CHEMICAL, CLINICAL, AND IMMUNOLOGICAL STUDIES ON THE PRODUCTS OF HUMAN PLASMA FRACTIONATION.

XXIII. THE EFFECTS OF FEEDING POSSIBLE BLOOD SUBSTITUTES ON SERUM PROTEIN REGENERATION AND WEIGHT RECOVERY IN THE HYPOPROTEINEMIC RAT [1]

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Because of disadvantages connected with the use of the hypoproteinemic dog for the evaluation of the biological potencies of *small* quantities of dietary proteins, we have utilized the hypoproteinemic rat; the present report describes experiments in which proteins of possible value as blood substitutes were fed to protein-depleted rats in order to ascertain their abilities to promote serum protein regeneration and weight recovery.

The method is, in principle, a modification of that used by Weech and others (1, 2). Instead of measuring only albumin regeneration, however, it determines regeneration of total serum protein. As we have used it thus far, the method may be summarized as follows: Healthy adult male albino rats are placed on a low-protein, low calorie diet of a protein content usually varying between 1.75 and 2.0 per cent (N × 6.25). The composition of the diet (3E) (for 10 kilograms) is as follows:

Carrots, finely ground, uncooked	3000 grams
Ruffex (Fischer)	500 grams
Lard	400 grams
Corn starch	4400 grams
Water	1000 grams
Salt mixture (3)	400 grams
Brewer's yeast	200 grams
Liver concentrate (Wilson and Company, 20 : 1)	100 grams
Choline chloride	10 grams
Oleum percomorphum	16 drops
Calcium pantothenate	20 mgm.
Pyridoxine hydrochloride	20 mgm.
Riboflavin	50 mgm.

In making up the diet, the dry ingredients are weighed and thoroughly mixed in a large rod mill, after which the melted lard containing the oleum percomorphum is added and thoroughly mixed into the main mass. Then the carrots and the water containing the dissolved purified vitamins are added and the mixing continued. Each mixing procedure takes about 15 minutes. The diet yields approximately 2.3 calories per gram. When 18 to 22 per cent of vitamin-test casein (Smaco) is substituted for an equal weight of corn starch and fed on an average daily intake of approximately 20 grams per rat, the rats gain weight and appear healthy. The vitamin supplements with respect to vitamin A, thiamin, riboflavin, pyridoxine, pantothenic acid, and vitamin D are well above those commonly considered as daily requirements for the rat. No additional ascorbic acid or niacin have been provided, as the rat has not been found to require them. In order to ensure an adequate supply of choline above the amount occurring in carrots, 0.1 per cent has been added to ration 3E.

METHOD OF DIETARY ASSAY

Healthy adult male albino rats, ranging between 200 and 300 grams in weight and of as nearly uniform size and age as possible, are weighed, ear-marked, and placed 6 to a cage in rabbit cages with wire-screen floors. One rat from each group is usually bled from a tail-vein for a total protein determination, after which most of the groups are fed a low protein, low calorie ration (2E or 3E) for 10 to 12 weeks, at an average daily intake of 20 grams per rat per day. A few groups are fed similar quantities of the control diet, *i.e.*, the low basal ration with from 18 to 22 per cent of vitamin-test casein (Smaco) substituted for an equal weight of corn starch. These animals serve as controls for later experiments on antibody-production and chemical fractionation of the livers; they also supply a group of well-nourished animals with which to compare the regeneration levels of weight and serum protein which the depleted rats attain after having been fed one of the test-proteins. All animals of both groups receive water *ad libitum* and about 15 to 20 grams of leaf lettuce per rat per week.

[1] The products of plasma fractionation employed in this work were developed by the Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts, under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University. The fractions of bovine plasma were prepared at the Armour Laboratories, Chicago, Illinois, under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Armour Laboratories. Study of the products was aided by the John and Mary R. Markle Foundation, the Douglas Smith Foundation for Medical Research of the University of Chicago, and the National Livestock and Meat Board.

