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GENESIS OF HEINZ BODIES AND METHEMOGLOBIN FORMATION

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Abstract—Phenylhydrazine hydrochloride, *p*-Aminopropiophenone and Methylene blue serve as models for the examination of the temporal connexions between the possible formation of methemoglobin and the appearance of Heinz bodies. It is proved that there is no correlation between the formation of ferrihemoglobin and precipitations in the erythrocytes. These findings and the application of the various substances and the appearance of the Heinz bodies lead to the conclusion, that different mechanisms of the metabolism are responsible for the appearance of the Heinz bodies. The appearance of ferrihemoglobin is not a prerequisite for the presence of precipitations in the erythrocytes but can be observed in certain cases contemporary with or prior to the formation of Heinz bodies.

IF MICE receive 1 per cent solutions of sulfanilamide as drinking water, they produce in the course of 3–4 days intracellular inclusion bodies in nearly all erythrocytes with a simultaneous rise in ferrihemiglobin (Moeschlin[1]). This phenomenon is also released by a series of other drugs and toxic acting substances. The inclusion bodies can be stained by vital dyes. Unstained they can be detected under the fluorescence microscope due to their green auto-fluorescence (Leonhartsberger and Pakesch;[2] Wittekind and Rentsch[3]). Since these structures in vital blood have been described by Riess[4] and also by Marchand[5] and were later more exactly examined and described by Heinz[6]—the reason they are now called Heinz Bodies (HB)—a series of authors have been of the opinion, that there might be a causal connexion between the oxidation of the hemoglobin iron and the appearance of HB. Although Heubner[7] and his school[8] proved as early as 1941 that this could not be the case and that both processes must be independent—findings which later authors could support (Beutler and Mikus;[9] Beutler and Baluda;[10] Finch;[11] Harley and Robin[12])—Jandl, Engle and Allen[13] in a recent publication on the theory of the HB held the view, that the pathophysiological background of the process, starting from new formed ferrihemoglobin, passes through the stages ferrihemoglobin → increase in electro negativity → formation of sulfhemoglobin—like hemochromes → frank denaturative precipitation as HB. This problem was also subject of a discussion between Sass-Kortsak, Thalme and Ernster[14] and Beutler.[15]

In connexion with our studies of the genesis of the Heinz bodies by vital dyes (Wittekind and Rentsch[16]), it seemed to be desirable to take up the problem again with appropriate model substances and under comparable conditions. For this purpose we first studied *in vivo* the temporal connexion between the formation of ferrihemoglobin and the appearance of Heinz bodies after equimolar doses of $NaNO_2$,

p-aminopropiophenone (PAPP), methylene blue (MB) and phenylhydrazine (PHH). Other papers will deal with enzymatic studies under *in vitro* conditions.

MATERIALS AND METHODS

The substances were applied to male stock Füllinsdorf albino mice from a closed randomized colony, weight 25–30 g by the i.p. route in concentrations of 2×10^{-4} mole/kg.

Solvents. PHH.HCl, $NaNO_2$ and MB are dissolved in Hanks solution, PAPP in a mixture of 0·5 ml of ethanol, 3 ml of propylene glycol and 6·5 ml of Hanks solution for 100 mg of substance. pH of the solutions for injection: 6·9.

Killing of the animals. After 3, 5, 10, 15, 20, 30, 60, 120, 150, 180, 240 and 300 min, as well as after 24 hr.

Determination of ferrihemoglobin. Under CO-protection (Heubner, Kiese, Stuhlmann and Schwartzkopf-Jung[17]), according to the cyanide method at λ550 nm.

Staining of the Heinz bodies. With nile blue sulfate in a wet chamber during 20 min.

$NaNO_2$, PHH.HCl and PAPP were available as analytically pure, MB was puriss for medical purposes FLUKA. Per unit of time at least 20 determinations were made. In the case of $NaNO_2$ an additional group of 10 animals received 2×10^{-4} mole/kg of $NaNO_2$ daily for 6 days by the i.p. route. These animals were bled every day from the tail vein to determine hemoglobin, ferrihemoglobin, Heinz bodies and reticulocytes.

