ALTERATIONS IN RAT SERUM PROTEINS IN RELATION TO DIETARY PROTEINS AND B VITAMINS

II. Studies during Protein Depletion and Repletion

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The literature on the influence of dietary protein quality and level on the rates of restoration of blood proteins in depleted animals is extensive (Madden and Whipple, 1940; Benditt *et al.*, 1941; Cannon *et al.*, 1943, 1944; Zeldis *et al.*, 1945; Wissler *et al.*, 1946; Chow *et al.*, 1948; Allison, 1955). Studies on the influence of dietary levels or deficiencies of B vitamins on these are, however, lacking. In the preceding paper of this series (Mulgaonkar and Sreenivasan, 1959) it was shown that B vitamins may influence the utilization of dietary amino acids (especially from low quality proteins) for the synthesis of serum proteins in the rat. The present work relates to observations on the effects of (i) certain B vitamins (thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, folic acid and vitamin B_{12}) on repletion of serum proteins and (ii) vitamin B_{12} and folic acid (PGA) on the degree of depletion of serum proteins and of hemoglobin, in the proteinfasted rat.

Experimental

Two series of experiments were carried out. Experiment 1 was aimed at ascertaining the influence of minimal and optimal levels of seven of the B vitamins on rates of repletion of serum proteins. Forty male Wistar rats, 115–120 g. in weight, were used. The animals originally subsisted on the laboratory stock diet consisting of (parts by weight): whole meal wheat flour 75, whole milk powder 2, casein 12, dried yeast 2, arachis oil 4, vitaminised sesame oil 1, sodium chloride 2 and calcium carbonate 2, the vitamin additions in the sesame oil providing vitamin A 500 I.U., α-tocopherol 5 mg., and vitamin K (Menadione, Merck) 1 mg.

Five animals were sacrificed to establish normal concentrations of serum protein fractions and the rest were reared on a protein-free diet consisting of (per cent.): maize starch 80, cellulose 3, sucrose 5, arachis oil 6, shark-liver oil 2, and salt mixture (U.S.P. No. XIV) 4. Vitamin additions carried by the sucrose corresponded to (in mg. per kg. of diet): thiamine hydrochloride 6, riboflavin 10, nicotinic acid 30, calcium pantothenate 20, pyridoxine hydrochloride 6, PGA 5, vitamin B_{12} 0·25, biotin 1, *p*-aminobenzoic acid 100, choline chloride 500, inositol 500, vitamin K 10, and α-tocopherol 50.

At the end of two weeks, five animals were sacrificed to establish the serum protein profile in the depleted state. The rest were divided into two groups of fifteen each and re-fed the protein-free diet modified by the addition of vitamin-free casein (10 g. per 100 g.) at the expense of an equal amount of starch and with additions of the seven B vitamins at optimal or minimal levels. These were, respectively, as follows (in mg. per kg. of the diet): thiamine hydrochloride 3 and 0·75; riboflavin 4 and 1, nicotinic acid 20 and 5, calcium pantothenate 10 and 2·5, pyridoxine hydrochloride 3 and 0·75, PGA 1 and 0·03 and vitamin B_{12} 0·15 and nil. Vitamins other than these were added at the same levels as in the protein-free diet. Regeneration studies, with three rats from each group, were made at the end of 2, 4, 7, 11 and 14 days of *ad libitum* feeding.

In experiment 2, the effects, specifically, of a combined deficiency of vitamin B_{12} and PGA on depletion of serum proteins and hemoglobin during protein-fasting were studied.

