Elimination of the Protein.

A striking feature of Bence-Jones proteinuria is the free passage of large quantities of the protein through a kidney which holds back the normal serum proteins.

Most previous investigators have found the kidney of animals impervious to the Bence-Jones protein, although Boggs and Guthrie found that sometimes rabbits excreted it after intravenous injection. We injected subcutaneously 40 cc. of Bence-Jones urine into each of two dogs, weighing respectively 8 and 9.5 kilos. All urine passed during the following 3 days was entirely free from protein of any kind (ferrocyanide and acetic acid, heat, HCl). On the 3rd day one of the dogs was etherized and into the femoral vein were injected 30 cc. of Bence-Jones urine. The urine of this dog for the next 2 days showed no protein of any kind. The amounts of Bence-Jones urine taken for injection represented in each instance a little more than 1 gm. of the protein.

To try the effect of larger quantities we etherized a 14 kilo dog and injected into a vein of the leg 5 gm. of Bence-Jones protein dissolved in 200 cc. of slightly alkaline Ringer’s solution. The urine of this dog before injection showed just a trace of albumin; but as this is not uncommon in dogs and as the animal was strong, active, and apparently perfectly well, we did not reject him on that account. When coming out of the ether the animal urinated, and again about 20 minutes later. Both of these urines were entirely similar to that obtained before injec-

tion, and free from Bence-Jones protein. The urine found the next (2nd) day produces a heavy white cloud when heated with a drop of acetic acid; this cloud almost disappears upon boiling and reappears upon cooling, and this can be repeated. The characteristic acid reactions are also given in strong typical fashion. Evidently we here have a marked excretion of Bence-Jones protein. Upon estimation it was found that the urine contained 0.16 per cent, or for the total amount of urine 1.47 gm. of Bence-Jones protein. When the urine was carefully coagulated at boiling, allowed to cool, and filtered, the clear filtrate (which could no longer at any reaction or temperature be made to give cloudiness on heating) gave some opalescence when saturated with ammonium sulfate; and also gave moderate reactions with ferrocyanide and Roberts' reagent. While these positive tests in the filtrate may have been caused by failure to remove all of the coagulable protein, it seems more probable that there was a small amount of proteose present. Whatever the nature of this filtrate protein, its total amount was evidently very small in comparison with the coagulable protein. The urine found on the 3rd day was nearly free from protein. Spiegler and ferrocyanide tests both indicated traces, but these reactions were little if any stronger than this dog normally showed; there was no reaction for Bence-Jones protein. The urine found on the 4th day showed only traces of albumin, no stronger than before the experiment, so the dog was removed from observation. It is evident that rather less than one-third of the injected Bence-Jones protein was eliminated during the first 24 hours, and that after that time practically no more was recovered. Even granting that a very small amount of proteose was present in the urine of the first period, not more at most than one-third of the protein is accounted for; what became of the other two-thirds we are unable to say; but as even when injected directly into the blood stream it was neither eliminated as Bence-Jones protein nor digested and eliminated as proteose, it does not seem unreasonable to assume that it is promptly fixed by the blood cells or combined with something in the plasma, and subsequently made use of or catabolized in the organism.

When the dog has previously been moderately poisoned by uranium nitrate, the injection of considerable quantities of the

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protein produces very different results. A healthy dog weighing 6.2 kilos was injected subcutaneously with 0.03 gm. of uranium nitrate, 4.8 mg. per kilo. By the next (2nd) day the urine showed a moderate amount of albumin, and on the 3rd day the dog received (under ether anesthesia) into a vein of the leg 8 gm. of Bence-Jones protein dissolved in quite alkaline Ringer's solution. After coming out of the ether he vomited twice. The filtrate of this vomitus could not be made to coagulate by heat and seemed free from Bence-Jones protein. The urine found on the 4th day contained some albumin by boiling, but hardly as much as before the protein was injected. There was no trace of coagulation when heated for an hour at 58° with a drop of weak acetic acid, and the HCl test was negative; evidently no Bence-Jones protein was present. There was, however, a very heavy reaction to ferrocyanide and acetic acid totally out of proportion to the amount of protein coagulated on boiling. The urine found on the 5th day had the same characteristics, and as the dog seemed to be moribund he was killed with chloroform, and about 30 cc. of urine were taken from the bladder. This gave the same reaction as before. Macroscopic examination of the stomach, liver, and gall-bladder revealed nothing, while the kidneys showed moderate inflammation.