Rats eating the low protein, low calorie basal diet gradually lose weight and become both anemic and hypoproteinemic, whereas those kept on the casein control diet gain weight and have normal serum protein and hemoglobin values. When the animals undergoing protein depletion have lost about one-third of their initial weight, a preliminary serum protein determination is made on each rat in the following way: Each animal is lightly anesthesized by ether, the tail is dipped into water for a few moments at a temperature of 45 to 50° C., and after having been dried and coated with a thin layer of vaseline, a vein is incised with a sharp lancet. The freely-flowing blood is collected in a small specially-made flanged vial which holds 0.5 cc. The bleeding is stopped by applying a small bandage of cotton and collodion. After the blood has clotted and the clot detached from the walls of the vial, the vial is tightly stoppered. Next morning, the retracted clot is withdrawn and, after centrifugation, protein determinations are made on the clear serum with a LaMotte densiometer, according to the method of Barbour and Hamilton. These determinations are always made between 12 and 24 hours after bleeding. The densiometer method is checked frequently with micro Kjeldahl analyses, utilizing larger quantities of serum; the results have checked consistently within 0.1 to 0.2 grams per cent over the range of 4.0 to 7.0 grams per cent. No attempt has been made to measure blood volume in view of the fact that Weech and his associates found a direct relationship between increase in concentration of serum albumin and increase in blood volume.

The values for total serum protein have varied as follows: For rats on the casein control diet, the levels have usually ranged between 6.5 and 7.0 grams per cent whereas those from rats kept on the basal ration have been between 3.5 and 5.50 grams per cent. The latter animals, which have values higher than 5.25 grams per cent, are eliminated from the current experiment and kept on the low-protein diet for use later. For the assays recorded in the present report, only protein-depleted rats whose serum protein values ranged between 4.0 and 5.25 grams per cent were utilized.

The rats selected for the repletion experiments, together with a few from the casein control diet group, are next placed in small individual cages and kept on their respective diets for about a week. Each rat in this selected group is weighed and, under ether anesthesia, 0.5 cc. of blood is removed from the heart by means of a 1 cc. syringe with a No. 24 gauge needle and the serum protein values are determined from this blood, using the densiometer method. After the serum protein determinations, the animals are immediately regrouped, on the basis of these serum protein values, for the repletion experiment. Usually from 4 to 6 animals are selected for each diet group and the groups are adjusted so that each contains animals comparable as to levels of serum protein, hemoglobin, and weight. Protein-depleted rats with serum proteins outside the 4.0 to 5.25 range are usually included in the group maintained on the low-protein diets as controls for the period of repletion.

Next morning the animals are weighed individually and are started on their respective repletion diets, supplied daily to each rat in 20-gram portions. The proteins to be assayed are mixed into the basal ration so as to make a final concentration, by weight (including the protein in the basal ration), of approximately 9 per cent (N × 6.25). The protein is substituted for an equal weight of corn starch. If the food being added also contains fat, carbohydrate, roughage, and moisture, these constituents are also substituted so that their final concentration in the test-diet will be the same as in diets 2E or 3E; in other words, the repletion diets are all approximately isocaloric, both with one another and with the basal and casein control rations. No attempt has been made to balance the diets so far as vitamins and minerals are concerned inasmuch as we assume that these accessory materials are already being fed in sufficient amounts and that added quantities would probably have no additive effect. Duplicate samples of each test-ration are analyzed for nitrogen content by the Kjeldahl method. At the end of each day during the repletion period, the amount of ration not eaten, if any, is weighed and the total amount eaten per rat per 24 hours recorded. With the exception of some of the incomplete proteins, the entire ration is usually consumed. During the repletion period, the rats are weighed on alternate days before being given their ration in order to determine gains or losses of weight. On the morning of the seventh day, the rats are weighed before feeding, and later in the day are again bled from the heart (0.5 cc.). The weights, and the values for serum proteins determined from this blood, are compared with the initial values, to establish gains or losses in one week.

In the present communication, only the results of the 7-day repletion are recorded. The data on the animals fed the casein control ration are not recorded inasmuch as they offer no information relevant to the present discussion.

The following experiments (I and II) present the detailed findings for the assay of the several proteins tested. Seven test rations containing the different proteins have been used as follows:

Ration 1 (2E and 3E). These two rations are the basal low protein mixtures described above. They differ only in that 2E contains no liver concentrate and only the amount of choline occurring normally in the dietary constituents. This ration was used in experiment I; ration 3E was used in experiment II. It should be noted, however, that, as judged by their inability to cause regeneration of serum protein, both were identical.

Ration 2—Crystallized bovine serum albumin. This highly purified serum albumin was prepared at the Armour Laboratories by the method of Cohn and Hughes, and in collaboration with the Department of Physical Chemistry, Harvard Medical School.