RESULTS

Of all the substances examined, PAPP lead to the highest ferrihemoglobin level in mice. Equimolar doses of $NaNO_2$ did not produce such a high rate of ferrihemoglobin. On the other hand, PHH is a relatively weak ferrihemoglobin-forming agent in the living animal. Its maximum is at ~4 per cent ferrihemoglobin of the total amount of hemoglobin. An increase of ferrihemoglobin was never found *in vivo* after the application of MB (Fig. 1). The ferrihemoglobin maximum of PAPP and $NaNO_2$ occurs later than with PHH. The maximum after PHH is already reached within about 5 min. It seems worth noting, that in all cases examined ferrihemoglobin was reduced very rapidly and reached the normal level after about 5 hr. Our graphs of ferrihemoglobin formation after $NaNO_2$ correspond to the values measured by Arbanat and Smith,[18] which have later been confirmed by Kiese and Weger.[19] PAPP is identical with the course of the graphs indicated by Beutler and Mikus,[9] as well as by Arbanat and Smith.[18]

In addition to these divergences in the faculty of oxidation against Fe^{2+}, there are noteworthy peculiarities in the temporal appearance of the HB. The complete incapacity of $NaNO_2$ to produce HB under our conditions, and above all the intervals until the formation of the precipitations are striking (Fig. 2). There is most probably no relationship between the quantity of ferrihemoglobin formed and the way and moment of formation of the Heinz bodies. The maximum of HB formation follows the steep ferrihemoglobin rise of PAPP with a retardation of about 4 hr. In the case of application of MB, the formation of HB takes 24 hr and in this case no ferrihemoglobin could be previously proved *in vivo*. For PHH the unimportant ferrihemoglobin maximum and the point of culmination of the Heinz body formation synchronize more or less. They lie in the range of 5 min post injection. Animals which were

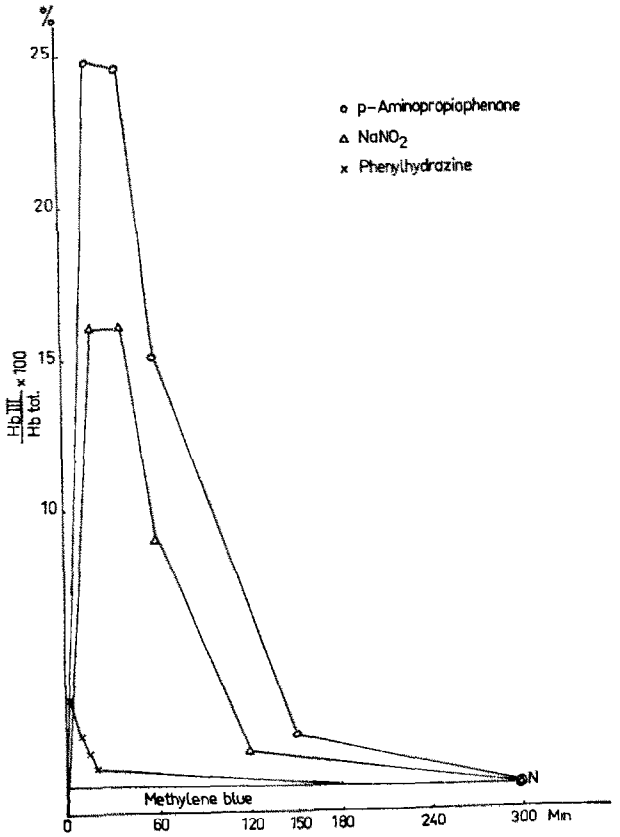

FIG. 1. Methemoglobin formation by equimolar doses of different drugs in mice.