This experiment was repeated substituting Bence-Jones urine for the separated protein. A dog weighing 12 kilos was injected subcutaneously with 0.075 gm. of uranium nitrate, 6.2 mg. per kilo. The next (2nd) day the urine contained a moderate amount of albumin by the heat, Spiegler, Roberts', and ferrocyanide tests. The urine found on the 3rd day reacted similarly and under ether anesthesia the dog received into a vein of the leg 250 cc. of Bence-Jones urine, representing nearly 7 gm. of protein. This dog also vomited after the ether, and the vomitus was, like that of the previous animal, free from Bence-Jones protein. The urine of the 4th day contained less albumin than before and no Bence-Jones protein. The reaction to ferrocyanide is very strong, and there is also a strong biuret test. To one part of the urine were added ten parts of absolute alcohol; this was allowed to stand an hour and filtered. To a portion of the filtrate was added water, evaporated down to small volume on the water bath, when it gave a strong biuret reaction. The
urine of the 5th day gave the same result. Also a portion of this urine was saturated with ammonium sulfate, and a drop of acetic acid added; the result was a heavy precipitate, and the clear filtrate gave a strong biuret reaction. Another portion of the urine was saturated with ammonium sulfate and allowed to stand with excess of the sulfate for 36 hours, when as before the clear filtrate gave a strong biuret reaction. The precipitate produced by the ammonium sulfate was in each instance many times heavier than the heat coagulum. The 6th day the dog died.

From these two experiments it would seem that the uranium dogs have lost their normal power of utilizing the Bence-Jones protein, and that the latter is so energetically hydrolyzed that a portion even escapes precipitation by ammonium sulfate.

It might be objected that the uranium itself or the etherization were in some way responsible for the appearance of digestion products in the urine. In order to test this point we injected another dog with uranium nitrate as before, but gave no protein. The urine for 2 days following showed no proteose. On the 3rd day the dog was etherized for 20 minutes, and the urine for the following 3 days still showed no proteose. This dog was the 14 kilo animal previously used in the experiment of injecting 5 gm. of protein. He was, however, entirely well as far as could be observed, and in the best of spirits. The amount of uranium nitrate used was 0.09 gm., 6.4 mg. per kilo; and it is interesting to note that although more uranium was used than in the other experiments this dog appeared to have practically recovered by the 6th day, the albumin having almost disappeared from the urine, and he was in the best of spirits with excellent appetite. He remained under our observation for a couple of weeks and was apparently absolutely well.

Another dog weighing 9.7 kilos was injected with uranium nitrate, using 0.06 gm., 6.1 mg. per kilo. On the 2nd day there was considerable albumin, and on the 3rd day also. On this day under ether anesthesia we injected into a vein 200 cc. of normal urine. The urine of the 4th, 5th, and 6th days showed no proteose. This animal recovered rather slowly, and it was 10 days before he again seemed in normal condition.
DISCUSSION.

These experiments indicate that the normal dog is able to utilize or catabolize a moderate amount of Bence-Jones protein, even when this is injected rapidly into the circulation; but that a limit is soon reached beyond which the excess protein is promptly excreted in unchanged condition. In dogs suffering from moderate uranium poisoning this power of utilization appears to be lost; though on the other hand no Bence-Jones protein is excreted as such, but only after being broken down to proteose. It is interesting that the dog receiving the largest dose of uranium (6.4 mg. per kilo) but no urine or Bence-Jones protein promptly recovered; that the dog receiving uranium nitrate and then normal urine showed more serious toxic symptoms but did recover; and finally, that of the two dogs receiving uranium nitrate and then Bence-Jones protein and freely excreting proteose, one died on the 6th day, and the other was moribund when he was killed on the 5th day. Since the Bence-Jones protein is not toxic to the healthy dog, and since, apart from our own results, this amount of uranium would not be expected to cause rapid deaths, it would seem that the uranium causes some change in the metabolic power such that the normally harmless or perhaps useful protein rapidly becomes hydrolyzed with unfavorable results. When dealing with uranium poisoning, one's attention is naturally directed toward the kidney; but in these experiments the peculiar effect of the uranium on the elimination of the protein might depend rather on a general toxic influence; possibly some powerful ferment may have appeared in association with the uranium intoxication, with the result of protecting the organism from the breakdown products of the poisoning; and this ferment, while successful in saving the animal from the uranium, is so active that as soon as an abnormal protein is introduced it causes him to be overwhelmed by proteoses formed from the injected protein faster than they can be eliminated. This is the

Thus W. de B. MacNider (J. Pharm. and Exp. Ther., 1913, iv, 491) in his studies concerning elimination by the uranium kidney after anesthetics and diuretics found that it required 6.7 mg. per kilo injected twice to cause the "desired changes in the kidney without the undesirable gastro-intestinal complications."
general type of explanation one would formulate on the basis of Vaughan's hypothesis.