Ration 3—Bovine serum gamma globulin (Fraction II). This fraction of serum globulin, another product of the plasma fractionation method (5) carried out on bovine plasma at the Armour Laboratories, when tested electrophoretically, assays slightly better than 90 per cent gamma globulin. This is of especial interest in view of the fact that usually it is the gamma globulin fraction of immune serum which contains most of the antibody.

Ration 4—Dehydrated beef. This material was prepared by Wilson and Company, Chicago, Illinois, as Batch 142. Its composition is as follows:

Moisture	7.6 per cent
Protein	62.5 per cent
Fat	27.6 per cent
Ash	2.3 per cent

Ration 5—Gelatin. Gelatin was tested because of its possible value both as a blood substitute and for parenteral alimentation. This sample was supplied by Wilson and Company, Chicago, Illinois, as No. B 5162. It is derived from third run pigskin and has a nitrogen concentration of 15.9 per cent.

Ration 6—Isinglass. This was supplied by Dr. N. B. Taylor of the University of Toronto. It is a fine powder which contained a total nitrogen value of 16.3 per cent.

Ration 7—Corn germ, defatted. This material was prepared by the VioBin Corporation of Monticello, Illinois, as Sample No. 3697, Lab. No. A 775, and was used for comparison with the proteins of animal origin. Its composition was:

TABLE I

The influence of different proteins upon the regeneration of serum proteins and the regaining of weight in the hypoproteinemic rat

Rat No.	Initial values		Dietary protein and percentage in diet	Grams of protein consumed in 7 days	Body weight Gain or loss in 7 days		Serum proteins Gain or loss in 7 days	
	Serum proteins	Body weight				Per gram of protein consumed		Per gram of protein consumed
	grams per cent	*grams*			*grams*		*grams per cent*	
45–6	4.83	194	Low basal 2 E, 1.78 per cent protein	2.38	+1		+0.14	
42–5	4.94	208		2.20	−1		+0.03	
45–1	4.48	162		2.08	±0		−0.20	
Av.	4.75	188		2.22	0		−0.01	
41–4	4.97	186	Crystallized bovine serum alb., 7.82 per cent	10.40	+12	+1.15	+1.33	+0.13
47–2	4.41	172		10.47	+19	+1.82	+1.01	+0.10
43–4	5.25	229		10.94	+22	+2.05	+0.98	+0.09
50–3	4.90	134		8.91	+19	+2.13	+0.74	+0.08
46–7	4.65	201		9.85	+17	+1.73	+0.42	+0.04
Av.	4.84	184		10.11	+18	+1.78	+0.90	+0.08
47–1	4.38	208	Bovine serum gamma globulin, 8.72 per cent	11.59	+18	+1.55	+1.88	+0.16
45–3	4.86	201		11.51	+25	+2.17	+1.61	+0.14
41–3	5.00	182		11.07	+24	+2.17	+1.50	+0.14
41–5	5.21	203		11.94	+32	+2.68	+1.29	+0.11
50–4	4.65	149		10.72	+26	+2.43	+1.23	+0.12
Av.	4.80	189		11.37	+25	+2.20	+1.50	+0.13
47–3	4.41	181	Dehydrated beef, 9.04 per cent	12.65	+23	+1.83	+1.33	+0.11
46–2	4.86	178		12.65	+42	+3.32	+1.30	+0.10
42–2	4.69	201		12.65	+39	+3.08	+1.11	+0.09
47–4	5.11	200		12.65	+28	+2.21	+0.66	+0.05
Av.	4.77	190		12.65	+33	+2.61	+1.10	+0.09
45–2	4.80	191	Gelatin, 8.76 per cent	9.20	−3		+0.48	+0.05
45–5	4.90	165		9.98	−1		+0.35	+0.04
47–5	4.45	167		10.07	+3		+0.28	+0.03
Av.	4.72	174		9.75	±0		+0.34	+0.04

Protein	20.3 per cent
Fat	1.1 per cent
Fibre	3.7 per cent
Moisture	10.3 per cent
Ash	8.1 per cent
NFE	56.5 per cent

EXPERIMENTAL RESULTS

The results of experiments I and II are recorded in Tables I and II. Table I demonstrates the rapid regeneration of serum proteins in hypoproteinemic rats, fed crystallized bovine serum albumin, bovine serum gamma globulin, and dehydrated beef. All 3 also engendered a rapid regaining of weight, although the crystallized bovine serum albumin was somewhat less effective in both respects than bovine serum gamma globulin. The latter and the dehydrated beef