Weanling male rats, 40 g. in weight, were divided into two groups of ten each, one of which received a basal vitamin B_{12}-deficient, Bengal gram (*Cicer arietinum*) diet. It has been observed in this and in other work from this laboratory (Lawate and Sreenivasan, unpublished) that a high-vegetable protein with Bengal gram is as effective as the corn-soybean ration (Register *et al.*, 1949) in producing vitamin B_{12} deficiency in rats. The basal diet consisted of (per cent.): Bengal gram 90, sesame oil 3, shark-liver oil 2, salt mixture (U.S.P. No. XIV) 4, and sucrose 1; vitamin additions were the same as in the protein-free diet of experiment 1 with omissions of vitamin B_{12} and PGA. The second group received this basal diet with supplements of vitamin B_{12} and PGA at levels of 200 μg. and 1 mg. respectively per kg. of the diet. Feeding was *ad libitum* for seven weeks. The average food intake of the deficient group over this period was about nine per cent. lower than that of the control group.

At the end of 7 weeks, five animals each from the deficient and control groups were sacrificed, while the rest were replaced on the original diets modified by withdrawal of Bengal gram and substitution by an equal amount of maize starch. Serum protein and hemoglobin changes were studied after one week of protein-fasting.

Preparation of serum

The animals were dissected under ether anesthesia. Blood was drawn from inferior vena cava using a dry syringe. A portion was immediately heparinized while the rest was allowed to clot at 37° for one hour in a sterile glass container. The separated serum was obtained by centrifugation at 2°.

Hemoglobin was estimated in 30 μl. portions of whole blood by acid hematin method.

Total serum protein was determined in 30 μl. portions of serum by the biuret method (Gornall *et al.*, 1949).

Serum protein fractionation was carried out by electrophoresis on Whatman No. 3 paper strips according to the procedure described earlier (Mulgaonkar and Sreenivasan, 1959). The relative concentrations of the protein fractions were estimated densitometrically using an 'EEL' Scanner (Evans Electroselenium Ltd., Harlow, Essex).

RESULTS

Alterations in serum proteins due to protein depletion and during repletion with minimal and optimal intakes of B vitamins

The changes in body weight and in serum proteins due to protein-fasting of animals on stock diet are given in Table I. Typical electrophoretic patterns of serum proteins obtained before and after depletion are illustrated in Fig. 1. These data demonstrate marked reductions ($p < 0{\cdot}001$) in serum levels of albumin, α_1-globulin and β-globulin due to protein-fasting. While there is also a small, but insignificant ($p > 0{\cdot}2$), reduction in serum α_2-globulin level, γ-globulin level remains unaltered or is slightly increased numerically though not significantly ($p > 0{\cdot}2$); the albumin-globulin ratio is unaffected. During repletion, there is a faster restoration of total serum protein concentration with minimal than with optimal intake of B vitamins but growth is retarded (Fig. 2). In either group the total serum protein rises above the normal, pre-fasting, level but drops thereafter to levels below normal. This drop is more pronounced with minimal than with optimal intake of the B vitamins and also precedes the latter.

Figure 3 relates to the levels of serum protein fractions attained at periods during repletion with optimal and minimal intakes of the vitamins. In either group, during the first two days of regeneration, there is a small rise in the levels of α- and β-globulins with reductions in albumin and γ-globulin levels. With minimal intake of the vitamins the restoration of

TABLE I

Effects of protein-fasting on body weight and serum proteins

Forty male adult rats reared on laboratory stock diet were fed a protein-free diet for two weeks. Ten of these were sacrificed, five before and five after protein depletion, for serum protein fractionation studies. Others were used for regeneration studies. Change in body weight due to protein-fasting are for all thirty-five rats. All figures are mean values ± standard deviations.

