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# BIOCHEMICAL EFFECTS OF AROMATIC AMINES—I. METHAEMOGLOBINAEMIA, HAEMOLYSIS AND HEINZ-BODY FORMATION INDUCED BY 4,4′-DIAMINODIPHENYLSULPHONE

M. HJELM and C.-H. DE VERDIER

Departments of Clinical Chemistry and Dermatology,
University Hospital, Uppsala, Sweden.

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**Abstract**—This investigation was made to clarify the mechanism of the increased methaemoglobin formation in erythrocytes that follows the administration of 4,4′-diaminodiphenylsulphone.

The drug itself does not induce the formation of methaemoglobin, but the results of incubation of erythrocytes with plasma obtained during administration of the drug indicate that a metabolite is responsible for this complication. The metabolite activates the pentose-phosphate shunt of the erythrocytes.

A hypothesis is put forward according to which the aromatic amino compound is converted to a nitroso- or hydroxylamino-compound. A coupled oxidation involving the hydroxylamino compound and haemoglobin results in the formation of a nitroso-compound and methaemoglobin, respectively. The hydroxylamino-compound is regenerated by means of a pyridine-nucleotide-dependent enzyme. Rapid methaemoglobin formation after addition of the drug to a system of erythrocytes, microsomes, and $NADPH_2$ supports the theory.

SINCE the early 1940's 4,4′-diaminodiphenylsulphone (Avlosulfon®, Dapson®. DADPS®) has been one of the most effective drugs in the treatment of leprosy.[1, 2] During recent years the drug has also turned out to be effective in dermatitis herpetiformis.[3–5] This disease usually takes a very chronic course and cannot be cured. As long as the substance is administered, however, the patients are more or less free from symptoms.

The drug itself was first synthesized in 1908, but was not used in the treatment of bacterial infections until 1937.[6] It soon appeared that it was very toxic when administered in doses comparable to those of a great many sulphones[7–12]. It was shown, however, that the drug had a therapeutic effect also in smaller amounts, and it was thus possible to diminish but not completely eliminate the toxic side effects.

Many reports have appeared on the toxic nature of 4,4′-diaminodiphenylsulphone.[5, 6, 10–12] Most of them deal with haematological disturbances. Cyanosis is a common finding during treatment with the drug, and minor amounts of methaemoglobin and of Heinz bodies in the erythrocytes are often present. In severe cases of intoxication haemolytic anaemia has developed.[10–12]

A preliminary report of this investigation was given at the Cambridge International Meeting, July 12–13, 1962.

Clinically the haematological side-effects are probably of minor importance in leprosy, which in most cases is completely cured in about six to twelve months. The toxic nature of the drug seems to be more serious in patients with dermatitis herpetiformis, owing to the need for prolonged medication. It is therefore important to study the disturbances induced by the drug more closely. If the mechanism of these disturbances could be established, it might also be possible to eliminate them.

This investigation is devoted to a study of the methaemoglobinaemia, the Heinz body formation and the haemolysis induced by the drug in erythrocytes from normal individuals and patients with dermatitis herpetiformis. Some experiments were performed *in vitro* to establish the mechanism of the changes in the erythrocytes.

## MATERIALS AND METHODS

### *A. Clinical experiments*

The trials were carried out on healthy male adults and patients with dermatitis herpetiformis. 4,4′-diaminodiphenylsulphone (Avlosulfon®, Imperial Chemical Industries Ltd., Wilmslow, Cheshire, England) was given in 100 mg tablets. Methaemoglobin was determined by the method of Evelyn and Malloy, modified by Hjelm and de Verdier.[13] The concentration of total 4,4′-diaminodiphenyl sulphone in plasma was determined by the method of Bratton and Marshall.[15] For preparing standards the pure substance was used. This substance was kindly supplied by the manufacturer. Lactate dehydrogenase (LDH) in plasma was determined as described in ref. 16 and haptoglobin in serum as described by Jayle.[17] Heinz bodies and reticulocytes were determined by staining blood smears with brilliant cresyl blue. Red-cell survival was determined by a $Cr^{51}$-method described by Foconi and Sjölin.[18]

### *B. Experiments with intact erythrocytes in vitro*

Freshly-drawn venous blood from healthy blood donors (blood-group O) was used. The erythrocytes were spun down, and the plasma was discarded. The erythrocytes were incubated in solutions of various compositions.