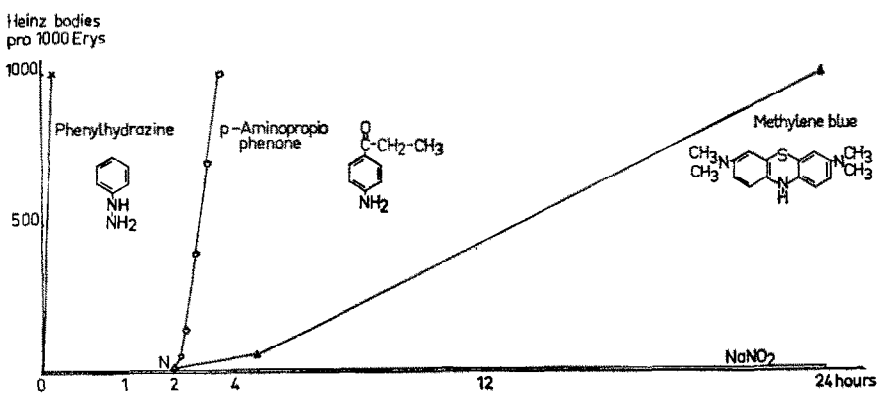

FIG. 2. Heinz body formation in the mouse in relation to time.

exposed over 6 days to an intermittent daily increase in ferrihemoglobin by $NaNO_2$ up to 16 per cent of the total amount of hemoglobin did not at any time show a formation of Heinz bodies exceeding the norm. The normal HB values of our animals were at 0·6 per cent. The only modification of blood we found was an increase of the reticulocytes from about 3 per cent at the beginning to about 18 per cent on the 6th day of the experiments.

The Heinz bodies had marked qualitative differences. PHH led to the formation of multiple, very small Heinz bodies, distributed irregularly over the cell. MB and PAPP provoked only solitary, in some cases very coarse polymorphous precipitations.

DISCUSSION

Until now, there has been little general agreement on the problem of the Heinz bodies (Fertman and Fertman[20]). It is considered as certain, that Heinz bodies consist of protein, probably of globulin.[21–30] But it is not yet clarified whether the electron-microscopically, rather uniform basal protein body of these precipitations represents the globin part of the hemoglobin or cell proteins (Jung;[28] Ninni;[29] Moser and Kreuzer;[31] Rozsa and Spicer;[32] Braunsteiner, Pakesch and Reimer;[33] Rifkind and Danon[34]).

In the theory of Jandl, Engle and Allen[13] the oxidation of the glutathione cysteine to the disulfide GS—SG plays a decisive role in the development of Heinz bodies. But Harley and Robin[12] could show, that, although there is ferrihemoglobin formation, no oxidation of glutathione can be observed in human erythrocytes with an active pentosephosphate pathway even in nitrite concentrations many times higher than the hemoglobin concentration. These findings will not support the oxidation theory of Heinz body genesis. If we admit that all noxae, which are capable of producing Heinz bodies, precipitate the same basal protein body, we must ask ourselves why phenylhydrazine causes multiple, and methylene blue and *p*-aminopropiophenone only solitary precipitations. The difference between the permeability of the cells does not seem to play a part—at least not for the substances PHH and PAPP examined by us. Besides, we have to find an explanation for the different time factor in the appearance of the Heinz bodies after the application of the different compounds, permitting at the same time a correlation with the equally different ferrihemoglobin level. If we take into consideration the chemically well-examined properties of PHH as regards reducing and oxidizing agents (Emil Fischer[35]), as well as the metabolic possibilities of the erythrocytes,[36–38] it seems most probable that there is an enzymatic degradation of all the substances we have examined to form the agent releasing the Heinz bodies. In certain cases, for example methylene blue, metabolic processes in the liver can also occur (Rentsch;[39] Rentsch and Wittekind;[40] Wittekind and Rentsch[16]). The different rates of degradation of the various substances would enable us to explain the differences in time in the appearance of the Heinz bodies. The formation of ferrihemoglobin would therefore be a function of metabolic intermediate phases and would occur additional to the formation of Heinz bodies. In the case of PHH one must primarily take into consideration the relation found by Warburg, Kubowitz and Christian.[23]

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