It will be observed that the healthy dog which eliminated a portion of the injected Bence-Jones protein received only 0.36 gm. of the protein per kilo, whereas the uranium dogs received respectively 1.29 and 0.58 gm. per kilo; so there is here at least no evidence in favor of the supposition that a kidney permeable to normal serum proteins is on that account more easily passed by the Bence-Jones protein. On the other hand, the conditions of uranium poisoning are not sufficiently similar to those of a chronic nephritis to make positive statements, and we have not succeeded in finding a dog with a natural nephritis upon which this question could be more exactly studied.

The ease with which Bence-Jones protein passes through a kidney impermeable to the normal proteins is almost certainly not due to the molecule being small in size, since nearly all the more recent studies indicate that we have to deal with a higher protein of large molecular weight. For example, Hopkins and Savory, after careful amino-acid determinations state as their opinion⁴ that: "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;" and this view is entirely in accord with the conclusions previously arrived at² on other grounds by two of us. What may be the nature of the physicochemical adjustment permitting this selective action on the part of the kidney we venture no surmise.

**Origin of the Protein.**

Two sources suggest themselves: The protein may be a special product of the tumor cells of the myeloma; or it may be a normal or possibly aberrant stage in the synthesis of some body protein, the completion of which is interfered with by deficiency of some necessary condition.

The first of these assumptions is doubtless the more natural, although there seems little direct evidence to support it. If the protein is in fact the product of the tumor cells we should expect to

find at least small amounts of it in the diseased marrow. Reach examined the bones with negative results, but found the protein in the spleen, which organ contained a neoplasm similar to those found in the bones. However, Hopkins and Savory found no trace of the protein in the bone marrow; and when the marrow was injected into rats none of the protein was found in the urine of these animals. When our case came to autopsy we were permitted to take only small specimens of the bones for microscopic examination, and were therefore not able to test this question ourselves. Rosenbloom has suggested an osseo-albuminoid origin for the protein. The strongest argument in favor of the protein being a special product is the unusual amino-acid composition, the phenylalanine and tyrosine together amounting to over 9 per cent of the whole, which, as Hopkins and Savory point out, is "considerably higher than any yet described for a blood or tissue protein. Judging, indeed, by our figures as a whole, the protein would seem to stand by itself." On the other hand, the surprisingly large amounts excreted would rather indicate some more general source. As was previously pointed out the biologic reactions of this protein relate it closely to the normal serum proteins, a conclusion further substantiated by Massini's complement fixation experiments. Our ignorance of tumor composition and of their products, if any, of the exact degree of foreignness experimentally limiting biologic reactions, makes it impossible to be dogmatic; but the conclusion may well be reserved whether a protein behaving biologically as does this is really a special, and to the organism previously unknown, substance.

If we were to assume that in the diseased marrow something was lacking which prevented the complete synthesis of a normal body protein, then by supplying to Bence-Jones protein normal bone marrow and excess of amino-acids we might, theoretically at least, observe the building up of a protein of higher coagulation point. We at first proposed to carry out such an experiment by perfusing normal dog bone with a solution of Bence-Jones protein and amino-acid solution. After a large amount of

preliminary experimentation we were forced to abandon this, as we were unable to devise and carry out any scheme that gave promise of reliable quantitative results; and since in this experiment we are sure to have both Bence-Jones and normal protein, it is necessary to operate quantitatively in order to learn whether the latter protein is being increased at the expense of the former. We then tried the following experiment in vitro. An amino-acid solution obtained by tryptic digestion of dog muscle was heated to boiling, filtered, and cooled, and to 200 cc. of it were added 2.25 gm. of Bence-Jones protein as well as the ground up bone marrow from the long bones of a large dog, removed as aseptically as possible and used perfectly fresh. A portion of this mixture was put into the incubator with toluene, and the other portion used to estimate under definite previously determined salt and acid relations the respective content in Bence-Jones and high coagulation point protein. The portion in the incubator was allowed to remain 7 days, then removed and analyzed as before. The results showed that only a trace of the Bence-Jones protein remained, but that nearly all of the serum protein had also disappeared. Therefore any possible synthetic action was entirely lost in the active digestion which had broken down nearly all of the coagulable protein. When we remember the comparative ease with which various ferments accomplish digestion and the well known difficulties surrounding the production artificially of biologic synthesis, this result will cause no surprise; and we feel that this experiment can only be regarded as leaving the question unsettled.

**Course of the Case Previously Reported.**

The improvement in the condition of the patient, previously reported,² up to March 1, 1916, proved to be only temporary. After this he failed at first slowly, then more rapidly, all the while maintaining the excretion of Bence-Jones protein at and finally above the previous high level. In addition there were soon added at first small then larger quantities of serum albumin. As we were unable to devise a process for separating with any precision the two kinds of protein without at the same time causing material denaturation, this change in the character of the excretion unfortunately compelled us to abandon a series of ex-
experiments which had been planned along biologic lines to obtain more exact information as to which of the proteins of the normal organism the Bence-Jones body is most closely related to. The patient died in October, 1916, and for several months before that time no work was done by us except an occasional examination of the urine.