TABLE II

The influence of different proteins upon the regeneration of serum proteins and the regaining of weight in the hypoproteinemic rat

Rat No.	Initial values		Dietary protein and percentage in diet	Grams of protein consumed in 7 days	Body weight Gain or loss in 7 days		Serum proteins Gain or loss in 7 days	
	Serum proteins	Body weight				Per gram of protein consumed		Per gram of protein consumed
	grams per cent	*grams*			*grams*		*grams per cent*	
62–4	4.10	103	Low basal 3 E, 2.14 per cent protein	2.82	−14		+0.31	
68–6	3.72	125		2.68	−27		+0.28	
66–4	4.69	133		2.40	+5		+0.21	
62–5	3.96	98		2.16	−8		−0.15	
67–6	3.90	125		2.16	−4		−0.15	
61–5	5.28	125		2.16	+11		−0.31	
Av.	4.28	118		2.40	−6		+0.03	
63–4	4.59	133	Crystallized bovine serum alb., 9.40 per cent	11.67	+21	+1.80	+1.29	+0.11
61–2	4.45	141		12.69	+22	+1.74	+1.11	+0.09
61–1	4.28	106		9.69	+16	+1.65	+0.97	+0.10
68–3	4.31	126		10.43	+14	+1.34	+0.90	+0.09
65–1	4.69	121		12.60	+26	+2.06	+0.42	+0.03
66–6	5.11	136		10.62	+15	+1.41	+0.35	+0.03
Av.	4.57	127		11.28	+19	+1.67	+0.84	+0.08
64–3	4.20	125	Bovine serum gamma globulin, 8.71 per cent	10.37	+24	+2.32	+2.00	+0.19
64–4	4.73	127		11.05	+23	+2.08	+1.88	+0.17
68–1	4.31	121		10.62	+27	+2.54	+1.70	+0.16
62–1	4.59	127		10.71	+14	+1.31	+1.61	+0.15
65–5	4.59	104		10.18	+30	+2.95	+1.50	+0.15
Av.	4.48	121		10.59	+24	+2.24	+1.74	+0.16
63–6	4.34	117	Dehydrated beef, 8.85 per cent	12.12	+35	+2.89	+2.02	+0.17
67–3	4.31	124		12.60	+36	+2.86	+1.90	+0.14
61–3	4.14	113		11.50	+35	+3.04	+1.95	+0.17
65–4	4.65	158		12.60	+31	+2.46	+1.92	+0.15
66–1	4.59	134		12.30	+38	+3.08	+1.74	+0.14
Av.	4.41	129		12.22	+35	+2.87	+1.92	+0.15
68–5	4.14	138	Isinglass, 8.69 per cent	9.13	−4		+0.20	+0.02
67–4	4.38	109		7.73	−3		+0.17	+0.02
64–2	4.04	163		6.17	−26		+0.06	+0.01
66–3	5.14	151		8.60	−6		−0.10	
Av.	4.43	140		7.91	−10		+0.08	
62–2	4.06	108	Corn germ, 8.34 per cent	10.33	+29	+2.80	+1.58	+0.15
67–5	4.16	124		11.34	+34	+3.00	+1.54	+0.14
65–3	4.24	149		11.68	+39	+3.34	+1.43	+0.12
66–2	5.18	177		11.68	+38	+3.26	+0.66	+0.06
Av.	4.41	140		11.26	+35	+3.10	+1.30	+0.12

were more nearly equal. Whereas greater increases in serum protein concentration resulted from the ingestion of gamma globulin, gains of weight were larger in the beef-fed animals. In contrast with these 3 proteins, gelatin was relatively ineffective in that the levels of the blood proteins were raised only slightly, and body weight was unaffected.

Table II records observations which, in general, confirm those of Table I. Here, too, crystallized bovine serum albumin was inferior to bovine serum gamma globulin and dehydrated beef. The dehydrated beef, moreover, produced greater gains in serum protein concentration than it did in experiment I. Nevertheless, the gamma globulin equaled dehydrated beef if measured by the grams per cent of serum protein gained per gram of protein consumed. This relationship, indeed, between the amount of protein consumed and the increase in concentration of serum proteins, would seem to be of particular significance.

