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The Effect of 'Glucose Cycloacetoacetate' on the Regeneration of Erythrocytes and Restoration of Haemoglobin Level in Experimental Anaemia in Rats

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That phenylhydrazine brings about experimental anaemia accompanied by liver damage has been shown by several workers (Damodaran & Vijayaraghavan, 1943; Chanutin, Lentz & Ludewig, 1953; Helbig, 1955; Imanishi, 1956). It was reported by Vijayaraghavan & Dunn (1950) that an experimental anaemia induced in mice by the injection of phenylhydrazine (6–8 mg./100 g. body weight) responded to the parenteral administration of vitamin B₁₂ by a regular increase in the erythrocytes. It has also been shown by Popper, Koch-Weser & Szento (1949) and Hove & Hardin (1951) that liver damage caused by phenylhydrazine can be prevented by vitamin B₁₂.

One of the important physiological properties of vitamin B₁₂ is its effect on the maintenance of reduced glutathione in liver as reported by Dubnoff (1950), and it has been shown by Nath and associates (Nath & Behki, 1958; Behki, Motlag & Nath, 1958) that 'glucose cycloacetoacetate', a crystalline compound formed by condensing glucose with acetoacetate, when hydrolysed with 2N-hydrochloric acid for 10–15 min. has a similar effect in sparing reduced glutathione in acetoacetate-induced diabetes as well as in scorbutic guinea pigs. Glucose cycloacetoacetate, on such hydrolysis, has been shown by Nath & Bhattathiry (1956*a, b*) to give rise to 1:2-dienol glucose. It has also been reported by Nath & Saikia (1958, 1959) that hydrolysed glucose cycloacetoacetate, like vitamin B₁₂, can prevent the rise of the ratio total cholesterol/lipid phosphorus in the blood and

tissues in experimental atherosclerosis, induced by diets either with a high content of saturated fat or of cholesterol. Therefore hydrolysed glucose cycloacetoacetate was studied to see if it could also behave like vitamin B₁₂ in the regeneration of erythrocytes and restoration of haemoglobin in experimental anaemia induced by phenylhydrazine.

MATERIALS AND METHODS

Male albino rats weighing 90–120 g. were selected and divided into three groups of six or seven each. All were fed on a normal stock diet consisting of wheat flour (75%), casein (10%), yeast powder (5%), Hawk & Oser (1947) salt mixture (5%), ground-nut oil (5%) and cod-liver oil (0.1%). Drinking water was given *ad lib*.

Before making the animals anaemic, the initial haemoglobin level and the erythrocyte count in all the animals were determined from the tail blood. Erythrocytes were counted by the usual method with the help of a Neubauer ruling counting chamber. Haemoglobin was estimated by the method of Wong (1928) with 0.1 ml. of blood, by converting it into Fe³⁺ ions by means of conc. H₂SO₄ and potassium persulphate and by using 3N-KSCN for colour development. The red colour formed was compared against that of the standard iron solution with a green filter in a Lange Universal Colorimeter. The haemoglobin value (in g./100 ml.) was obtained by multiplying the total blood iron (mg.) by the factor 1/3.4.

The experiment was performed for 3 weeks. All the animals were first injected with a single dose of 80 mg. of phenylhydrazine hydrochloride/kg. body wt. The solution of phenylhydrazine for injection was prepared as described by Chanutin *et al.* (1953): a weighed amount of recrystallized phenylhydrazine hydrochloride was dissolved in aq.

0.9% NaCl so that the desired dose/100 g. body wt. was present in 0.25 ml. This solution was adjusted to approx. pH 6.0 by dilute NaOH. The solution was injected into the animals subcutaneously.

Group 1 animals were kept as control. Group 2 animals were daily given an injection of 100 mg./100 g. body wt. of a solution of hydrolysed glucose cycloacetate (GCA) subcutaneously. The hydrolysis was done as follows: to 1 g. of crystalline GCA was added 5 ml. of 2N-HCl and the

mixture heated in a boiling-water bath for 20 min. It was then cooled and neutralized with NaOH to pH 7.0 and the whole extracted with three times its volume of ether. The aqueous layer was then collected, ether removed and the pH adjusted to 7.2 by adding a few drops of dil. NaOH soln. by the help of Merck's special indicator paper (pH 6.6-8.0). Group 3 animals were daily given an injection of 10 μ g. of vitamin B₁₂ (BE-DOUZE; Laboratories Grimault, Paris, France)/100 g. body wt. subcutaneously.

