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SOME EFFECTS OF PROTEIN DEFICIENCY IN YOUNG GROWING PIGS

III. FERROKINETIC INVESTIGATIONS**

By

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Disturbances in iron metabolism have been found to occur
in animals and man after consumption of diets containing in-
sufficient protein. Thus, an increased storage of iron in the liver
and a decreased rate of absorption were observed in rats on
protein free diets (*Aschkenasy* 1966, *Conrad et al.* 1967). These
rats also showed a low rate of plasma iron turnover accompanied
by an increase in serum iron levels. However, the serum iron
concentrations of pigs on a low protein diet were found to be
diminished (*Cartwright & Wintrobe* 1948) although some data
on only a few animals indicated a slight elevation (*Platt et al.*
1964). All of the afore-mentioned authors have reported reduc-
tions in serum total iron binding capacity and the occurrence of
a normocytic, normochromic anaemia during protein malnutri-
tion. It seems that a similar pattern occurs in man although
data are less reliable because of the presence of various infec-
tions and the lack of precise dietary control (*Adams & Scragg*
1965, *Tandon et al.* 1968).

Studies on the iron status and turnover in protein deprived pigs, which are more comparable to man than rats, do not appear to have been performed. Thus, an investigation into the effects of long term protein deficiency on the iron metabolism of young growing pigs was undertaken. Low levels of serum β-globulin, which contains the iron-binding protein transferrin as a major component, have already been observed in these animals (*Nikolić et al.* 1969). Ferrokinetic parameters, blood constituents and organ iron storage have been examined in protein depleted, rehabilitated and protein repleted animals.

MATERIALS AND METHODS

Animals

Two Swedish Landrace pigs were selected from each of Groups A, B and C (control, rehabilitated and protein deprived respectively) after 77—78 days of the different dietary treatments (*Nikolić et al.* 1969).

Iron deficiency had been prevented by intramuscularly injecting each pig with Ironex (1 ml; 100 mg Fe) (Løvens Kemiske Fabrik, København) before the start of the experimental period.

Experimental

At the start of the ferrokinetic studies the pigs were weighed, and blood samples (20 ml) were taken from the jugular vein into heparinized tubes. Plasmas were prepared by centrifugation, and ferric chloride ($^{59}FeCl_3$) of specific activity 10.7 Ci/g Fe (Radiochemical Centre, Amersham, England) was added to a sufficient quantity of each plasma so that the iron was bound without a sizeable increase in the saturation of transferrin. Small portions of the plasma solutions were reserved as standards, and gravimetric aliquots of the remainder were reinjected into each pig through the marginal ear vein. Each animal received about 0.8 μCi ^{59}Fe/kg body weight.

At intervals of 15 and 30 min., 1, 2 and 4 hrs. and 3 and 7 days after injection samples of blood (2—3 ml) were taken from the jugular vein and assayed. The animals were killed by exsanguination under ether narcosis. The liver, heart, spleen and a kidney were removed and weighed. Samples of the gastrocnemius muscle were taken.

Estimation of radioactivity

Radioactivity measurements were made in a "Well-type" scintillation counter (Nuclear Chicago) with an accuracy of ± 2 %. The counts recorded were corrected for radioactive decay to day 0 of the study.

After determination of whole blood and plasma radioactivity, the activity of the red cells from 1 ml of blood was estimated after washing three times with cold saline (0.9 %). The radioactivity contents of weighed samples of liver, heart, spleen, kidney and muscle were measured.

Analyses

Haematocrit determinations were made regularly at fortnightly intervals from start of the different dietary treatments and on each day of blood sampling during the ferrokinetic studies. Blood samples were centrifuged in capillary tubes (Clay-Adams Inc. New York) at 3.500 r.p.m. for 90 min.

The blood haemoglobin content was measured on day 0 and day 7 of the ferrokinetic studies according to *Berkeš & Terzić* (1963).