Group	Body weight (g.)	Serum proteins					
		Total protein	Albumin	α_1-globulin	α_2-globulin (g./100 ml.)	β-globulin	γ-globulin
Normal	119·0 ± 3·0	6·80 ±0·11	2·82 ±0·02	1·31 ±0·09	0·67 ±0·05	0·89 ±0·01	1·09 ±0·02
Depleted	88·0 ± 6·4	5·40 ±0·34	2·12 ±0·16	0·86 ±0·04	0·56 ±0·16	0·69 ±0·03	1·16 ±0·11

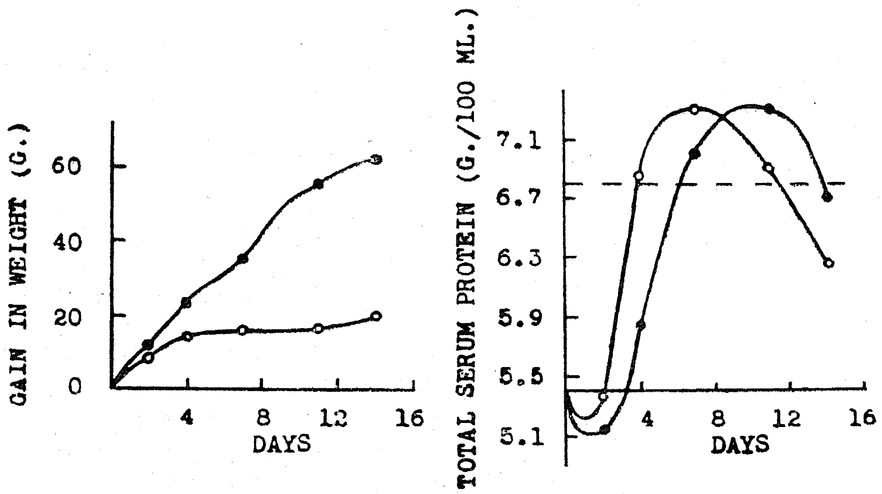

FIG. 2. Repletion of serum proteins and growth. Normal rats fed a protein-free diet for two weeks (see Table I) were re-fed a 10 per cent. casein diet at optimal (●) or minimal (o) levels of thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, folic acid and vitamin B_{12}. The broken line indicates the serum protein level of animals before protein depletion; the origin denotes the level after depletion. Each of the plots on the curves represents the average value of at least 3 determinations.

albumin and α_1-globulin is incomplete; on the other hand, there is a marked rise in β- and γ-globulins to abnormal levels. However, at the end of two weeks, the β-globulin level returns to normal, while there is some drop in the γ-globulin level also. The restoration of α_2-globulin is rapid and complete.

With optimal intake of the vitamins the serum protein fractions attain normal levels in the course of two weeks. The albumin concentration, in good parallelism with the total protein level, decreases after 11th day of repletion to below normal level following an initial increase. α_1-Globulin is almost completely restored after 14 days. α_2-Globulin increases slightly above normal in 4 days and maintains this level during subsequent period. β-Globulin, which initially shows an increment similar to, but less marked than, that observed in the low B vitamins-fed group, returns to normal at the conclusion of the regeneration period. The γ-globulin level fluctuates within the normal range. Figure 4 demonstrates the differences in the electro-phoretic profile due to variations in intake of the B vitamins, as at the end of the repletion period.

