Why this should be so is not clear, and can only be a matter for speculation. The fact that the production of choleic acid enteroliths requires the concurrence of a number of factors explains their rarity. Nevertheless, certain considerations suggest that they may not be so rare as the small number of reported cases would indicate. Of the ten previously reported cases, five have been recorded by one man (Mörner, 1939) and three by another (Helström, 1936). That one keen observer should have identified half the known cases does seem to show that others must have been missed; there is in fact much evidence in the literature that some enteroliths have not been examined at all, while some have received insufficient examination to identify their constituents with certainty. Any real knowledge of the incidence of bile-acid enteroliths—or indeed of any type of enterolith—can only be obtained if all stones found in the intestine are submitted to careful chemical analysis.

SUMMARY

1. Two enteroliths removed from the intestine of a woman have been analyzed, and it has been shown that they contained over 90% of bile acids. Of these

acids approximately 90% was choleic acid, and 10% was cholic acid.

2. The pH at which certain bile acids are precipitated from solutions of their salts has been determined, and it has been shown that, of the bile acids usually present, choleic acid is much the most likely one to be precipitated at the reaction normally found at the site of formation of these enteroliths. This is believed to explain why all the bile acid enteroliths so far reported, except one, consist mainly of choleic acid.

3. The factors which might be responsible for the formation of choleic acid enteroliths have been discussed and some deficiencies in our knowledge of the bile acid composition of human bile have been pointed out.

4. While bile acid enteroliths are undoubtedly of rare occurrence, it is considered that they are probably commoner than the number of reported cases indicates. This is believed to be due to insufficient examination of intestinal stones in some cases, and lack of any examination in others. Careful chemical examination of all intestinal stones is recommended.

REFERENCES

- Armitage, G., Fowweather, F. S. & Johnstone, A. S. (1949). *Brit. J. Surg.* (in the Press).
 Chitre, R. G. & Puradare, B. N. (1944). *Indian Physician*, **3**, 174.
 Doubilet, H. (1936). *Proc. Soc. exp. Biol., N.Y.*, **34**, 86.
 Gregory, R. & Pascoe, T. A. (1929). *J. biol. Chem.* **83**, 35.
 Helström, J. (1936). *Hygiea, Stockh.*, **98**, 480.
 Irvin, J. L., Johnston, C. G. & Kopala, J. (1944). *J. biol. Chem.* **153**, 439.
 Josephson, B. A. (1933). *Biochem. Z.* **263**, 428.
 Kawaguchi, S. (1938). *J. Biochem., Tokyo*, **28**, 445.
 McGee, L. C. & Hastings, A. B. (1942). *J. biol. Chem.* **142**, 893.
 Mörner, C. T. (1923). *Hoppe-Seyl. Z.* **130**, 24.
 Mörner, C. T. (1939). *Hoppe-Seyl. Z.* **259**, 35.
 Raper, H. S. (1921). *Biochem. J.* **15**, 49.
 Reinhold, J. G. & Wilson, D. W. (1932). *J. biol. Chem.* **96**, 637.
 Saba, G. (1940). *J. Biochem., Tokyo*, **31**, 43.
 Sobotka, H. (1937a). *The Chemistry of the Sterids*. Baltimore: Williams and Wilkins Company.
 Sobotka, H. (1937b). *Physiological Chemistry of the Bile*. London: Baillière, Tindall and Cox.
 Wieland, H. & Revere, G. (1924). *Hoppe-Seyl. Z.* **140**, 186.
 Wieland, H. & Sorge, H. (1916). *Hoppe-Seyl. Z.* **97**, 1.

The Bence-Jones Protein of Multiple Myelomatosis: its Methionine Content and its Possible Significance in Relation to the Aetiology of the Disease

By C. E. DENT AND G. A. ROSE, *Medical Unit, University College Hospital, W.C. 1*

(Received 5 October 1948)

Few chemical investigations have been made on Bence-Jones protein in recent years. Reports on the methionine content of the protein are particularly scanty since, at the time of the earlier work (Hopkins & Savory, 1911; Abderhalden & Rostoski, 1905), this amino-acid was unknown. The values reported for methionine (Calvery & Freyberg, 1935; Devine, 1941; Harvier & Rangier, 1943) show considerable

variation, but agree upon a low figure. As a patient with multiple myelomatosis, who was excreting large quantities of Bence-Jones protein, had come to our notice, it was decided to investigate again the content of methionine and other amino-acids. The quantities excreted by the patient permitted purity tests on the intact protein, and the use of five independent methods for determining the methionine content.

It was also hoped that the material might provide a source of methionine-free protein in rat feeding experiments designed to produce anaemia and hepatic necrosis.