Weighed organ samples were placed in Kjeldahl flasks and sulfuric acid (10 ml) and 70 % perchloric acid (1 ml) were added. The flasks were heated until the solutions were clear. After neutralization to bromthymol blue with 25 % ammonia solution, aliquots of the mixture were used for the determination of iron according to *Fischl* (1960).

The plasma iron content and total iron binding capacity (TIBC) were estimated by the methods of *Fischl,* and *Fischl & Cohen* (1962), respectively.

Ferrokinetic calculations

Maximal plasma and whole blood radioactivities were obtained by extrapolation of the lines representing the disappearance of radioactive iron from these media to zero in a semilogarithmic plot. Blood and plasma volumes were calculated according to the isotope dilution principle (*Nordén* 1960).

From the data obtained the following results can be calculated:

time taken for the radioactive iron to reach half the original concentration (T 1/2);

total circulating plasma iron;

fraction of iron removed from the circulation per hour;

plasma iron turnover per 24 hrs.;

plasma iron turnover (mg/day/100 ml whole blood);

percent incorporation of radioactive iron by the red blood cells.

The results were also related to body weight to make comparisons between individual pigs more valid.

RESULTS

Changes in the blood haematocrit of all the animals undergoing the different dietary treatments are given in Fig. 1. Each group showed an initial increase during the first fortnight of the experimental period. Thereafter, levels in the control group were
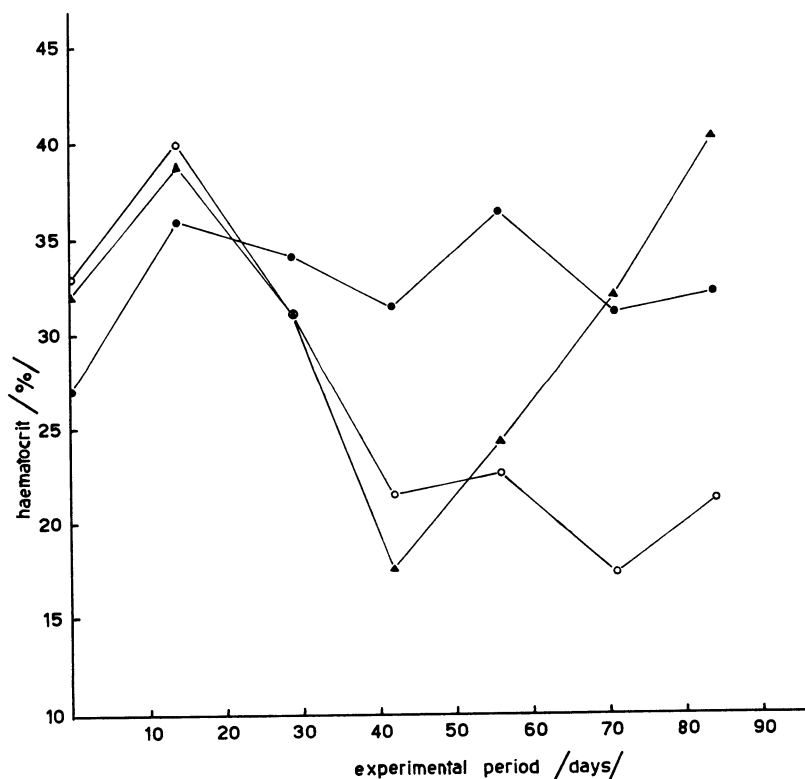

F i g u r e 1. Haematocrit levels of pigs subjected to different dietary treatments. Group A, control, ● ; Group B, protein deprived 42 days, rehabilitated 42 days, ▲ ; Group C, protein deprived 84 days, o.

T a b l e s 1. Some blood parameters after 77—78 days of different dietary treatments.