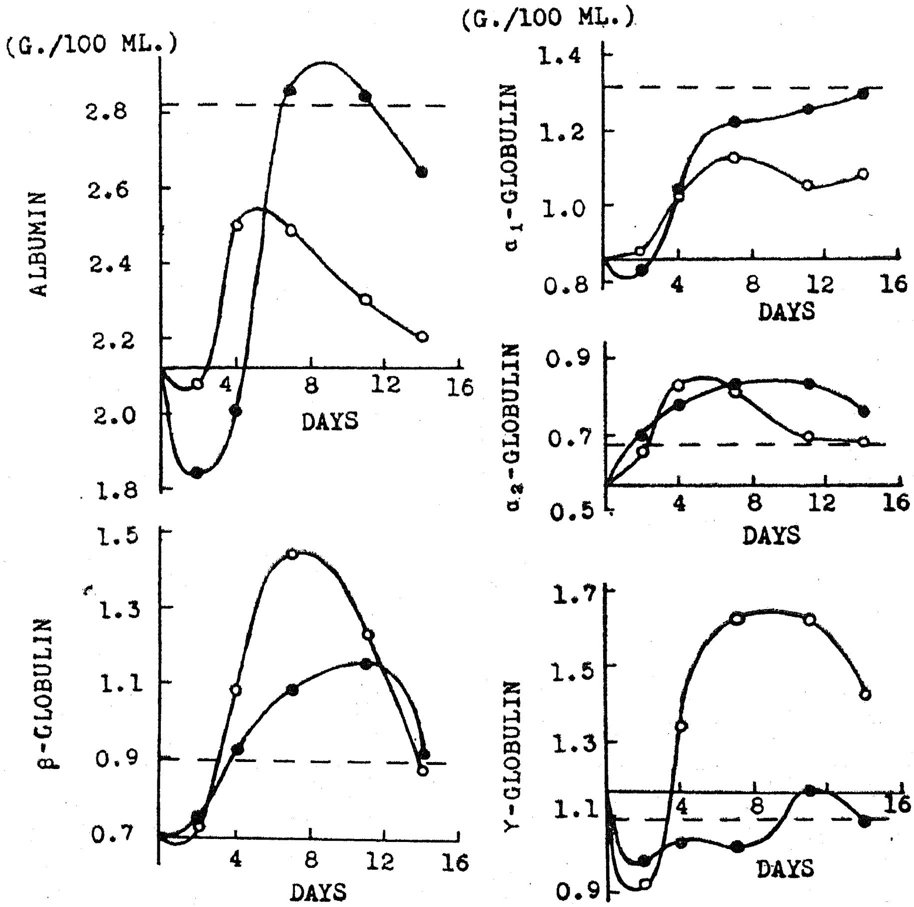

FIG. 3. Changes in electrophoretically separated serum protein fractions at various stages in the course of regeneration in protein depleted rats fed a 10 per cent. casein diet with optimal (●) or minimal (o) levels of seven B vitamins. Details as in Table I and Fig. 2. The broken line indicates the level of the protein fraction before depletion; the origin denotes the level after depletion. Plots represent in each case the average of three determinations.

TABLE II

Effects of vitamin B_{12} and PGA on the depletion of blood proteins

Weanling rats were reared on a vitamin B_{12}-deficient basal Bengal gram diet with or without supplements of vitamin B_{12} and PGA for seven weeks. The animals were then protein-depleted for one week. Blood hemoglobin and serum protein levels were determined before and after depletion. Figures represent mean values ± standard deviation. There were five rats in each series.

Group	Body weight (g.)	Hemo-globin (g./100 ml.)	Serum proteins (g./100 ml.): Total protein	Albumin	α_1-globulin	α_2-globulin	β-globulin	γ-globulin
Before Depletion								
Basal	213·0 ± 24·0	11·80 ± 0·62	6·27 ±0·28	2·26 ±0·06	1·18 ±0·06	0·72 ±0·11	1·10 ±0·07	0·99 ±0·11
Basal + Vitamin B_{12} + PGA	280·0 ± 16·6	11·81 ± 0·47	7·20 ±0·24	2·67 ±0·10	1·39 ±0·10	0·68 ±0·09	1·33 ±0·06	1·11 ±0·12
After Depletion								
Basal	198·8 ± 19·2	10·62 ± 0·34	5·00 ±0·36	1·67 ±0·07	0·81 ±0·06	0·48 ±0·06	0·85 ±0·04	1·18 ±0·10
Basal + Vitamin B_{12} + PGA	266·5 ± 11·8	11·46 ± 0·38	6·45 ±0·08	2·37 ±0·08	1·15 ±0·06	0·57 ±0·10	1·17 ±0·05	1·17 ±0·08

Influence of vitamin B_{12} and PGA on blood proteins during protein-fasting

In Table II are shown the changes in body weight, hemoglobin and serum proteins due to protein-fasting of rats on the basal Bengal gram diet and the effects on these of vitamin B_{12} and PGA. It is seen that supplements of the vitamins have positive effects on growth and on serum protein concentration but not on the hemoglobin level. The higher serum protein concentration of the vitamin supplemented group is reflected in elevated levels of albumin ($p < 0 \cdot 001$), α_1-globulin ($p < 0 \cdot 005$) and β-globulin ($p < 0 \cdot 001$).