Case summary

The patient, a man aged 44, was admitted to University College Hospital, under Prof. H. P. Himsworth on 3 August 1946, complaining of weakness, loss of weight and pain in the lower ribs on the left side. On examination he appeared pale and the pain in the ribs was found to be accentuated by direct pressure. The urine on warming gave a heavy curdy precipitate at about 60°, which completely redissolved when it was heated above 80°, and reprecipitated on cooling below 80°. No other abnormal constituents were found. A further examination of the urine for Bence-Jones protein was made with the tests described by Osgood & Haskins (1931). Typical reactions were given with salt and acetic acid as well as with sulphosalicylic acid. Investigations during the next few days showed: blood urea 36 mg./100 ml., plasma proteins 6.2 g./100 ml., albumin 4.7 g./100 ml., albumin/globulin ratio = 3.1, haemoglobin 66%, white cells 6700/cu.mm. with normal differential. Sternal marrow examination showed that 55% of the nucleated cells were plasma cells. Radiography of the skeleton showed a few clearly defined decalcified areas in the skull and vertebrae consistent with multiple myeloma deposits. Similar areas were much more widespread in the lower ribs, humeri, femora, pelvis and clavicles. The patient was put on a high-protein (200 g. daily) diet and treated with nitrogen mustard (methyl-bis-(β -chloroethyl)amine hydrochloride). His anaemia worsened, however, and the haemoglobin fell to 42% in about 6 weeks, when he was given blood transfusions and allowed to go home.

He was admitted again on 9 December 1946 with further loss of weight, lassitude and pain in the ribs. He was beginning to become breathless on exertion. His blood urea was now 78 mg./100 ml., haemoglobin 32%, red cell count 1.6×10^6 /cu.mm., white cell count 6000/cu.mm., of which 2% were myelocytes and 1% plasma cells. Plasma proteins were 6.2 g./100 ml., albumin 4.5 g./100 ml., albumin/globulin ratio 2.7; sedimentation rate 65 mm. in 1 hr. He was treated by means of blood transfusions and given a course of methionine, 5 g. by mouth daily for 10 days. His condition continued to get worse, he began to be irrational, and, when very ill, insisted on going home where he soon died.

Unfortunately, the plasma proteins were not examined by electrophoresis.

METHODS

Collection of urines. Urine was collected over periods of 24 hr. and stored at 5°, and preserved by thymol.

Determination of protein content. The volumes of 24 hr. collections of urine were measured. The urine was filtered, and 5 ml. introduced into a centrifuge tube. It was made just acid to litmus with acetic acid, and heated on a water bath to 70°. The coagulated precipitate of protein was spun down, washed once with distilled water, twice with 90% ethanol and once with absolute ethanol. Finally the precipitate was dried in the oven at 100° for 2 hr., weighed and reheated to constant weight.

Preparation of large sample of protein. Urine was treated, in 7 l. amounts, with 90 g. of active charcoal for 15 min., and filtered through fluted filter paper. The clear, pale yellow

liquid, made just acid to litmus with acetic acid, was heated with mechanical stirring to 55–65° to produce precipitation and coagulation of the protein. The precipitate was filtered and washed with distilled water until chloride free, and spread out on Petri dishes, which were placed on top of an oven at 90° overnight. The dried protein was put through a mincer and gave a white powder, quite tasteless and odourless. The yield was about 95 g./7 l. urine. The protein contained no phosphorus.

By this procedure, a total of 1350 g. of protein was collected and thoroughly mixed. This denatured protein was later used for the feeding experiments and analyses.

Analysis of protein by salting-out methods. The filtered urine in volumes of 280 ml. was dialyzed against running tap water for 48 hr., and against a large volume of static distilled water for a further 48 hr. It was filtered from the slight precipitate and the filtrate used for salting-out experiments.

A constant volume of the dialyzed protein solution was treated with varying quantities of saturated Na_2SO_4 solution and distilled water to give a series of solutions having a volume of 10 ml., a nitrogen content of 10.5 mg., a pH of 7.0, and with the Na_2SO_4 concentrations ranging from 30 to 50% saturation at 37°, at which temperature the whole subsequent procedure was also carried out. Having stood for 22 hr., the mixtures were filtered, and the filtrates analyzed for nitrogen.

To isolate the first fraction of protein precipitated by this method, 1700 ml. of urine were dialyzed by the above method, and the protein solution treated with Na_2SO_4 to bring to 38% saturation. Under these conditions 29% of the nitrogen was precipitated. The mixture was filtered, and the precipitate taken up in distilled water and dialyzed as above to give fraction A.

To investigate further the upper part of the salting-out curve, 10 ml. fraction A, containing 10.0 mg. N, were treated with varying quantities of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ at 37°. The protein left in solution was estimated as in the salting-out experiment.

Electrophoretic analysis of protein. Urine (250 ml.) was freeze dried and sent to Dr A. L. Alling of the Strong Memorial Hospital, Rochester, N.Y., U.S.A., to whom we are indebted for the electrophoretic study. After suitable dialysis of the urine against sodium diethylbarbiturate buffer of pH 8.5 and ionic strength 0.1 the electrophoresis was carried out for 3 hr. in the tall form of the standard 11 ml. Tiselius cell at a field strength of 6.8 V./cm. Boundary patterns were obtained by the Longworth scanning method (Longworth, 1939).