Treatment	Pig no.	Haematocrit (%)	Haemoglobin (g/100 ml)	Mean corpuscular haemoglobin concentration (MCHC)	Serum iron (μg/100 ml)	TIBC (μg/100 ml)	Saturation of trans-ferrin (%)
control	7	40.8	—	—	231	534	43.2
	18	35.7	12.9	36.1	241	496	49.8
protein starvation 42 days	8	31.6	10.1	32.0	169	535	31.6
rehabilitation 35—36 days	14	43.4	12.0	27.6	173	470	36.8
protein starvation 77—78 days	3	25.4	7.7	30.3	44	305	14.4
	17	21.6	5.9	27.3	88	359	24.6

more or less even while levels in both protein deprived groups fell steeply. After 42 days the haematocrit of Group C remained about steady at a low level whereas Group B, which was refed a protein supplement, showed a rapid increase. Control levels were reattained within one month of refeeding.

After 77 days of the different dietary treatments the individual haematocrit values of the six pigs used for the ferrokinetic studies were as shown in Table 1. Haemoglobin levels in the two protein starved animals were about half the control level. Total iron binding capacities of the serum and serum iron concentrations were also greatly reduced in these animals. The latter parameter was so low that the saturation percentage of transferrin was only half that of the control pigs. Percentage saturation levels in the rehabilitated pigs were also lower than control values due to a lower serum iron content in these animals. However, the mean corpuscular haemoglobin concentrations was similar in all the pigs indicating that the red cells were normochromic.

The effect of the different dietary treatments on the blood and plasma volumes is summarized in Table 2. The results from two separate experiments are included. Lower total blood and plasma volumes were found in all the protein starved animals when compared with control groups, but this is largely an effect

Table 2. Effect of diet on blood and plasma volumes in pigs.

Treatment	Pig no.	Body weight (kg)	Blood volume (ml)	Plasma volume (ml)	Blood volume (ml/kg)	Plasma volume (ml/kg)
Experiment 1						
control	B	28.2	2490	1520	88.2	53.9
	H	26.2	2440	1470	93.1	56.2
protein starvation 38 days	C	23.9	1580	960	66.0	40.3
	E	23.4	1370	1030	58.7	43.9
Experiment 2						
control	7	67.7	4470	3600	66.1	53.2
	18	65.2	4260	2690	65.3	41.2
protein starvation 42 days	8	48.6	3640	2480	74.9	51.1
rehabilitation 35—36 days	14	53.2	3720	2100	69.9	39.6
protein starvation 77—78 days	3	25.7	1540	1190	59.9	46.2
	17	23.8	1850	1150	77.6	60.8

of the different body weights. However, when the protein starved animals from both experiments are compared with the control group from Experiment 1, the blood volumes per kg body weight are still lower. The rehabilitated animals showed similar blood and plasma volumes per kg body weight as the control group in the same experiment.

The ferrokinetic data are shown in Table 3. The time for half the injected radioactive iron to be cleared from the plasma (T 1/2) is shorter in the protein starved animals by a factor of about two compared with the controls. A tendency for T 1/2 to be less in rehabilitated animals is also noticeable. Total circulating plasma iron per se and when related to body weight is reduced in both the rehabilitated pigs and the protein starved pigs particularly in the latter case. Although the protein starved pigs showed a lower rate of turnover of iron per day, the values for the rehabilitated group were similar to those of the controls.

In Fig. 2 the pattern of whole blood radioactivity over a period of seven days after the intravenous injection of iron-59 is shown. Up to four hours the curve indicates the rate of clearance

T a b l e 3. Ferrokinetic data from pigs subjected to different dietary treatments.

