Protective Influence of Hydrolysed Product of 'Glucose Cycloacetoacetate' in Experimental Anaemia Resulting from Necrogenic Diet

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Nath & Prasannan (1959) have shown that hydrolysed 'glucose cycloacetoacetate' can restore the erythrocyte count and haemoglobin level with the same efficacy as vitamin B₁₂ in experimental anaemia induced by phenylhydrazine. The occurrence of anaemia of some type or other due to the liver damage is well known (Shumacker & Wintrobe 1938; Koszewski, 1952).

During the development of dietary liver necrosis it is recognized that depletion of reduced glutathione in liver occurs (Leaf & Neuberger, 1947; Lindon & Work, 1953).

Nath & Behki (1958) have shown that the depletion of glutathione occurring in experimental diabetes induced by acetoacetate in rabbits can be checked by administering the sodium salt of glucose cycloacetoacetate. The same substance also checked the depletion of glutathione in scorbutic guinea pigs (Behki, Motlag & Nath, 1958).

The effect of glucose cycloacetoacetate, its hydrolysed product and vitamin B₁₂ on haemoglobin level and erythrocyte count has been studied during the feeding of necrogenic diet to rats by the methods of Lindon & Work (1953), with slight modifications.

EXPERIMENTAL

Animals and diets. Weanling male albino rats weighing 40–60 g. were divided into the following five groups in respect of their diet and experimental treatment. Each group contained six or seven animals. The grouping of the animals was carried out in such a manner that the average body weight of the animals for all groups was approximately the same. Group I animals were fed normal stock diet (%): casein 12, cane sugar 36, groundnut oil 6.4, arrowroot 40, cod-liver oil 1.6, salt mixture 3.5 (Hawk, Oser & Summerson, 1947), L-cystine 0.3 and choline chloride 0.2. The following vitamins were added/kg. of the above diet: thiamine hydrochloride 5 mg., riboflavin 5 mg., pyridoxine 5 mg., *p*-aminobenzoic acid 5 mg., niacin 5 mg., calcium-D-pantothenate 25 mg., inositol 50 mg., biotin 0.1 mg., folic acid 1 mg., vitamin B₁₂ 25 µg., α -tocopherol acetate 25 mg. and vitamin K 5 mg. All the animals in the remaining groups were fed on the necrogenic diet (Lindon & Work, 1953) with minor modification as follows (%): baker's yeast 6, cane sugar 36, arrowroot 46, cod-liver oil 1.6, ground-nut oil 6.4, salt mixture 3.5 (Hawk *et al.* 1947). All the vitamins except α -tocopherol and vitamin B₁₂ were

added as mentioned for the diet of group I animals. Group III animals were given together with their diet crystalline glucose cycloacetoacetate at a concn. of 0.3%. Group IV animals were injected subcutaneously with 1 g. equiv. of glucose cycloacetoacetate (hydrolysed)/kg. body wt. on alternate days. Group V animals were injected subcutaneously, on alternate days, with vitamin B₁₂ (BE-Douze Lab. Grimault, Paris, 200 µg./kg. body wt.).

Analytical methods. Blood was taken from the tail before the animal was fed. The tip of the tail was shaved clean, washed with saturated solution of sodium citrate and dried with sterile cotton. A small longitudinal incision (1 mm.) was made on one of the tail veins, and the first drop of blood was wiped off with moistened cotton. By applying gentle pressure with fingers, the freely flowing blood was collected into the clean pipettes. The erythrocyte count was made by the usual procedure with a Neubauer Ruling (improved double Neubauer Ruling Depth 0.100 mm., 0.01 sq. mm.) Haemoglobin was estimated by the method of Wong (1928) with 0.2–0.5 ml. of blood by converting it into Fe³⁺ ions with conc. H₂SO₄ and potassium persulphate and by using 3N-KSCN for colour development. The extinction of the coloured sample was compared with that of the standard iron solution with a green filter in a photoelectric colorimeter (Unicam, Cambridge, England). The haemoglobin was calculated from the corresponding blood iron value taking 0.34% iron content for haemoglobin.