Chromatographic analysis for amino-acids. The denatured protein was hydrolyzed for 24 hr. at 100° with 6N-HCl, and, after removal of the HCl by distillation *in vacuo*, the residue was dissolved in water. An amount of the solution containing 50 μg . of N was analyzed for amino-acids on the two-dimensional phenol-'collidine' paper chromatogram of Consden, Gordon & Martin (1944). A further similar quantity, previously treated with 10 μl . of 30% (w/v) H_2O_2 , was analyzed similarly for the specific purpose of revealing methionine and cystine if present (Dent, 1947). These two analyses were later repeated with 10 times the above amounts of hydrolysate with the object of detecting constituents present in small quantities only. One-dimensional chromatograms were also carried out in collidine, for subsequent spraying with the Pauly reagent (Pauly, 1904) for detection of histidine and tyrosine, and in *tert*-amyl alcohol (Work, 1948) to detect the two leucines, which overlap in the other solvents.

Analyses for cystine (other than by paper chromatography). *Qualitative.* To test qualitatively for cystine in a protein hydrolysate, the nitroprusside test may be inconvenient on account of the colour of the hydrolysate itself, and the quantity of protein required. The following spot test was used.

A drop of solution which may contain about 0.1% (w/v) cystine is applied to a filter paper, which is allowed to dry, immersed in 5% $\text{Hg}(\text{NO}_3)_2$ solution, thoroughly washed with distilled water and dried at room temperature or in an oven at 100°. The same concentration $\text{Hg}(\text{NO}_3)_2$ is striped across the paper from a micropipette, and is followed by 0.112N-KI applied in the same way. The background appears red on account of HgI_2 formation, whilst the area of cystine impregnation appears colourless for low concentration of cystine, and pale yellow for higher concentrations.

The following substances give a positive reaction to the test: cystine, cysteine, glutathione, homocysteine, cystine disulphoxide, thiolhistidine, *S*-benzylcysteine. The following substances give negative results: *S*-methylcysteine, taurine, cystathionine, methionine sulphone, methionine sulphoxide, lanthionine, $\beta\beta\beta'$ -tetramethylcysteine, ergothionine, homocysteine thiolactone, glycine, alanine, valine, leucine, isoleucine, serine, threonine, aspartic acid, glutamic acid, arginine, lysine, histidine, phenylalanine, tyrosine, tryptophan, proline and methionine.

Cystine, however, can be distinguished from all other substances which give positive reactions by repeating the test, this time omitting the preliminary immersion in $\text{Hg}(\text{NO}_3)_2$ and subsequent washing and drying. Under these conditions cystine gives a negative result, but the other substances continue to give the same reaction as before.

The test will detect 2 μg . of cystine with ease.

Quantitative. Cystine and inorganic sulphur were determined gravimetrically by the method of Lugg (1938).

Analyses for methionine (other than by paper chromatography). *Chemical.* The nitroprusside colorimetric method of McCarthy & Sullivan as modified by Horn, Jones & Blum (1946) was used. The method is not, however, very accurate for low concentrations of methionine in protein hydrolysates. Methionine was also determined by subtracting cystine and inorganic sulphur (as determined above) from total sulphur as determined gravimetrically by a wet-ashing method (Masters, 1939).

Microbiological. This was carried out by Mr J. G. Heathcote at the Cereals Research Station, St Albans, by the method of Dunn, Shankman, Camien, Frankl & Rockland (1944) as modified by Barton-Wright (1946) with the organism *Leuconostoc mesenteroides* P60.

Biological. Two animal-feeding experiments were carried out. In both cases white Wistar rats were housed separately in metabolism cages, and a constant intake of dietary protein N (123 mg./rat/day) was provided, the carbohydrate supplied being appropriately reduced when the appetite was poor, so that minimal amounts of food were left uneaten. The cages, donations of carbohydrate, salt, arachis oil, cod-liver oil, yeast extract, aneurin, riboflavin, pyridoxine, calcium pantothenate, choline and tocopherol were exactly as described by Dent & Rimington (1947), except where otherwise stated.

In the first experiment, two male rats of about 100 g. were fed with casein as the sole source of protein N. After 20 days the casein was replaced by Bence-Jones protein. After a further 14 days, 19 mg. α -aminobutyric acid/rat/day were included in the diet, as part of another experiment not

reported here. After a further 14 days, instead of this supplement, 27 mg. DL-methionine/rat/day were given.

In the second experiment, three male and three female rats, all litter mates, and weighing about 115 g. if males and about 93 g. if females, were fed with casein for the first 14 days as above, except that the carbohydrate was mixed with CaHPO_4 in the proportion of 99.2% corn starch and 0.8% CaHPO_4 . The salts, apart from the CaHPO_4 , were supplied by adding a solution of the composition of the salt mixture of Osborne & Mendel (1919), except that the metallic sulphates were replaced by the equimolecular amounts of their chlorides. In this way an adequate salt mixture completely free of sulphur was provided.

For the rest of the experiment rats 1 and 2 remained on the same diet, to serve as controls, while all the other rats were given Bence-Jones protein instead of casein. After the first 14 days, however, rats 3 and 4 received the Bence-Jones protein alone, while rats 5 and 6 received the same protein and in addition 27 mg. DL-methionine/rat/day. After a further 18 days the methionine supplement was switched from rats 5 and 6 to rats 3 and 4.