| Treatment	Pig no.	T½ (min.)	Total plasma iron (mg)	Total plasma iron (mg/kg)	Plasma iron turnover (mg/day)	Plasma iron turnover (mg/kg/day)	Plasma iron turnover (mg/kg/ 100 ml blood)
control	7	57	8.3	0.122	142	2.09	2.39
	18	67	6.4	0.098	95	1.45	3.59
protein starvation 42 days	8	54	4.2	0.086	78	1.60	2.14
rehabilitation 35—36 days	14	36	3.6	0.068	101	1.89	2.60
protein starvation 77—78 days	3	24	0.5	0.020	22	0.85	1.36
	17	38	1.3	0.053	33	1.38	1.81
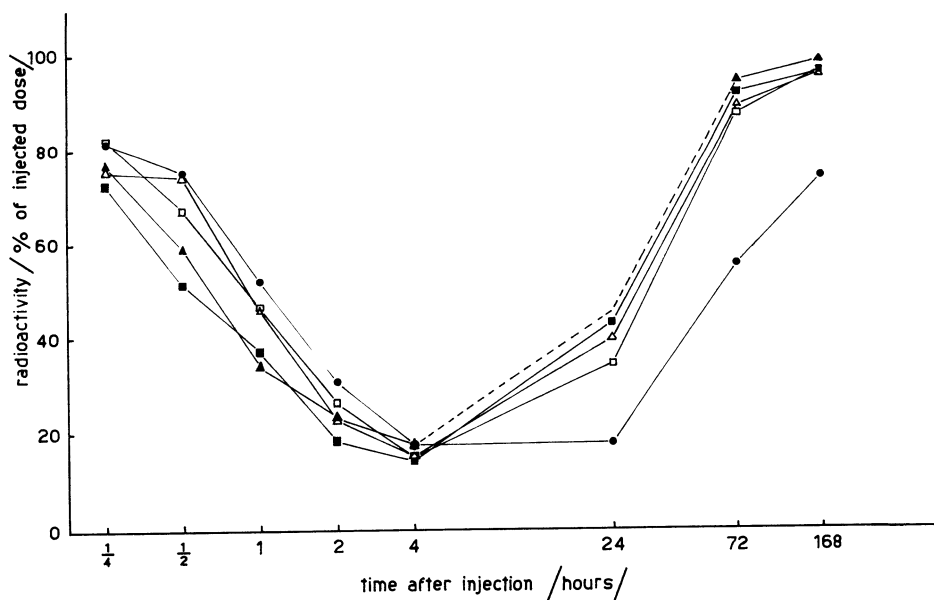
F i g u r e 2. Changes in the whole blood content of radioactivity after intravenous injection of plasma labelled with iron-59. Pig no. 18, • , control; Pigs nos. 8, △, and 14, ▲, protein deprived 42 days, rehabilitated; Pigs nos. 3, ■, and 17, □, protein deprived.
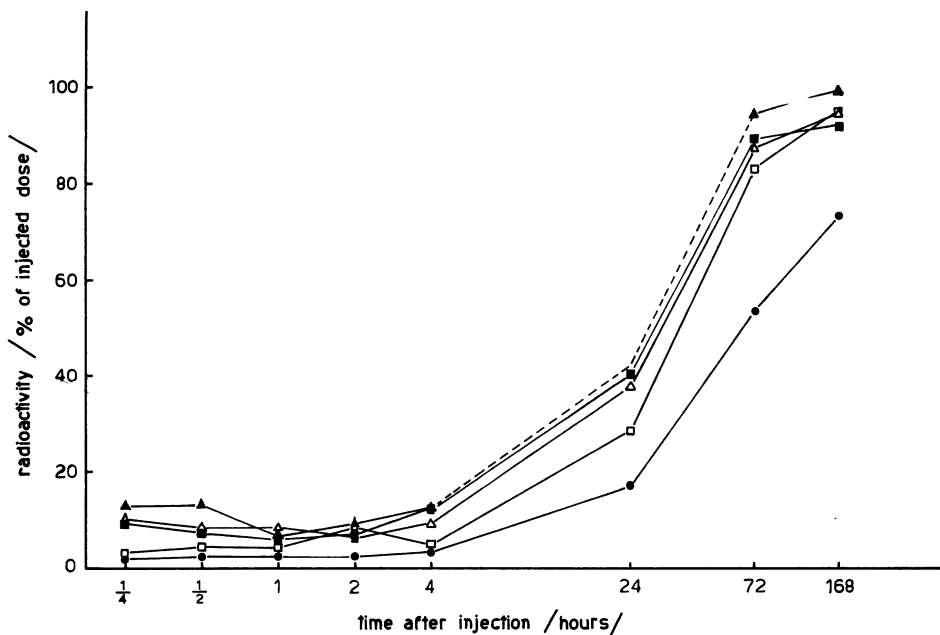
F i g u r e 3.   Uptake of radioactivity into the washed red blood cells
of pigs after intravenous injection of plasma labelled with iron-59
(cf. Figure 2).