Glucose cycloacetoacetate was first prepared according to a method of West (1927) modified by Nath, Chitale & Belavady (1952), which was then hydrolysed as follows: In a 50 ml. conical flask, 2 g. of crystalline glucose cycloacetoacetate was hydrolysed with 10 ml. of 2N-HCl over a boiling-water bath for 20 min. The flask was cooled over ice, and the contents were then neutralized to about pH 7. It was then extracted three times with ether; the lower aqueous fraction was separated and adjusted carefully to pH 7.2 with a few drops of 0.1N-NaOH, by using Merck special indicator paper (pH range 6.6–8.0).

RESULTS AND DISCUSSION

In the course of 35 days, the animals fed on normal stock diet showed a slight increase in erythrocyte count and haemoglobin, whereas those fed on necrogenic diet showed a considerable decrease, the erythrocyte count and haemoglobin being decreased to levels corresponding to 64 and 66% of the initial average values respectively (see Table 1). The presence of glucose cyclo-

Table 1. *Antianaemic effect of hydrolysed glucose cycloacetoacetate and vitamin B₁₂ in rats receiving the necrogenic diet*

Group no.	RBC, red blood corpuscles; Hb, haemoglobin. Mean values from seven rats of RBC (10 ³ /mm ³) and Hb (g./100 ml.) and standard deviation after time			
	0	1 week	3 weeks	5 weeks
I. Normal stock diet				
RBC	7.68 ± 1.12	8.30 ± 0.65	8.74 ± 0.66	8.89 ± 0.69
Hb	12.5 ± 1.21	13.1 ± 0.89	13.5 ± 0.94	14.3 ± 1.25
II. Necrogenic diet				
RBC	8.57 ± 1.13	8.37 ± 1.08	7.84 ± 0.85	5.55 ± 0.57
Hb	14.4 ± 1.15	13.8 ± 0.82	12.4 ± 1.21	9.6 ± 1.03
III. Necrogenic diet + glucose cycloacetoacetate at a concn. of 0.3 % in the diet				
RBC	7.44 ± 0.83	7.13 ± 1.27	6.28 ± 0.83	5.39 ± 0.75
Hb	13.5 ± 1.85	13.2 ± 1.55	11.4 ± 1.19	10.2 ± 1.16
IV. Necrogenic diet + hydrolysed glucose cycloacetoacetate injected on alternate days (100 mg./100 g. body wt.)				
RBC	7.93 ± 1.03	8.32 ± 0.75	8.02 ± 0.94	8.00 ± 0.35
Hb	13.4 ± 1.16	13.5 ± 1.02	13.8 ± 1.93	13.5 ± 1.78
V. Necrogenic diet + vitamin B ₁₂ injected on alternate days (20 µg./100 g. body wt.)				
RBC	8.43 ± 0.96	8.22 ± 0.91	8.10 ± 0.98	8.50 ± 1.16
Hb	12.9 ± 1.18	13.2 ± 0.97	13.2 ± 1.44	13.0 ± 1.65

acetoacetate (0.3 %) in the diet did not check the fall in erythrocyte count and haemoglobin appreciably, although the hydrolysed product of glucose cycloacetoacetate was almost as efficacious as vitamin B₁₂ in maintaining the erythrocyte count and haemoglobin level. The meagre haematopoietic activity of glucose cycloacetoacetate as compared with its hydrolytic product may be attributed mostly to its poor absorption through the gastrointestinal tract because of the oral administration. Antianaemic behaviour of the hydrolysed product of glucose cycloacetoacetate and perhaps also of vitamin B₁₂ may be, broadly, due to the protection offered by these substances against liver damage resulting from necrogenic diet. During the first phase of the development of dietary liver necrosis, depletion of glutathione occurs (Lindon & Work, 1953) and both vitamin B₁₂ and hydrolysed product of glucose cycloacetoacetate have been found to check the depletion of glutathione (unpublished data). Further, the hydrolysed product of glucose cycloacetoacetate also facilitates the biosynthesis of methionine in rabbits in experimental atherosclerosis (Nath & Saikia, 1959). Methionine, being the precursor of cystine, checks the glutathione depletion in dietary liver necrosis (Leaf & Neuberger, 1947). The turnover of labelled haemoglobin formed from injected radioactive glycine is also increased in animals supplied with additional methionine in the diet (Cohen & Berg, 1956).