Throughout both experiments, 16 mg. L-tryptophan/rat/day were added to the casein diet. Water, distilled in the second experiment, was supplied *ad lib*. The rats were weighed individually at least every other day.

Other analyses. *Ash.* A small quantity of protein was ignited at red heat for 45 min. and the residue weighed.

Total nitrogen. This was determined by the micro-Kjeldahl method, with CuSO_4 and SeO_2 as catalysts.

Carbohydrate. This was determined by the orcinol method of Rimington (1940).

RESULTS

Daily output of Bence-Jones protein. The output varied by about ± 10 g./day (Fig. 1). On normal hospital diet, with a protein intake of about 100 g.,

Fig. 1. Patient's excretion of Bence-Jones protein from 14 to 29 August 1946, representative of the 62 days on which it was determined at various stages of the illness. On 26 August (indicated by an arrow) he was given by mouth 50 g. of his own Bence-Jones protein in addition to his normal diet.

the daily Bence-Jones protein output averaged 34.5 g. over a 5-day period. For the next 9 days, on a diet containing about 200 g. protein, the daily

output averaged 38.4 g. The average output remained at about the same level throughout the period during which the patient was studied, and was 36 g. when averaged over the 62 recorded days. After eating 50 g. of his own recovered Bence-Jones protein, in addition to his normal diet, the patient showed no change in the output. (A healthy volunteer also ingested 50 g. of the protein, without producing proteinuria or other detectable effects.) After the patient had been transfused with 2 pints of blood the output was halved for 2 consecutive days, but after a second transfusion about 2 months later a similar result was not reproduced. The feeding of 5.0 g. of DL-methionine/day for 10 days did not affect the output, and methionine could not be detected in the daily urines by the one-dimensional chromatographic technique of Dent (1946) using phenol as the solvent. Since the method would detect 2.5 μ g. of methionine, and the average urine volume was 2500 ml., the daily output of this amino-acid, while it was being given by mouth, must have been less than 250 mg. α -Aminobutyric acid was also not detected in the urine. Methionine is readily detected in this way in the urine of a normal person who ingests 5 g./day.

Fig. 2. Salting-out curve of Bence-Jones protein by Na_2SO_4 at 37° . The arrow indicates the point at which the bulk fractionation was made (see text).

Salting-out curves. Fig. 2 shows the effect of salting out the dialyzed Bence-Jones protein with sodium sulphate. To find the number of constituents, the log of the solubility of the protein was plotted against salt concentration (Fig. 3; see also Cohn, 1925). The precipitation graph then follows along two straight lines, representing two constituents, with the point of inflexion when 87.8 % of the nitrogen has been precipitated. The arrows in Figs. 2 and 3 indicate the point at which the fractionation described on p. 611 was made. The salting-out curve of

this fraction (fraction A) with zinc sulphate is shown in Fig. 4.

Fig. 3. The results of Fig. 2 plotted on the log scale to show the number of components present. The arrow indicates the point on the curve above which it was more carefully examined in another experiment (see text and Fig. 4).

Fig. 4. Salting-out curve of fraction A of Bence-Jones protein by ZnSO_4 at 37° .

Electrophoretic diagrams. Dr Alling reported: 'The electrophoretic mobility of different samples of Bence-Jones proteins varies somewhat, but the mobility in this case corresponds to that which we have found most frequently for Bence-Jones protein. The pictures (Fig. 5) show the usual boundary anomalies and one large slightly asymmetrical peak moving with the mobility of fibrinogen. The asymmetry of the main peak probably represents a small amount of protein travelling slightly faster than the main component.'

When this undenatured protein was added to a sample of normal plasma, the peak migrated with the fibrinogen.

Analysis by paper chromatography. The results of one of the two-dimensional chromatograms are shown diagrammatically in Fig. 6. It shows all the

Fig. 5. Electrophoretic patterns of Bence-Jones protein. (a) and (b) are the ascending, (c) and (d) the descending patterns. In (a) and (c) the protein has been mixed with plasma proteins from a normal man. A = albumin. α_1 , α_2 , β and γ = the corresponding globulins. ϕ = fibrinogen. δ and ϵ are the usual boundary anomalies. BJP = Bence-Jones protein.

Fig. 6. Diagram of chromatogram obtained from acid hydrolysate of Bence-Jones protein. An amount containing 50 μ g. of N was taken and placed at the bottom right-hand corner of the filter paper. Phenol was run from right to left followed by 'collidine' in an upward direction. The latter was run for half as far again as the phenol. The sizes of the spots have been drawn to represent the relative strengths of ninhydrin colour reaction according to an arbitrary scale. The identifications are: 1, cysteic acid (from cystine); 2, aspartic acid; 3, glutamic acid; 4, serine; 5, glycine; 6, threonine; 7, tyrosine; 8, alanine; 9, histidine; 10, phenylalanine; 11, leucine and/or isoleucine (an independent method showed that both were present); 12, valine; 13, proline; 14, arginine; 15, lysine.

common amino-acids except tryptophan (destroyed in acid hydrolysis), methionine, histidine and

Table 1. *Analysis of Bence-Jones protein*

(All values are expressed as % (w/w) of dry ashless protein except in the case of values for ash itself which are corrected for dry material only.)