Protein fasting brings about marked reductions in hemoglobin ($p < 0 \cdot 01$) and in total serum protein ($p < 0 \cdot 001$) concentrations in the deficient animals. The effects are less severe in the group receiving vitamin B_{12} and PGA with no significant change in hemoglobin ($p > 0 \cdot 2$), and a smaller, though significant, reduction in total serum protein ($p < 0 \cdot 001$), even though animals in the two groups have lost equally in weight. There are marked reductions ($p < 0 \cdot 005$) in serum levels of albumin, α_1-globulin and β-globulin in either group. Additionally, there is a decrease in α_2-globulin level ($p < 0 \cdot 005$) and a small but significant rise in γ-globulin level ($p < 0 \cdot 02$) in the deficient animals. These changes bring about marked alterations in the relative distribution of protein fractions in the deficient animals with decrease in the relative concentrations of albumin, α-globulins and β-globulin and an increase in γ-globulin proportion. On the other hand, the serum protein pattern of rats receiving vitamin B_{12} and PGA show a striking constancy of distribution.

Discussion

The present observations on the effects of protein-fasting on rat serum proteins are in accord with those of Allison (1955) and also with our previous findings in rats on 18 and 10 per cent. casein diets (Mulgaonkar and Sreenivasan, 1959) that a reduction in dietary protein level chiefly affects serum levels of albumin, α_1-globulin and α_2-globulin. Moreover, it is seen that the reductions are most striking in the albumin and α_1-globulin levels. This supports our earlier postulation that the dependence of albumin and α_1-globulin fractions on dietary amino acids is greater than that of other serum protein fractions. On the other hand, failure to observe any appreciable change in γ-globulin concentration with variations in dietary protein quality and level (Mulgaonkar and Sreenivasan, 1959), the latter amounting to protein-fasting in the present experiments, would suggest that its synthesis is least susceptible to qualitative or quantitative variations in dietary amino acids. Although similar conclusions have been reached by other workers with respect to antibodies and γ-globulin production in rats on low protein diets (Metcoff *et al.*, 1948; Wertman and Sarandria, 1952; Wertman *et al.*, 1952; Allison, 1955), and in under-fed humans (Bieler *et al.*, 1947; Balch, 1950; Keys *et al.*, 1950), it is possible that the degree of depletion is an important factor here, severe protein depletion having been found to affect both γ-globulin and antibody synthesis in dogs (Cannon *et al.*, 1943, 1944; Zeldis *et al.*, 1945; Chow *et al.*, 1948).

Since the animals received adequate amounts of B vitamins in the protein-free diet it is understandable why, during regeneration, growth and serum protein repletion progressed for a considerable period even though the intake of the B vitamins was minimized. Thus, the rise in the levels of albumin and α-globulins regressed only after 4 days of protein re-feeding.

The faster replenishment of total serum protein level with minimal than with optimal intake of B vitamins is associated with retarded growth and may indicate a preferential channelling of amino acids into serum protein synthesis in this condition. It is to be noted, however, that this faster repletion occurs through abnormal rise in β- and γ-globulin levels. A similar behaviour of these globulins has been recorded earlier in protein depleted dogs re-fed an enzymatic hydrolyzate of casein (Chow *et al.*, 1948). That this is a specific property of casein was evident as a lactalbumin hydrolyzate failed to produce a similar effect whereas an alcoholic extract of casein had a globulin-regenerating action when added to the lactalbumin hydrolyzate (Albanese *et al.*, 1950). Other investigators (Holman *et al.*, 1934; Madden and Whipple, 1940; Seeley, 1945) have also noted a similar behaviour of the plasma globulin fraction upon feeding casein to protein-depleted dogs.