SUMMARY

1. The fall in the erythrocyte count and haemoglobin level in rats during 5 weeks of feeding necrogenic diet and the effect of glucose cycloacetoacetate, its hydrolysed product and vitamin B₁₂ in restoring the haemoglobin level and erythrocyte count was studied.

2. The erythrocyte and haemoglobin levels in control animals kept on a necrogenic diet were lowered to 64 and 66 % of the average values for normal animals respectively, whereas, in the animals kept on a necrogenic diet but receiving injections either of vitamin B₁₂ or of hydrolysed glucose cycloacetoacetate, no significant fall in erythrocyte count and haemoglobin level was observed.

3. The nature of the antianaemic effect of vitamin B₁₂ and hydrolysed product of glucose cycloacetoacetate has been discussed.

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The Lipotropic Action of some Halogen Derivatives of Acetic Acid

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In previous work it was found that ethyl trichloroacetate was effective in lowering the high amount of liver lipid which accumulated when rats were fed a choline-deficient diet (Kratzing & Windrum, 1957, 1959). It seemed likely that other halogen derivatives of acetic acid might also possess this lipotropic action.

The investigation reported here shows that ethyl trifluoroacetate was able to prevent accumulation of excess of lipids in the liver of choline-deficient rats. Ethyl acetate, ethyl dichloroacetate and ethyl tribromoacetate were without demonstrable lipotropic action.

METHODS

Animals. The present experiments were carried out in separate laboratories; two different locally bred strains of young rats were used. The animals were housed separately in wire cages with wire-mesh floors. Strain 'A' rats were from the Physiology Department colony and were the same strain used for the experiments described in the initial papers (Kratzing & Windrum, 1957, 1959). Strain 'B' rats were from the Brisbane Hospital colony and had been used for experiments described in another paper (Windrum & Kratzing, 1960).

Diet. Strain 'A' rats were fed *ad lib.* on the diet used in previous experiments (Kratzing & Windrum, 1959). Strain 'B' rats were fed on the same diet to which 0.4% of cystine was added in order to increase the requirement of choline as discussed previously (Windrum & Kratzing, 1960). The amount of food fed to strain 'B' rats was adjusted to maintain the growth of all groups at an approximately uniform rate. Choline was fed to the control groups as choline chloride.

Esters. Ethyl trichloroacetate was prepared as described by Kratzing & Windrum (1959). Ethyl tribromoacetate,

ethyl trifluoroacetate and ethyl dichloroacetate were prepared from the corresponding acids and the fractions which boiled between 120° and 125° (at 35 mm. Hg), 62° and 64°, and 156° and 159° respectively were used for injection. Ethyl acetate was a redistilled commercial sample.

Treatment. The esters were given for a 3- or 4-week period from the time of commencement of the deficient diet, except that strain 'A' rats, given ethyl dichloroacetate, were fed the choline-deficient diet for 3 weeks before commencing injections. The animals were injected subcutaneously once daily 5 days a week. Series 'B' rats were given the ethyl acetate and ethyl trichloroacetate as a 1:1 dilution in liquid paraffin for 4 weeks. Ethyl trifluoroacetate was injected as such because it was not completely miscible with paraffin. In order to minimize any lipotropic effect of the injection itself (see Kratzing & Windrum, 1959), strain 'A' choline-deficient control rats received injections of 0.9% sodium chloride and strain 'B' animals were given subcutaneous injections of liquid paraffin.

The animals were killed 24 hr. after the last injection and exsanguinated, and the livers and kidneys were excised and weighed.

Analysis. The liver-lipid content of strain 'A' rats was determined by the method described by Wheeldon & Collins (1957). The lipid content of strain 'B' rat liver was determined by the method used by Windrum & Kratzing (1960).

RESULTS

Ethyl acetate. Expt. 1 of Table 1 shows that strain 'A' rats fed on the choline-deficient diet did not respond with lowered lipid content of the liver when given repeated doses of ethyl acetate. The dosage given was that previously found effective with ethyl trichloroacetate (on an equimolar basis) in reducing excess of liver lipids (Kratzing & Windrum, 1959).