	This paper	Calvery & Freyberg (1935)		Devine (1941)	Harvier & Rangier (1943)
		Sample 1	Sample 2		
Ash	0.16	5.1	1.2	0.4	—
Total nitrogen	16.2	18.0	18.1	14.7	—
Total sulphur	0.76	1.0	1.0	1.34	1.15
Cystine	2.47	3.0	3.0	2.9	—
Inorganic sulphur	0.10	—	—	—	—
Carbohydrate	0.17	—	—	—	—
Methionine (chemical methods)	0.00*	0.81†		0.58	1.80‡
Methionine (microbiological method)	<0.02	—		—	—
Glucose/nitrogen ratio	0.0106	—		—	0.07

* The gravimetric method for methionine gave a small negative value, cystine + inorganic sulphur averaging 0.768 (0.753, 0.771, 0.778, 0.776) and total sulphur 0.761 (0.760, 0.758, 0.765). The reason for this is not known.

† This value does not appear in the paper by Calvery & Freyberg (1935), but is attributed to them by Block & Bolling (1947, p. 178).

‡ The authors do not indicate which chemical method was used. The protein was obtained not from a case of myelomatosis, but from the urine of a patient with multiple secondary carcinoma of bone.

hydroxyproline. There is also a spot in the position occupied by leucine and/or isoleucine. After oxidation with hydrogen peroxide the cysteic acid spot showed strongly, indicating the presence of a fair quantity of cystine, but no methionine sulphone was seen as would have been found had methionine been present in the original protein. The same result was also obtained from a sample of the protein passed in the urine during the period of methionine administration. The chromatograms run with 10 times the usual quantity showed histidine, but still no methionine as its sulphone, nor any hydroxyproline. As the technique readily detects 5 μ g. of methionine there must have been less than 5 μ g. in the 3 mg. of protein taken, i.e. less than 0.16 g./100 g. of protein. The presence of histidine in small amounts was confirmed by the one-dimensional collidine chromatogram, tryptophan by the Hopkins-Cole reaction, leucine and isoleucine (much more of the former) in the *tert*.-amyl alcohol chromatogram.

DISCUSSION

The recognition of the protein in our case was based on the clinical diagnosis and on its giving the classical reactions on heating. The salting-out and electrophoretic properties indicated that about 90% of the material consisted of a single component. Nearly all the protein was precipitated within the concentration range of 38–46% saturation with regard to sodium sulphate (Figs. 2 and 3). The salting-out curve with zinc sulphate of the less soluble fraction (Fig. 4) fully confirmed that the upper point of inflexion was as sharp as has been drawn and that the upper part of the curve was a straight line.

With regard to the chemical composition of the protein, the paper chromatograms (Fig. 6) clearly displayed the many amino-acids present in the acid hydrolysate. All the 'essential' or 'indispensable' amino-acids (Rose, 1938) were found with the

Fig. 7. Growth curves in first rat experiment. Before arrow *A* both rats received casein as sole source of protein in the diet. From *A* till the end of the experiment Bence-Jones protein containing the same amount of nitrogen was substituted for casein. After arrow *B* methionine was added to the diet, between *C* and *B* α -aminobutyric acid was added. The weights of the two rats at the beginning and end of the experiment are marked on the curves.

Chemical analyses (including microbiological). These are shown in Table 1.

Biological results. The growth curves from the two rat experiments are shown in Figs. 7 and 8. In all cases rats given Bence-Jones protein diet without added methionine developed marked loss of appetite so that instead of eating quickly the maximum daily allowance of 11 g. they ate only 7 or 8 g. On the addition of methionine, appetite rapidly returned.

Fig. 8. Growth curves in second rat experiment. Before arrow *A* all rats received casein as sole source of protein. Rats 1 and 2 continued to receive casein throughout the experiment. After *A* and until the end of the experiment rats 3–6 received an equivalent amount of Bence-Jones protein instead of the casein. Between arrows *A* and *B* rats 5 and 6 received the methionine supplement, at *B* the supplement was transferred to rats 3 and 4. The weights of the rats at the beginning and end of the experiments are shown on the curves. The six rats were litter mates.

notable exception of methionine. The latter could not have been present to the extent of more than 0.2% or it would have been detected by this highly specific technique. In addition, the common 'non-essential' (or 'dispensable') amino-acids were all found except hydroxyproline. Compared with those obtained from a casein hydrolysate these chromatograms showed the presence of much higher relative amounts of serine and threonine. In this feature the

protein shows some resemblance to the γ -globulin of human serum (Brand, Kassell & Saidel, 1944).