The observed parallelism between growth rate and serum albumin level brings out an interesting relationship between protein nutriture and serum albumin concentration. The final level attained by the different protein fractions in the control group is probably a function of dietary protein quality and level. It is of interest to note that a similar trend in albumin and β-globulin levels is observed in rehabilitating starving subjects (Keys *et al.*, 1950).

In our studies mentioned above, growth retardation in rats on an 18 per cent. casein diet with minimal supplements of B vitamins is attended by a decrease in serum levels of α_1- and γ-globulins. The partial restoration of α_1-globulin and the ultimate drop in the level of γ-globulin encountered presently, during repletion with minimal B vitamins, are in accord with these findings. It is possible that the incomplete restoration of albumin, observed additionally, is due, at least in part, to the low level of protein in the diet.

The improved growth and blood picture resulting from supplementation of the basal Bengal gram diet with vitamin B_{12} and PGA may be attributed to the known sparing effect of the vitamins on methionine, which is the limiting amino acid in Bengal gram protein (Vijayaraghavan and Srinivasan, 1953). The failure to obtain any increase in hemoglobin level upon vitamin supplementation, as against a definite increase in serum protein concentration, is similar to the findings of Hsu *et al.* (1953) that in the young chick fed a 65% soybean protein diet dietary vitamin B_{12} increased the plasma protein level but not the hemoglobin level.

Vitamin B_{12} and PGA apparently moderate the effects of protein-fasting on hemoglobin and serum proteins. A similar effect of vitamin B_{12} on blood amino acids has been described by Charkey and co-workers (1954) who found that the rise in the free amino acids of the blood in fasting chicks was moderated by vitamin B_{12} at levels in the diet above that required for maximal growth.

Summary

Protein-fasting for two weeks of rats on the laboratory stock diet results in a decrease in serum level of albumin, and of α_1-, α_2- and β-globulins. The albumin-globulin ratio remains unaltered.

Repletion on a 10 per cent. casein diet with minimal as compared to optimal intake of the B vitamins—thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, folic acid and vitamin B_{12}—causes a faster restoration of total serum protein but growth is retarded. With minimal intake of the vitamins there is an incomplete restoration of albumin and α_1-globulin; an abnormal initial increase in β- and γ-globulins is followed, upon prolonged regeneration up to two weeks, by a drop in all fractions and in total serum protein. With optimal intake of B vitamins, the fractions attain normal levels at or before two weeks of regeneration. An initial abnormal increase in serum β-globulin resembles that observed at minimal intake of the vitamins. Following prolonged regeneration there is a drop in albumin and in total serum protein to subnormal levels, which effects are attributable to low level of protein in the diet.

Rats on a basal, vitamin B_{12} deficient, Bengal gram diet show reductions in blood hemoglobin, serum albumin and in serum α_1-, α_2-, and β-globulins upon protein-fasting for one week. The effects are moderated with supplements of vitamin B_{12} and folic acid to the basal diet. Incidental observations also point to growth-promoting and serum protein-generating effects of the vitamins in the growing rat.