(1) 0·5 ml of the packed erythrocytes (haematocrit 95–98%) was incubated in 5 ml of plasma collected before or during administration of 4,4′-diaminodiphenylsulphone to three healthy male students. For purposes of control the cells were incubated in plasma drawn before taking the drug, but with 4,4′-diaminodiphenylsulphone added to a final concentration of $0{\cdot}5 \times 10^{-5}$ M. The concentration of glucose in the plasma samples was determined by the method of Hjelm and de Verdier,[19] and the concentration in the plasma samples adjusted to 200 mg/100 ml with small volumes of M glucose solution. The incubation temperature was 37°. Samples were taken at intervals for determination of methaemoglobin.

(2) 0·5 ml of the packed erythrocytes were incubated with about 2 $\mu$C of 6-$^{14}$C-glucose (The Radiochemical Centre, Amersham, England). The final concentration of glucose in the plasma samples was adjusted to 200 mg/100 ml, and the samples were incubated at 37° for one hr. The incubation mixture was deproteinized with 0·8 ml of 3·2 M perchloric acid. 2 ml of the neutralized extract was applied to a Dowex 1 (chloride form) column and was eluted as described by de Verdier and Killander.[20] The collected fractions were analysed on aluminium planchettes for radioactivity. The packed

erythrocytes were washed three times with buffered saline of the following compositions: 0·15 M NaCl 361 ml, 0·15 M KCl 13 ml, 0·1 M $NaH_2PO_4$ 19 ml, 0·1 M $Na_2HPO_4$ 82 ml (pH 7·4).

(3) 1 ml of the packed erythrocytes was incubated with 1 $\mu$C of 1-$^{14}$C-glucose in 2 ml of plasma obtained from healthy male adults before or during treatment with 4,4′-diaminodiphenylsulphone. The final concentration of glucose in the incubation mixture was 180 mg/100 ml, and the pH of the plasma samples was adjusted to 7·2 with M $NaH_2PO_4$. The incubation was carried out at 37° for two hr in stoppered 25 ml Erlenmeyer flasks equipped with a centre well and was stopped by injecting 0·4 ml of 3·2 M perchloric acid. $^{14}CO_2$ was absorbed into 0·2 ml M hyamine hydroxide, which was injected into the centre well. To make certain that absorption was complete the flasks were shaken for one hr. The radioactivity was measured in an Automatic Liquid Scintillation System (Packard Instrument Comp., Inc., Illinois, U.S.A.).

(4) The erythrocytes were washed three times at 4° with buffered saline, pH 7·4. 5 ml of the packed erythrocytes (haematocrit 75%) were incubated in the following mixture:

TABLE 1.

| | |
|---|---|
| 4,4′-diaminodiphenylsulphone | 2·48 mg |
| or aniline-HCl | 1·29 mg |
| $MgCl_2$, 0·1 M | 0·6 ml |
| nicotinamide-HCl, 0·2 M | 0·6 ml |
| NADP, 0·01 M | 0·24 ml |
| glucose 6-phosphate, 0·1 M | 0·48 ml |
| glucose 6-phosphate dehydrogenase (C. F. Boehringer & Soehne, GmbH) | 1·4 U/10 $\mu$l |
| buffered saline, pH 7·4 | 1·07 ml |
| rat liver microsomes (prepared according to Kiese and Rauscher[22]) | 2·0 ml |

The incubation experiment was started with the addition of the microsomes. Samples were taken at intervals for determination of methaemoglobin. As control the same incubation mixture without 4,4′-diaminodiphenylsulphone or aniline-HCl was used.

*C. Experiments with Sephadex filtrate from haemolysates*

10 ml of erythrocytes from the healthy blood donors was mixed with 2 ml of 1% $NaNO_2$ solution. The mixture was allowed to stand in ice-water for 15 min. The cells were washed twice with buffered saline, pH 7·4, and haemolysed by rapid freezing-thawing. The hemolysate was filtered through a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column with 0·15 M Tris-Cl buffer, containing mM Mg-EDTA, pH 7·2. The red-coloured eluate containing haemoglobin and enzymes (the high-molecular-weight fraction, h.m.w.f.) was collected. The solution was diluted with the buffer to a haemoglobin concentration of 1·5 g/100 ml. 2 ml of the h.m.w.f. was suspended in 4 ml of the buffer. 0·4 ml of 0·1 M $MgCl_2$, 0·1 ml of $4 \times 10^{-5}$ M methylene blue, 0·1 ml of Tween 20 (to obtain a clear solution), and 0·1 ml of 0·01 M $NADH_2$ were added. The solution was divided into two equal parts and transferred to pairs of 1 cm silica cells for photometric registration. Nitrosobenzene in 0·05 ml of acetone in varying amounts was added to one cell and the same volume of the solvent to the other. The difference

in absorbancy at 635 mμ between the sample and reference was recorded in a Beckman DK2 spectrophotometer. In some experiments plasma obtained from healthy male students before and during administration of 4,4′-diaminodiphenylsulphone replaced the buffer and the nitrosobenzene. The plasma samples were buffered with small amounts of M $Na_2HPO_4$ to pH 7·2. The plasma sample obtained before ingestion of the drug was used as reference.