The feeding experiments with rats confirmed closely the chromatographic results. As would be expected from the complete absence of an essential amino-acid the Bence-Jones protein failed to support growth or even to maintain the body weight of the animals. Supplementation, however, with the amount of methionine contained in the same weight of casein enabled growth to occur almost as well as in the control rats fed with casein as sole source of protein (Figs. 7 and 8). The other essential amino-acids must therefore have been present in adequate amounts. Glynn, Himsworth & Neuberger (1945) describe experiments in which rats were fed pure amino-acids in place of protein. Some of their diets were otherwise very similar to ours. They showed that a mixture of amino-acids of the composition of a casein hydrolysate which provided daily only 8 mg. of methionine, instead of the required 23 mg., allowed appreciable growth in the rats. The loss of weight on the unsupplemented Bence-Jones protein diet is therefore further evidence that there was much less than 8 mg. of methionine in the daily allowance of about 0.8 g. of protein, i.e. much less than 1% of methionine in the protein.

The two independent quantitative chemical methods also failed to detect any methionine in the protein. The microbiological assay led to the same result. More exactly, the latter showed that there could not have been more than 0.02% of methionine in the protein. If this maximal amount were actually present in the protein it would demand a minimum molecular weight of 650,000 for Bence-Jones protein which is far higher than the figure of about 35,000 generally accepted by most workers (Svedberg & Sjögren, 1929).

The material thus belongs to that small group of proteins devoid of methionine (e.g. insulin, tobacco mosaic virus) and differs conspicuously in this respect from the proteins present in normal plasma (Brand *et al.* 1944). Only three other analyses for methionine in Bence-Jones protein have been found in the literature (Table 1). The results of these do not agree with ours or with each other. The methods used are, however, either not mentioned or else are now not accepted as satisfactory, nor were any of the results checked by independent methods as in the present work. In view of these facts and of the possible differences between different samples of the protein, we think, therefore, that these results in no way detract from our findings that the Bence-Jones protein, at least in our particular case, contains no methionine. All the other amino-acids, except for the biologically unessential hydroxyproline, were present. Our findings support strongly the work of Abderhalden (1940) who claims, as have others before him, that Bence-Jones protein is quite distinct from

proteins of normal human plasma. The physico-chemical and immunological differences between different samples of the protein such as have been shown to occur by other workers (Hewitt, 1929; Abderhalden, 1940; Hektoen & Welker, 1940) still remain a puzzling feature. However, if the absence of methionine is confirmed in pure samples excreted by other patients a most definite point of similarity will be available, which may become useful for purposes of identification.

The above facts may be of significance in regard to the aetiology of the disease. The urinary protein has been shown to be present in the plasma of myeloma patients (Kydd, 1934; Moore, Kabat & Gutman, 1943; Blackman, Barker, Buell & Davis, 1944) and there is some evidence that the plasma cells may be the seat of its formation (Martin, 1947). In our case (and in one reported by Hewitt, 1929) there was a disease process at work resulting in the formation, by a very ill man, of at least 30–40 g./day of a fairly pure protein. The output of urinary protein was independent of changes in the diet and clinical condition of the patient, even being unaffected by adding to his diet 50 g. of his own Bence-Jones protein, and it was maintained at this level over a period of months. For the reasons stated above, this protein was either foreign to the body or else present normally in only undetectably small quantities. Such a situation cannot be explained as an overaction of a normal process such as could occur as the result of excessive stimulation or of malignant change. For purposes of comparison, the rate of formation of other body proteins can be discussed. A healthy dog under the best conditions of diet and under maximal stimulus of hypoproteinaemia can produce some 10 g./day of mixed plasma proteins (Whipple, 1948). A human being with a liver weight 4 times greater could be expected to produce under similar optimal conditions 40 g./day. In severe nephrosis, the disease in which the largest proteinurias occur, it is rare for more than 30 g./day to be excreted and this may be largely serum albumin. The total protein formation in the regenerating rat liver, which grows much faster than most malignant tumours (Brues & Drury, 1936) is only about 60 mg./100 g. body weight/day under the best conditions and during the peak growth period. At this rate the healthy human subject of 70 kg. could produce about 40 g./day of liver proteins, but there is still no analogy here for the production of similar amounts of a single protein constituent. The other theories also put forward (summarized by Calvery & Freyberg, 1935) likewise cannot explain the facts.

The tentative suggestion is, therefore, put forward that multiple myelomatosis is due to invasion of the body by a virus which lives and multiplies in the plasma cells of the bone marrow. It is assumed to stimulate their growth as is the case with the white

cells in fowl leukaemia. It is further suggested that the Bence-Jones protein, when combined with nucleic acid in the plasma cells, is the virus itself.

This suggestion would be quite compatible with our knowledge of viruses. Tobacco mosaic virus, for instance, can multiply very rapidly almost up to the moment when the leaf dies, by which time the bulk of the total protein is in the form of virus protein (Stanley, 1937). Markham, Mathews & Smith (1948) have shown that the yellow mosaic virus growing in the turnip can produce protein free from nucleic acid as well as its own nucleoprotein. Such work as has been done on animal viruses shows a more complex mechanism as several proteins, physicochemically distinguishable from each other, may be produced by the one virus.