of radioactive iron from the plasma pool which, although similar
in all animals, is the slowest in the control pig. The second part
of the curve shows the appearance of the radioactive iron in the
red cells which is verified in Fig. 3, where the uptake of radio-
activity into washed red cells is given. The slowest uptake of
radioactive iron was observed in the control animal, whereas the
rates in the protein starved and rehabilitated pigs were similar.
These pigs showed a higher initial content of radioactive iron in
the red cells.

The distribution of iron-59 in various tissues after intra-
venous administration is presented in Table 4. Almost all of the
injected radioactive iron is circulating in the blood, the lowest
proportion (74 %) being found in the control animal. The values
for the specific activity of the iron in the blood are similar in
the control and rehabilitated animals, but the two starved pigs
show specific activities twice as great. In the liver the rehabil-
itated animals had lower iron contents than the control pig,
whereas the protein starved pigs showed an iron concentration

T a b l e 4. Iron content of various tissues seven days after intravenous administration of iron-59.

Tissue	Pig no.	Radioactive material (counts/ min/g or ml)	Percentage of injected dose (%)	Iron content (μg/g or ml)	Organ weight (g)	Total iron content (mg)	Specific activity (counts/ min/μg Fe)
blood	18*	3590	73.9	449	—	1920	7.94
	8	3840	95.4	394	—	1480	9.69
	14	3540	98.4	449	—	1680	7.85
	3	4460	95.7	238	—	366	18.70
	17	3850	95.8	209	—	388	18.30
liver	18	3180	19.50	117.0	1270	149	27.60
	8	734	5.30	66.6	1060	70	12.60
	14	919	7.69	92.2	1120	103	11.10
	3	1760	8.13	743.0	340	253	2.52
	17	3140	23.30	383.0	550	211	8.90
spleen	18	673	0.36	103.0	111	11.4	8.62
	8	1110	0.80	134.0	105	14.1	9.63
	14	658	0.49	153.0	100	15.3	5.16
	3	4170	2.83	539.0	50	27.0	7.95
	17	—	—	—	25	—	—
heart	18	359	0.44	46.9	258	12.1	9.29
	8	204	0.31	38.0	223	8.5	6.95
	14	197	0.30	42.9	206	8.8	5.99
	3	355	0.55	54.3	115	6.2	8.45
	17	—	—	—	90	—	—
kidney	18	550	0.56	56.6	105	5.9	11.61
	8	565	0.92	73.9	120	8.9	9.87
	14	735	1.68	84.8	153	13.0	10.15
	3	418	0.36	205.2	32	6.6	2.51
	17	—	—	—	35	—	—
muscle	18	38	—	16.6	—	—	9.98
	8	24	—	10.5	—	—	16.70
	14	26	—	18.2	—	—	10.90
	3	28	—	23.0	—	—	6.01
	17	18	—	101.0	—	—	2.43

* Pig no. 18: control.
  Pigs nos. 8 and 14: rehabilitation.
  Pigs nos. 3 and 17: protein starvation.

more than three times as great. The total iron content of these livers was greater than that of the much larger control liver and was almost the same in both animals despite a twofold variation in liver weight. Similarly spleen, kidney and muscle showed increased concentrations of iron in the protein starved pigs.

In the control pig most of the non-circulating radioactive iron seems to have been incorporated into the liver whose specific activity is much higher than the specific activity of blood or other organs. In the rehabilitated animals the specific activity of the liver is only slightly higher than that of the blood indicating a very small amount of storage. The protein starved animals show very low specific activities because of the very high liver iron content.