## RESULTS

### *A. Clinical investigations*

1. *Methaemoglobinaemia and Heinz-body formation after ingestion of* 4,4′-*diaminodiphenylsulphone.*

a. 400 mg of Avlosulfon® was given to each of two healthy male individuals over a a period of 48 hr. Figure 1 shows the concomitant increase in the concentration of methaehaemoglobin and of 4,4′-diaminodiphenylsulphone during loading with the

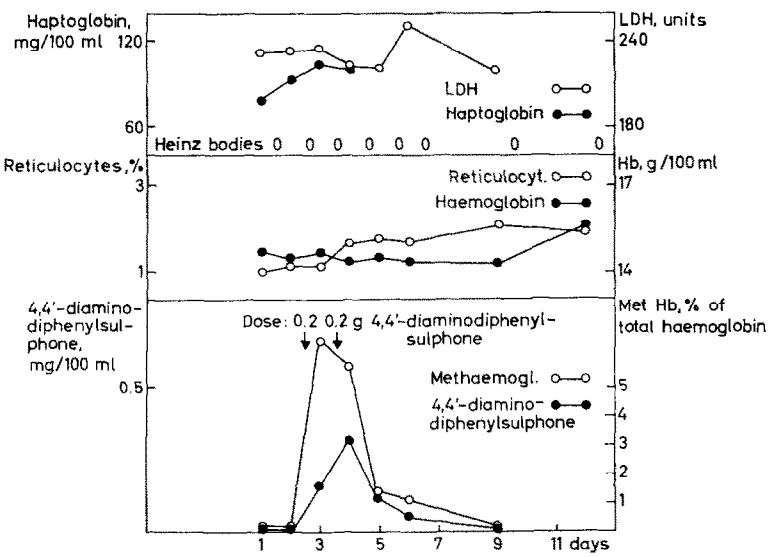



FIG. 1. Results obtained from a normal individual after treatment with 4,4′-diaminodiphenylsulphone for two days.

drug. There was a slight increase in the reticulocyte counts, but no significant changes in the haemoglobin or haptoglobin concentrations or in lactate dehydrogenase activity in serum. No Heinz bodies could be detected in these experiments.

b. A patient with dermatitis herpetiformis and receiving 200 mg of Avlosulfon® daily was followed up for three weeks with the parameters shown in Fig. 2. The same results were obtained as for the healthy individual in Fig. 1. In this case Heinz bodies were demonstrable in minor amounts. One interesting finding is the rapid drop in the methaemoglobin concentration between the 9th and 11th days, and the corresponding drop in the concentration of the drug. The patient did not take the drug on the 10th day while on leave at home.