It may be relevant to allude here to the work of Snapper & Schneid (1946, 1947) and of Snapper, Mirsky, Ris, Schneid & Rosenthal (1947). They have shown that administration of stilbamidine to patients with myelomatosis may produce a temporary clinical improvement. During the treatment the plasma cells may show the presence of basophil inclusion bodies which they interpret as resulting from the reaction between the stilbamidine and a 'foreign nucleoprotein' present in the cells. It appears to the present writers that this could be the virus in question. Inclusion bodies of this type may also be found in proved virus diseases (Findlay & Ludford, 1926).

Further support for this theory comes from the fact that virus proteins, so far as they have been analyzed to date, are usually methionine free (Block & Bolling, 1947, p. 304; Chandler, Gerrard & du Vigneaud, 1947; Knight, 1947).

SUMMARY

1. A case of multiple myelomatosis is described in which Bence-Jones proteinuria was found.

2. The average daily output of protein was about 36 g. The output was not increased on adding 50 g. of the protein to the usual diet.

3. A salting-out curve suggests that the protein consisted of two constituents in the proportion of about 90 and 10%. On electrophoretic analysis it gave one slightly asymmetrical peak which migrated with fibrinogen.

4. The protein was shown to be free of methionine by paper chromatographic, microbiological, biological and two different chemical methods. The hydrolysate of protein, on chromatographic analysis, showed the presence of all the other common amino-acids, except hydroxyproline. When supplemented with methionine, the protein was similar to casein in its ability to support the growth of rats.

5. A new spot test for cystine is described.

6. The qualitative and quantitative data presented are considered incompatible with the view that this Bence-Jones protein is synthesized by the body tissues. Their similarity to those in certain virus diseases is indicated, and it is suggested that multiple myelomatosis could be explained as a virus infection and the abnormal urinary protein as a constituent protein of that virus.

The writers wish to thank Prof. H. P. Himsworth, Prof. C. Rimington and Dr M. Rosenheim for their great interest and encouragement, Dr J. G. Heathcote for the microbiological assay and Dr E. L. Alling for the electrophoretic studies.

REFERENCES

- Abderhalden, E. (1940). *Fermentforschung*, **16**, 324.
 Abderhalden, E. & Rostoski, O. (1905). *Hoppe-Seyl. Z.* **46**, 125.
 Barton-Wright, E. C. (1946). *Analyst*, **71**, 267.
 Blackman, S. S., Barker, W. H., Buell, M. V. & Davies, B. D. (1944). *J. clin. Invest.* **23**, 163.
 Block, R. J. & Bolling, D. (1947). *The Amino-acid Composition of Proteins and Foods*. Illinois, U.S.A.: Thomas.
 Brand, E., Kassell, B. & Saidel, L. J. (1944). *J. clin. Invest.* **23**, 439.
 Brues, A. M. & Drury, D. R. (1936). *Arch. Path. Lab. Med.* **22**, 658.
 Calvery, H. O. & Freyberg, R. H. (1935). *J. biol. Chem.* **109**, 739.
 Chandler, J. P., Gerrard, M. W. & du Vigneaud, V. (1947). *J. biol. Chem.* **171**, 823.
 Cohn, E. J. (1925). *Physiol. Rev.* **5**, 349.
 Conden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
 Dent, C. E. (1946). *Lancet*, ii, 637.
 Dent, C. E. (1947). *Biochem. J.* **41**, 240.
 Dent, C. E. & Rimington, C. (1947). *Biochem. J.* **41**, 253.
 Devine, J. (1941). *Biochem. J.* **35**, 433.
 Dunn, M. S., Shankman, S., Camien, M. N., Frankl, W. & Rockland, L. B. (1944). *J. biol. Chem.* **156**, 703.
 Findlay, G. M. & Ludford, R. J. (1926). *Brit. J. exp. Path.* **7**, 223.
 Glynn, L. E., Himsworth, H. P. & Neuberger, A. (1945). *Brit. J. exp. Path.* **26**, 326.
 Harvier, P. & Rangier, M. (1943). *C.R. Acad. Sci., Paris*, **216**, 131.
 Hektoen, L. & Welker, W. H. (1940). *Biochem. J.* **34**, 487.
 Hewitt, L. F. (1929). *Biochem. J.* **23**, 1147.
 Hopkins, F. G. & Savory, H. (1911). *J. Physiol.* **42**, 189.
 Horn, M. J., Jones, D. B. & Blum, A. E. (1946). *J. biol. Chem.* **166**, 313.
 Knight, C. A. (1947). *J. biol. Chem.* **171**, 297.
 Kydd, D. M. (1934). *J. biol. Chem.* **107**, 747.
 Longworth, L. G. (1939). *J. Amer. chem. Soc.* **61**, 529.
 Lugg, J. W. H. (1938). *Biochem. J.* **32**, 2114.
 Markham, R., Mathews, R. E. F. & Smith, K. M. (1948). *Nature, Lond.*, **162**, 88.
 Martin, N. (1947). *J. clin. Invest.* **26**, 1189.
 Masters, M. (1939). *Biochem. J.* **33**, 1313.
 Moore, H., Kabat, E. A. & Gutman, A. B. (1943). *J. clin. Invest.* **22**, 67.