Autopsy.—The only noticeable abnormality of the viscera was several old healed lesions of the lungs. The pleural cavities contained considerable clear straw-colored fluid. The long bones were not affected; but in the bones of the pelvis, ribs, and vertebrae the marrow occupied a much greater space than usual with marked thinning of the walls; the body of the sacrum was so spongy as scarcely to resemble bone at all, and the bony structure of the ribs in some places was not over 0.5 mm. in thickness. The subsequent histologic examination showed undoubted myeloma, a fact of interest to clinicians since the x-ray had indicated no osseous abnormality.

Besides the specimens for histologic examination, which will elsewhere be reported, we obtained urine from the bladder, blood from the heart, and fluid from the pleural cavities. The urine presents the same depth of color and other appearance as usual, but contained 3.6 per cent of Bence-Jones protein, which was more than we had ever before found; and there was also considerable albumin. The pleural fluid gave well marked reactions for Bence-Jones protein, and upon estimation was found to contain 0.11 per cent. The blood after rubbing down the clot with water and pressing through the filter gave a well marked test with HNO₃ and heat, also the filtrate from boiling coagulation clouded strongly upon cooling. The amount of blood available was not sufficient to make satisfactory determinations, but the protein appeared to amount to about 0.2 per cent, which is confirmed by the fact that the qualitative tests were definitely stronger than in the case of the pleuritic fluid similarly diluted. Because hemoglobin is coagulated or decomposed at about the same temperature at which Bence-Jones protein coagulates, the latter cannot be estimated in the presence of the former by the usual method; it is necessary to bring the whole to boiling and then filter through a hot filter and weigh the protein which separates on cooling. This process requires a large amount of material, gives
results which do not agree well, and apparently the figures are always too low.

From these autopsy findings it is clear that the protein circulates freely throughout the body.

**SUMMARY OF BOTH PAPERS.**

1. Brief clinical notes of the case are given.
2. A number of reactions of the Bence-Jones protein found in this case, together with methods of identification, are described.
3. A method of separating the protein and of preparing a protein-free urine is also described.
4. Anaphylactic sensitization is accomplished best by the separated protein, less well by the same amount of protein as found in the native urine. Some factor exists in the native urine which prevents the protein contained in it from exerting its normal sensitizing action; and this factor is thermostable at 55°, but is destroyed by heating to near the boiling point or by such chemical manipulation as described in our method for preparing protein-free urine. Subsequent anaphylactic intoxication is shown equally by the native urine and by the separated protein.
5. The protein shows no direct toxicity.
6. The separated protein is digested with great ease by both pepsin and trypsin.
7. The protein is no proteose, but a higher protein of definite biologic stamp.
8. It is of endogenous origin, and might be derived either from the tumor cells of the myeloma, or produced through an interrupted or aberrant synthesis of some normal body protein. The biologic indications of close relationship to the normal blood proteins, and the enormous quantities produced would seem to favor the second alternative. The question remains obscure.
9. When we allowed Bence-Jones protein, normal bone marrow, and an amino-acid solution to remain together for 7 days nearly all coagulable protein had disappeared. In the presence of this active digestion we were unable to learn whether any synthesis had occurred or not.
10. Normal dogs can utilize or catabolize moderate quantities of Bence-Jones protein, but a limit is soon reached beyond which the protein is promptly excreted in unchanged condition.
11. In dogs suffering from moderate uranium poisoning this power of utilization is lost, and the Bence-Jones protein is energetically hydrolyzed and eliminated as proteose.

12. Moderate doses of uranium nitrate which provoke only moderate symptoms rapidly become fatal when Bence-Jones protein is injected into the circulation. An explanation of this in harmony with the Vaughan hypothesis would be that some ferment arises in association with the uranium intoxication protecting the organism from breakdown products of the poisoning, and that the ferment is so active that as soon as an abnormal protein is introduced the animal is overwhelmed by the proteose formed from the injected protein.

13. At autopsy the bones of the pelvis, ribs, and vertebrae were found involved in extensive myeloma notwithstanding that the clinical findings during life were negative.

14. Urine taken from the bladder at autopsy showed 3.6 per cent of Bence-Jones protein, besides considerable albumin; the pleural fluid showed 0.11 per cent of Bence-Jones protein; and the blood appeared to contain about 0.2 per cent of Bence-Jones protein. Evidently the protein circulates with great freedom throughout the body.
STUDIES IN BENCE-JONES PROTEINURIA. II
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