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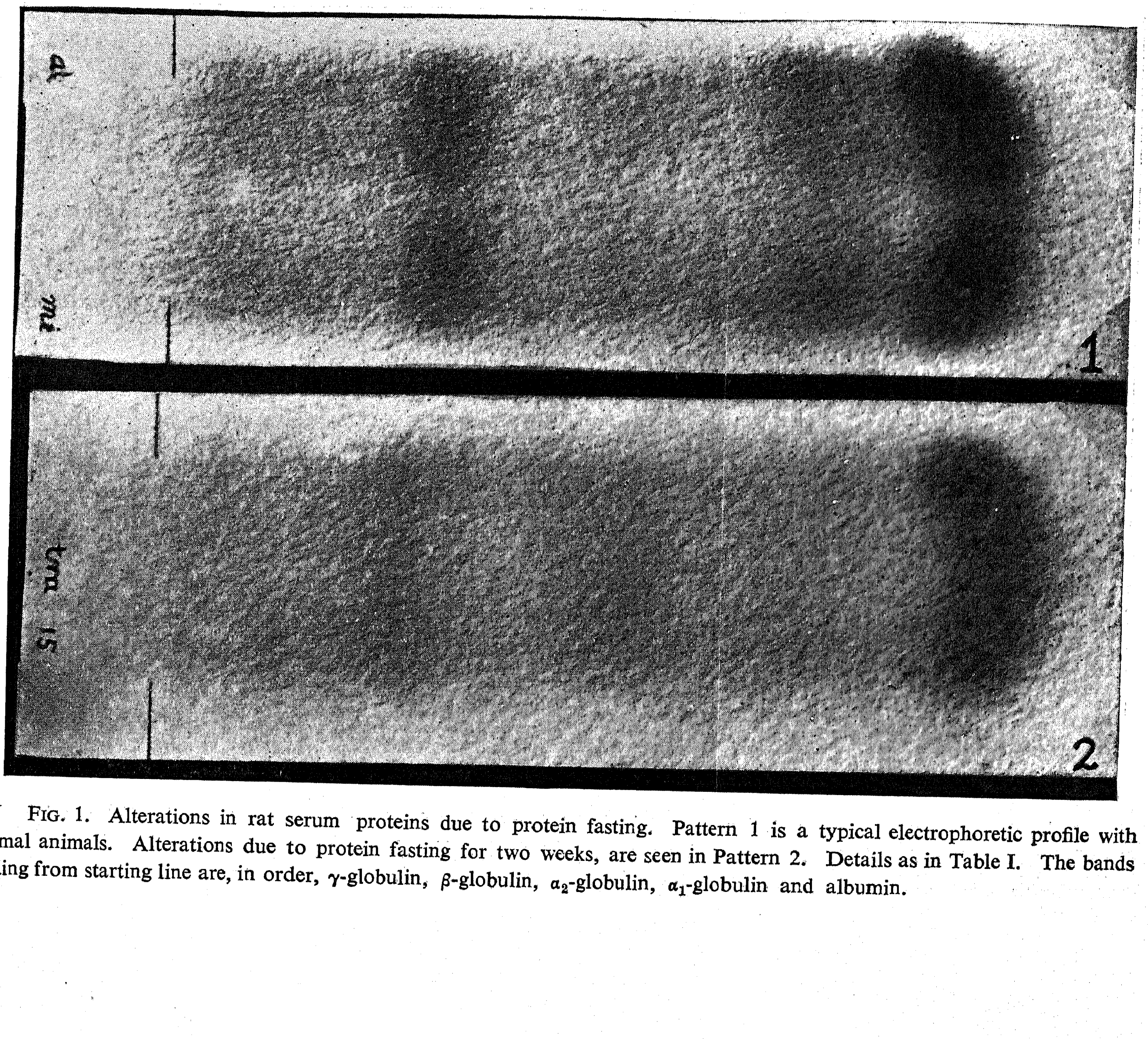

FIG. 1. Alterations in rat serum proteins due to protein fasting. Pattern 1 is a typical electrophoretic profile with rmal animals. Alterations due to protein fasting for two weeks, are seen in Pattern 2. Details as in Table I. The bands ding from starting line are, in order, γ-globulin, β-globulin, α_2-globulin, α_1-globulin and albumin.