- Osborne, T. B. & Mendel, L. B. (1919). *J. biol. Chem.* **37**, 572.
 Osgood, E. E. & Haskins, H. D. (1931). *J. Lab. clin. Med.* **16**, 575.
 Pauly, H. (1904). *Hoppe-Seyl. Z.* **42**, 508.
 Rimington, C. (1940). *Biochem. J.* **34**, 931.
 Rose, W. C. (1938). *The Physiology of Amino-Acid Metabolism*. 14th Ludwig Hektoen Lecture. Chicago, U.S.A.: Frank Billings Foundation.
 Snapper, I., Mirsky, A. E., Ris, H., Schneid, B. & Rosenthal, M. (1947). *Blood*, **2**, 311.
 Snapper, I. & Schneid, B. (1946). *Blood*, **1**, 534.
 Snapper, I. & Schneid, B. (1947). *Ann. Intern. Med.* **27**, 541.
 Stanley, W. M. (1937). *J. biol. Chem.* **121**, 205.
 Svedberg, T. & Sjögren, B. (1929). *J. Amer. chem. Soc.* **51**, 3594.
 Whipple, G. H. (1948). *Haemoglobin, Plasma Protein and Cell Protein*. Springfield, U.S.A.: Thomas.
 Work, E. (1948). *Biochem. J.* **42**, xlix.

The Fate of Certain Organic Acids and Amides in the Rabbit

7. AN AMIDASE OF RABBIT LIVER

By H. G. BRAY, SYBIL P. JAMES, ISABEL M. RAFFAN, BRENDA E. RYMAN
 AND W. V. THORPE

Department of Physiology, Medical School, University of Birmingham

(Received 16 November 1948)

In an earlier investigation in this laboratory (Bray, James, Ryman & Thorpe, 1948) it was shown that extracts of rabbit liver are capable of hydrolyzing the carbamyl group of several carboxylic acid amides, notably *p*-nitrobenzamide. The results of this study are summarized in Table 1. Subsequent

'amidases'. Since the most convenient substrates so far examined are *p*-nitrobenzamide and benzamide, it is convenient to refer to it provisionally as a benzamidase. It should be emphasized that the use of this term does not necessarily imply identity with the benzamide-synthesizing enzyme called benzamidase by Waelch & Busztin (1937). We have found that rabbit-liver extracts also hydrolyze certain aliphatic amides (e.g. propionamide (Table 1) and, more readily, butyramide and valeramide), but we have not yet sufficient evidence to say whether or not these compounds are hydrolyzed by the same enzyme as the aromatic amides.

It was, therefore, of interest to investigate the relationship of the benzamidase to the known amidases. Enzymes have been described which hydrolyze compounds containing a simple or substituted carbamyl group, thus

Thus glutaminase and asparaginase, which hydrolyze respectively the monoamides of glutamic and aspartic acids (glutamine and asparagine) are amidases acting upon substrates in which R'' is H. Hippuricase (histozyme) hydrolyzes hippuric acid ($R' = \text{C}_6\text{H}_5$, $R'' = \text{CH}_2.\text{COOH}$) to benzoic acid and glycine. Deacetylases which hydrolyze acetamido compounds ($R' = \text{CH}_3$, $R'' = \text{C}_6\text{H}_5$, $\text{CH}_2.\text{COOH}$, etc.) may also be regarded as amidases. There is the further possibility that some peptidases or proteases which hydrolyze the 'peptide' link might be capable of hydrolyzing amides. In the above-mentioned study (Bray *et al.* 1948) it was observed that, although pepsin and trypsin were without action upon the amides studied, papain hydrolyzed hippuramide readily and benzamide to a slight extent.

Table 1. *Hydrolysis of amides by rabbit-liver extract at pH 7.4*

Amide	Percentage hydrolysis	
	After 10 hr.	At equilibrium
Benzamide	24	45
<i>o</i> -Hydroxybenzamide	0	0
<i>m</i> -Hydroxybenzamide	0	1
<i>p</i> -Hydroxybenzamide	3	6
<i>o</i> -Aminobenzamide	5	9
<i>m</i> -Aminobenzamide	2	7
<i>p</i> -Aminobenzamide	3	13
<i>o</i> -Nitrobenzamide	13	23
<i>m</i> -Nitrobenzamide	15	31
<i>p</i> -Nitrobenzamide	73	82
<i>o</i> -Bromobenzamide	4	21
Formamide	0	0
Acetamide	1	2
Propionamide	14	25
Phenylacetamide	5	10
Nicotinamide	8	13
Asparagine	30*	—
Glutamine	22*	—

* Equilibrium reached.

investigations have shown that, in most cases, and especially with aromatic amides, these results are in good agreement with those obtained with the intact animal (see Bray, Thorpe & Wood, 1949).

The enzyme responsible for this reaction may be included in the group of enzymes often termed