Fig. 1. Effect of daily injections of hydrolysed glucose cycloacetate and of vitamin B₁₂ on the regeneration of erythrocytes in experimental anaemic rats. Δ , Phenylhydrazine hydrochloride (control; six animals); $\circ$, phenylhydrazine hydrochloride and glucose cycloacetate (seven animals); $\bullet$, phenylhydrazine hydrochloride and vitamin B₁₂ (six animals). The time axes for the three sets of results have been displaced slightly in order to avoid confusion. The vertical lines represent standard deviations from the mean values plotted.

Fig. 2. Effect of daily injections of hydrolysed glucose cycloacetate and of vitamin B₁₂ on the haemoglobin levels in experimental anaemic rats. Details are as given for Fig. 1.

The erythrocyte count and haemoglobin estimation were done simultaneously, from the same animal, every alternate day for 21 days. The estimations were made from 8 to 12 noon, before giving them food.

RESULTS AND DISCUSSION

The average values and standard deviations are indicated in Figs. 1 and 2.

After the injection of a single dose of 80 mg. of phenylhydrazine hydrochloride/kg. body wt., a rapid fall in both erythrocyte count and haemoglobin level was noticed within 24 hr. The minimum erythrocyte counts occurred in all three groups at about the fifth day, as observed by Vijayaraghavan & Dunn (1950) and Chanutin *et al.* (1953), and the minimum haemoglobin level was observed at about the same time. Regeneration of erythrocytes and increase in haemoglobin level were observed thereafter in all three groups, the improvement in the experimental groups being slightly but significantly better than that in the control group.

There was a considerable scatter in the individual results in each group, but this was notably least marked in the group receiving vitamin B₁₂.

Some animals receiving 100 mg. of hydrolysed GCA/100 g. body wt. reached a normal erythrocyte count after about 11 days, but none of those receiving 10 µg. of vitamin B₁₂/100 g. body wt. returned to normal before about 15 days. All the control animals showed a slower rate of regeneration of erythrocytes and had cell counts below normal even on the twenty-first day.

No protective effect was found with vitamin B₁₂ initially, and the average haemoglobin levels in the group of animals receiving the vitamin were not significantly above those of the control group until the end of the first 2 weeks. Hydrolysed GCA, however, showed a protective effect in a number of cases, and the average haemoglobin levels in the group of animals receiving GCA were slightly but significantly above those in the control group throughout the experiment. There was no significant difference in the effects of hydrolysed GCA and vitamin B₁₂ on erythrocyte regeneration or on the haemoglobin level after the first 2 weeks. The experimental animals mostly had normal haemoglobin levels on the twenty-first day but the levels in the control animals remained below normal in spite of the increase noticed day by day. The cell counts appear to be reduced more strongly than the haemoglobin; this is presumably due to the use of the Wong method for estimating haemoglobin, which determines total Fe³⁺ ions.

The beneficial effect of hydrolysed GCA in preference to vitamin B₁₂ in the anaemic animals is evident from these results.

Further, it has been shown by Bertolani,

Bendandi, Berganini & Chierago (1954) that methionine given to partially hepatectomized rats caused an increase in the rate of hepatic regeneration. Hydrolysed GCA has been found by Nath & Saikia (1959) to help in the biosynthesis of methionine, which might be helpful in the regeneration of hepatic cells damaged by phenylhydrazine.

It was reported by Whipple, Robscheit-Robins & Hooper (1920) and Whipple & Robscheit-Robins (1925) that raw liver, which exerts a beneficial role in pernicious anaemia, contains a blood-forming substance. This is now known to be formed in the stomach from an extrinsic factor (vitamin B₁₂) and Castles' gastric intrinsic factor and is ultimately absorbed through the intestine and stored in the liver. Any substance therefore which can help in the regeneration of the damaged cells of the liver will necessarily help in the restoration of the haematopoietic property.

Further, the recent work of Cohen & Berg (1956) on the formation of haemoglobin from [2-¹⁴C]-glycine has indicated that the turnover of labelled haemoglobin is much more in the animals supplied with additional methionine in the diet. The haematopoietic activity of GCA may therefore be accounted for through its effect on the sparing and maintenance of GSH and its help in the biosynthesis of methionine, which might cause an increased rate of hepatic regeneration accompanied by increase in numbers of erythrocytes and turnover of haemoglobin.

SUMMARY

1. The effect of hydrolysed glucose cycloacetoacetate and vitamin B₁₂ on the experimental anaemia induced by phenylhydrazine has been studied.