DISCUSSION

Chow et al. (1945) studying the effect of protein depletion in dogs found that the plasma volume decreased by 20 % after six to eight weeks of a protein free diet. Our results also indicate a reduction in plasma volume, but the data are complicated by the fact that the pigs were growing. *Bush et al.* (1955) reported that normal pigs show a progressive reduction in blood and plasma volume per kg body weight during growth. When the blood volumes per kg of the protein deprived pigs from both experiments (Table 2) are compared with the control pigs from Experiment 1, which had similar body weights, it can be seen that there is a definite reduction in intravascular fluid under conditions of protein starvation.

The initial haemoconcentration observed during the first 14 days of the experiment may be an effect of the semisynthetic diet. *Conrad et al.* (1967) noted a similar delay in the fall of haematocrit values in rats on a protein free diet. Since a greatly reduced utilization of iron-59 for red blood cell formation was found by these authors and *Aschkenasy* (1966) during this period, it seems that haemoconcentration masks the initial effects of protein deficiency on erythropoiesis.

Although the data in Table 1 indicate a reduction in the number of circulating red cells, the mean corpuscular haemoglobin concentrations show that the cells contain a normal concentration of haemoglobin, as found by *Cartwright & Wintrobe* (1948), *Conrad et al.* and *Platt et al.* (1964). The fact that serum β-glo-

bulin-2 concentrations in the protein deprived pigs were only 37 % of those of the control animals (*Nikolić et al.* 1969) while transferrin levels were 60 % of control values suggests that transferrin is less affected by the protein free diet than other proteins in this electrophoretic fraction.

Serum iron levels of representative samples measured during the course of the experiment were below control values after one month of protein deprivation, although they were within the range reported as normal for swine (*Hristić* 1967). However, after 70 days the values were below 100 µg/100 ml serum. This can be explained by the greatly reduced food intake in these pigs, a lower rate of intestinal absorption, as has been found in rats and man (*Aschkenasy; Conrad et al.; Tandon et al.* 1968) and a block on the use of the iron stores by some unknown mechanism. Our results support those of *Cartwright & Wintrobe* (1948), who fed pigs a low protein diet. It should be mentioned that the studies of *Conrad et al.*, who found a rise in serum iron levels, were relatively short term. Increases in serum iron levels have been reported for dogs (*Platt et al.*), but the measurements of these authors in pigs showed very small effects among only three animals on three different diets. The possibility of species differences in the reaction to a protein free diet exists.

The ferrokinetic data shown in Table 3 indicate a diminished metabolic turnover of iron in the protein starved animals. However, the turnover in pigs of a similar weight (*Hristić* 1966) is comparable, which suggests that the development of metabolic processes is arrested by protein starvation. Enhanced catabolism compared to synthesis is indicated by the low haematocrit and haemoglobin concentrations and the high content of iron in spleen, when compared to controls. Reduction in the rate of synthesis before an appropriate reduction of catabolism would lead to decreasing plasma concentrations followed by a new steady state as a balance is reached at a new low level, as has been found with serum albumin (*Kirsch et al.* 1968, *Nikolić et al.*). Thus, after long term protein deprivation the haematocrit levels of the protein deprived pigs were fairly stable (Fig. 1). *Aschkenasy* found a renewed erythropoietic activity at below normal levels after prolonged protein deficiency in rats.

The utilization of serum iron for haemoglobin synthesis appears to be very efficient in the protein starved pigs for, despite the low transferrin levels, the saturation percentage is even more

greatly reduced and yet 95 % of the injected dose of iron-59 is incorporated into the red cells after seven days compared to 73 % in the control animal. This suggests that marrow stores of iron are low as has been reported for children with kwashiorkor by *Adams & Scragg* (1965).

The rehabilitated pigs show typical symptoms of recovery with a high rate of incorporation of iron into red cells and slightly lower serum iron concentrations and percentage saturation of transferrin than the control pig. The metabolic turnover of iron in these animals is comparable with that of the controls, but organ data indicate a depletion of the liver iron stores. This iron has obviously been drawn into the red cell synthesis and degradation cycle according to the *Pollycove & Mortimer* (1961) model.