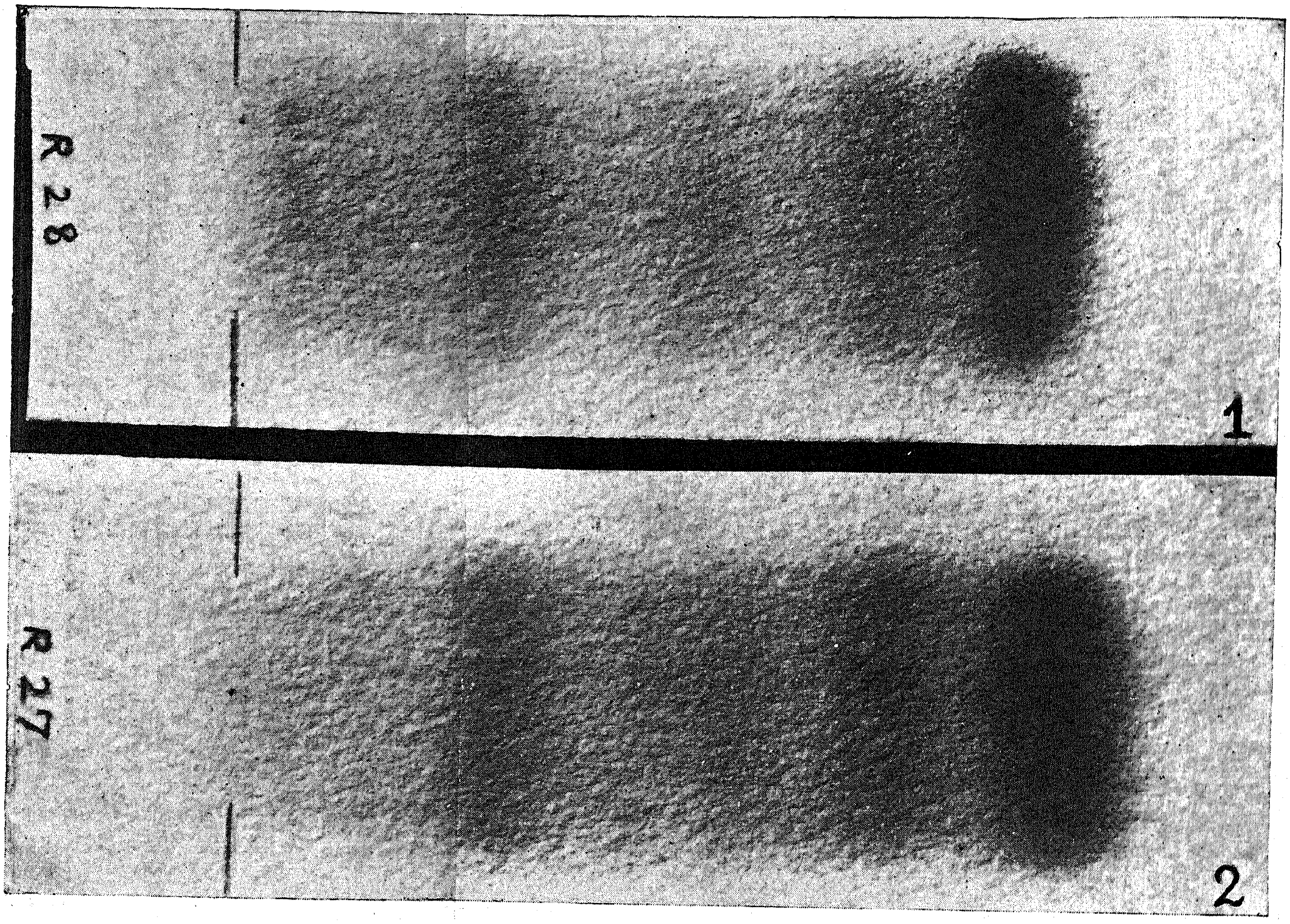

FIG. 4. Restoration of serum proteins in protein-depleted rats upon feeding a 10 per cent. casein diet, at minimal (Pattern 1) or optimal (Pattern 2) levels of seven B vitamins for 2 weeks. For details of the experiment refer Fig. 2. The disposition of the protein bands in the patterns follows the order given in Fig. 1.