2. Hydrolysed glucose cycloacetoacetate and vitamin B₁₂ (100 mg. and 10 µg./100 g. body wt. respectively) have been found to increase the erythrocyte count and haemoglobin level.

3. The mechanism of antianaemic activity of hydrolysed glucose cycloacetoacetate has been discussed.

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Formate Metabolism in the Vitamin B₁₂-Deficient Rat

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It was reported earlier (Fatterpaker, Marfatia & Sreenivasan, 1955; Lavate & Sreenivasan, 1956; Kasbekar, Lavate, Rege & Sreenivasan, 1959) that the protective action exerted by vitamin B₁₂ in experimentally induced hyperthyroidism in the rat is also observable in terms of a restoration of the uncoupling of oxidative phosphorylation caused by thyroxine. These studies were extended to ascertain whether the incorporation of formate into the purines of liver acid-soluble nucleotides is impaired in the thyrotoxic rat and whether vitamin B₁₂ protection is reflected in altered purine synthesis from administered formate.

METHODS

Animals. The basal diet employed was a modification of the corn-soya diet of Ericson, Harper, Williams & Elvehjem (1956) known to cause vitamin B₁₂ deficiency, and consisted of (percentages): corn meal 46.2, defatted pea-nut meal 46.2, salts (Ericson *et al.* 1956) 2, L-cystine 0.3, sesame oil 5.0, and vitamin mixture (Ericson *et al.* 1956) 0.3. Rats of either sex (Wistar strain) weighing 70–80 g. were divided into four groups and fed *ad lib.* on the basal diet either as such or with supplements of iodinated casein (Protomone: Cerophyll Laboratories, Kansas City, Mo., U.S.A.) or vitamin B₁₂ or both. Iodinated casein known to enhance vitamin B₁₂ deficiency (Lewis, Tappan, Register & Elvehjem, 1950) was added at 0.1 g./100 g. and vitamin B₁₂ at 20 µg./100 g. Feeding was continued for 7 weeks, during which period the animals on the basal diet with iodinated casein showed some 10% loss in weight when they were considered hyperthyroid (Du Toit, 1952).

Incorporation of formate into adenine and guanine of liver acid-soluble nucleotides. To animals from each group were administered intraperitoneally 0.5 ml. of a solution of

0.1M-sodium [¹⁴C]formate (2.25 × 10⁶ counts/min.). The animals were operated on under ether anaesthesia 2 hr. later and their livers perfused with cold aq. 0.09% NaCl, excised, weighed and extracted with 40 ml. of ice-cold 6% (w/v) trichloroacetic acid solution for 30 min. The protein precipitate was separated and washed by centrifuging in the cold at 5000 rev./min. The supernatant and washings were combined, neutralized and treated with 5 ml. of 20% (w/v) mercuric acetate solution to precipitate the acid-soluble nucleotides (Kerr, 1942). After separation and washing of the precipitate with 0.5% (w/v) mercuric acetate solution, the nucleotides were freed from Hg by H₂S and the excess of H₂S was removed by aeration. The nucleotides were then hydrolysed in N-H₂SO₄ for 1 hr. over a boiling-water bath. The liberated purines were precipitated at pH 1 with 1 ml. of M-AgNO₃ solution (Hayder, Chernigoy, Khatchadourian & Kerr, 1957) and extracted from their silver salts with hot N-HCl. The purine hydrochlorides were freeze-dried and dissolved in 0.2 ml. of N-HCl. The solutions were then chromatographed on Eaton-Dikeman (Mt. Holly Springs, Pa., U.S.A.) no. 613 paper strips, with 65% (v/v) propan-2-ol in 2N-HCl (Hayder *et al.* 1957) as the developing solvent. The separated purines were then identified as their mercury complexes made visible by ammonium sulphide (Vischer & Chargaff, 1948). Segments from the air-dried strips corresponding to the positions of the separated purines were directly eluted in 0.1N-HCl for adenine and N-HCl for guanine. They were estimated according to the procedure recommended by Vischer & Chargaff (1948) with a Beckman DUV spectrophotometer. Measured portions were evaporated to dryness on 25 mm. diameter Pyrex-glass planchets by heat lamps. Their activities were determined at infinite thinness in a helium-filled SC-16 Flow gas counter with an SC-51 Autoscaler (Tracerlab Inc., Waltham, Mass., U.S.A.) operating at 40–50% efficiency.

Folic acid and vitamin B₁₂. Other animals from each group were killed for determinations in liver of total folic