At first sight the results for pig no. 17 in Table 4 appear to be anomalous. The fact that the sum of the blood and organ contents of radioactive iron is greater than 100 % can be explained, if almost all the iron-59 is assumed to be in the blood circulation. Livers were not perfused before measurement of radioactivity and a low rate of uptake of iron into cells would be expected in protein deprived animals in view of the necessity of protein synthesis for iron storage (*Yoshino et al.* 1968) and the low rate of iron uptake when the liver iron content is high (*Charley et al.* 1960). Also, since the liver weight per kg body weight was 80 % higher in pig no. 17 than pig no. 3, the proportion of circulating blood in the liver of the former animal would be expected to be correspondingly greater.

According to the Pollycove & Mortimer model it may be assumed that red cell synthesis is reduced in the protein starved animals by either or both of two mechanisms. Utilization of iron from the liver or spleen stores may be prevented by some defect in the transfer of iron to transferrin molecules, as indicated by the high iron contents of these organs and the low percentage saturation of transferrin. However, the low iron levels are unlikely to be the controlling factor in causing the anaemia in view of the normochromicity of the red cells, which would probably be hypochromic if iron-shortage played a dominant role. Erythropoiesis in the bone marrow may be radically slowed because of a shortage of amino acids for protein synthesis. Plasma free essential amino acids have been shown to be much reduced in the protein starved pigs (*Čuperlović & Stošić* 1970). The shortage of amino acids may not act directly on the bone marrow. Thus

Reissman (1964 a, b) postulated that the depression of erythro-
poiesis is the result of a diminished formation of erythropoietin,
since the anaemia of protein deficiency could be completely pre-
vented in rats by administration of erythropoietin preparations
over a period of 35 days.

From the data presented it can be concluded that the protein
starved animals have adjusted their metabolic processes to a
minimum turnover to survive as long as possible and to conserve
essential elements such as nitrogen and iron.

ACKNOWLEDGMENT

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SUMMARY

Ferrokinetic investigations have been carried out in pigs which had undergone protein starvation and rehabilitation. After long term protein deprivation haematocrit levels, blood haemoglobin levels, serum iron concentrations, serum total iron binding capacity and blood volume per kg body weight were all reduced in the deficient

animals. The values for the rehabilitated animals were in the normal range. The erythrocytes of the protein starved pigs showed an increased rate of uptake of radioactive iron compared with the control pig. However, the turnover of iron as a whole was reduced greatly in the protein deprived group compared to the rehabilitated and control pigs. Accumulation of iron occurred in the livers, spleens and kidneys of the protein starved animals. The rehabilitated pigs had lower liver stores of iron than the control pig. On the basis of the data presented it has been concluded that these effects are mainly due to a shortage of amino acids for protein synthesis.

SAMMENFATNING

Nogle virkninger af proteinmangel hos voksende svin.

III. Ferrokinetiske undersøgelser.

Ved undersøgelser over jernomsætningen hos svin under protein-hunger fandtes, at hæmoglobinkoncentrationen, serumjernkoncentrationen og serums totale jernbindende evne blev betydelig nedsat. Samtidig formindskedes blodvolumenet. Under proteinrehabiliteringsperioden blev blodets sammensætning og blodvolumen normal. Under proteinhunger og i rehabiliteringsperioden var de røde blodlegemers optagelse af transferrinjern højere end normalt, i øvrigt var plasma jernpuljens „turnover rate" stærkt nedsat hos de proteinmanglende dyr, men disse havde en betydelig jernakkumulering i lever, milt og nyrer. Under proteinrehabiliteringsperioden svandt disse jerndepoter og nåede subnormale værdier. De beskrevne fund tyder på, at de patofysiologiske ændringer i jernstofskiftet under proteinhunger skyldes den nedsatte proteinsyntese.