it were possible to view the excretion of Bence-Jones protein from this standpoint—to look upon its appearance in the urine as due to a failure at a higher level in the metabolic series than that which leads to such conditions as cystinuria or alkaptonuria, we might arrive at the conception that it is a token of an interruption in the normal autolytic processes in a tissue. This interruption might be due either to the positive influence of a toxine from the growth, or to the loss of some normal function of the bone marrow.

Our general results may be summarised as follows:

The characteristic solubility at 100° of the heat-coagulated protein, is due to the influence of associated substances, in particular to that of electrolytes, in solution.

[1] *Zeitsch. f. physiol. Chem.* XXIV. p. 449. 1898.

[2] *ib.* XXXVI. p. 268. 1902.

The solvent power of a salt in neutral solution rises with increase of valency in either its acid or basic constituent. Solution is probably due to the formation of a molecular compound between protein and salt which is more stable at temperatures near 100° than at lower temperatures.

In solutions which are acid or alkaline the solvent influence of salts is complicated by the effect of ionic charges on the charged colloid particles of the protein. Ions with a charge opposite to that of the particle exert an aggregative effect in accordance with Hardy's rule. A salt, therefore, yielding a polyvalent ion of sign opposed to the sign of the particle exhibits in acid or alkaline solutions a marked diminution in the solvent power which it displays in neutral solution where the particles are uncharged. But as the tendency to molecular association increases with temperature and with salt-concentration more rapidly than the ionic effect, such a coagulative salt may nevertheless dissolve the protein at 100°.

With increase of valency in the acid or basic constituent of a salt the solvent capacity shows also a greater relative increase than does the ionic, aggregative (coagulative) power.

Salts are graded similarly in their solvent capacity for the Bence-Jones protein at 100° and for globulins at ordinary temperatures. The essential difference is found in the temperature relation. It is because the stability of its compounds with neutral salts increases rapidly with temperature that the case of the Bence-Jones substance is special among proteins. This property makes it easy in the case of this substance to bring to light two relations between salt and protein in solution—the relation of the ions to the colloid particle (*qua* particle) and that of the salt as a whole to the protein molecule.

A comparison of the amino-acid yield strongly suggests that the protein in two cases examined was identical, and the general characters, elementary analysis, etc., of the protein of the third case also suggest its identity with that of the others.

The Bence-Jones protein contains all the amino acids characteristic of a typical protein, and is itself characterised by containing a high proportion of the aromatic groupings.

No protein having the peculiar characters of the excreted substance could be detected in the growth or tissues of Case A (the spleen and muscles were not examined). Rats could not be infected by intraperitoneal injection of the growth.

The urinary protein was found to be less powerful than serum proteins as a precipitinogen; but its solutions reacted slightly with an anti-human serum.

The three individuals whose cases form the material of this paper differed greatly in the grade of their total metabolism. The amount of protein excreted was proportionate to the amount of metabolism rather than to any other factor. The protein-nitrogen, when the diet was uncontrolled, amounted to nearly one-third of the total nitrogen excreted.

A study of the metabolism (prolonged in one case), and of the effects of diet, indicates that the protein is of endogenous origin.

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