As with gelatin in experiment I, isinglass was also an inadequate protein in that all 4 rats lost weight and their serum protein concentrations remained essentially unaltered. In fact, the influence of isinglass was not different from that of basal diet (3E) alone. In sharp contrast to the findings for isinglass were those for the corn germ protein. It was not quite the equal of dehydrated beef in producing serum protein but was as effective in increasing body weight.

A few fractions from human plasma have also been tested but, due to the small amounts of materials available, their effects were ascertained for a period of only 5 days, and in only a few rats. Nevertheless, the results indicate that the fibrinogen fraction was the best protein, nutritionally, followed by the gamma globulin, and a mixture of alpha and beta globulin.

COMMENT

The differences in results of experiments I and II require brief comment because of variations in conditions. The basal ration in experiment II contained additional choline, as well as liver concentrate. Furthermore, the rats in this experiment were younger when the depletion diet was begun and were more hypoproteinemic and uniform in size at the start of the repletion period. Because of the lack of uniformity among the rats in these 2 experiments, it is obvious that during the period of repletion, some variability is inevitable. This is to be expected, particularly when proteins of closely similar biological potencies are tested in only a few animals. Averages, therefore, should be interpreted merely as suggesting trends rather than affording statistically valid estimates of biological quality.

There is still uncertainty concerning the degree of protein depletion necessary in an animal in order to demonstrate most effectively the abilities of different foods to cause the regeneration of serum proteins. Whipple and his associates (4) assume that, in general, the greater the degree of hypoproteinemia, the greater is the stimulus for protein regeneration. It is for this reason that they maintain their hypoproteinemic dogs at a plasma protein level of approximately 4.0 grams per cent. An added advantage of a marked hypoproteinemia is that it affords a wider range in which to measure variations in protein potencies. Furthermore, a more marked reduction of the protein reserves may serve to reveal the incompleteness of a test protein which might otherwise be masked by the complementing action of protein materials available in the reserve stores. For these reasons, therefore, we have subjected our rats to prolonged protein depletion (60 to 94 days) before using them for a dietary assay.

Despite this long period of protein depletion, we have seen no evidence of injury to the mechanism which produces the serum proteins. Thus far, we have assayed some 21 different proteins of animal and vegetable origin in more than 200 hypoproteinemic rats. In general, the proteins which, by other standards, are usually rated as proteins of good biological quality have caused a rapid regaining of weight and a quick increase in the concentration of serum proteins. In contrast, proteins of poor biological quality have yielded poor results. For example, 7 rats fed dehydrated beef in previous experiments have shown an average increase in percentage concentration of serum protein of around 2.0 grams, whereas 9 rats fed gelatin have shown an average increase of only 0.06 gram. It is possible, therefore, by this type of assay, to establish a rating of biological potencies for many varieties of proteins.

The theoretical implications of these experiments are of particular interest with respect to their demonstration of the superiority of gamma globulin as an effective stimulus in a starving animal, both for the regeneration of serum protein and for the rapid recovery of weight loss. We conclude that this means that beef serum gamma globulin contains a large assortment of readily available essential amino acids. Furthermore, in view of the fact that most antibodies are gamma globulins, these findings suggest that the process of antibody production must also require an ample supply of essential amino acids, either in the diet or in the protein reserves, in order to permit the fabrication of antibodies in normal quantities and at a normal rate (6). Without these amino acids, it is unlikely that a hypoproteinemic animal can regain its ability to synthesize antibody-globulin when fed only incomplete proteins. Experiments now in progress in this laboratory tend to confirm this assumption.

SUMMARY

A method is described for the quick determination of the biologic quality of a protein when fed to the hypoproteinemic rat. The efficiency of the protein is evaluated by its capacity to increase the concentration of serum proteins and to produce a gain in weight within a 7-day period. Variations due to small differences in degrees of weight and of hypoproteinemia, as well as to natural differences between animals, are compensated for by the use of groups of rats. This method of assay permits a separation of biologically adequate and inadequate proteins and gives information about differences of quality. A protein is considered to be good when the concentration of serum proteins is significantly increased and is high in relation to the quantity of protein ingested.

According to these criteria, bovine serum gamma globulin is highly efficient, especially with respect to serum protein regeneration. In fact, it practically equals dehydrated beef, the best protein so far tested. Crystallized bovine serum albumin, although a fairly adequate protein, is less effective, and corn germ protein compares favorably with both dehydrated beef and bovine serum gamma globulin. In contrast, the incomplete proteins, gelatin and isinglass, seem to possess no distinct ability in either respect.

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