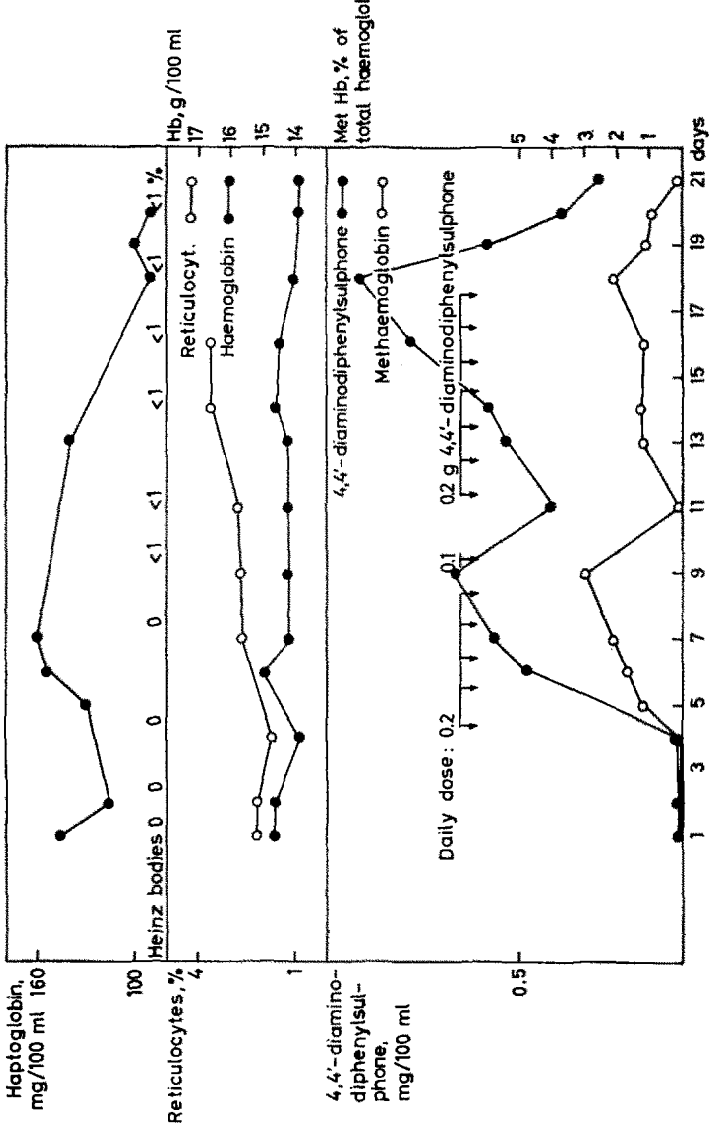



FIG. 2. Some parameters studied in a patient during treatment with 4,4'-diaminodiphenylsulphone for 14 days.

2. *Survival of erythrocytes labelled with $^{51}Cr$ during long-term administration of 4,4′-diaminodiphenylsulphone.* Erythrocytes from a patient with dermatitis herpetiformis receiving 0·2 g of 4,4′-diaminodiphenylsulphone per day were labelled with $^{51}Cr$. The $T_{1/2}$ for the injected radioactivity, 14 days, is considerably below the normal range for this method (23–32 days).

B. *Experiments in vitro*

1. *Incubation of erythrocytes with 4,4′-diaminodiphenylsulphone.* 0·5 ml of packed erythrocytes was incubated in 5 ml of buffered saline, pH 7·4, in 25 ml flasks to which 10 mg of 4,4′-diaminodiphenylsulphone was added. The mixture was incubated at 37° for 4–5 hr under gentle rocking. Samples were taken from the flasks immediately and at intervals of about 30 min for the determination of the methaemoglobin concentration. No significant increase could be detected during the incubation time.

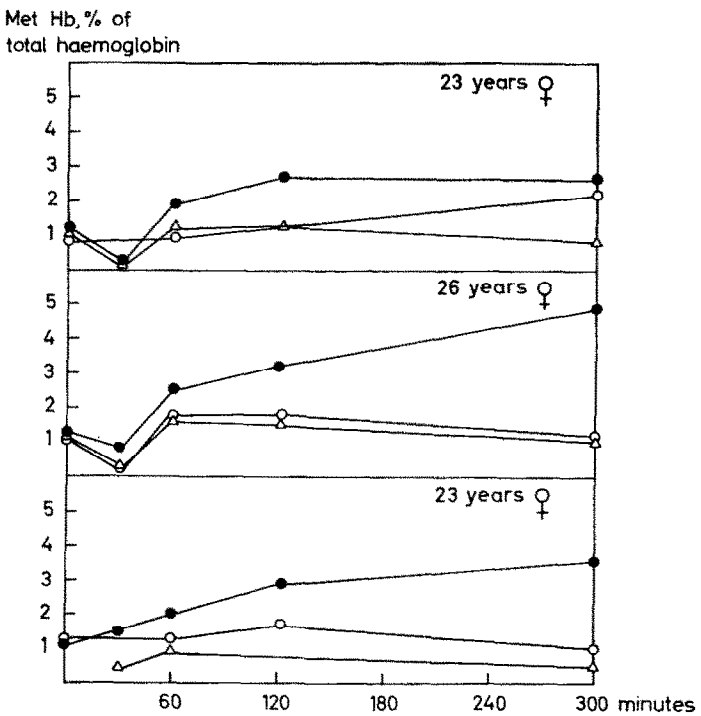



FIG. 3. Methaemoglobin formation in erythrocytes incubated in different plasma samples.

2. *Incubation of erythrocytes in plasma from three individuals treated with 4,4′-diaminodiphenylsulphone.* Erythrocytes were incubated as described in Methods. The result presented in Fig. 3 shows that methaemoglobin is generated only in erythrocytes incubated in plasma taken from the three adults during administration of the drug. This indicates that a metabolite of the drug is responsible for the methaemoglobin formation. The highest concentrations of methaemoglobin formed in the erythrocytes from the normal individuals were 2·7, 4·9, and 3·0% respectively.

3. *Determination of $^{14}CO_2$-production in erythrocytes incubated in plasma collected during treatment with the drug.* To make certain that the first step of the pentose phosphate shunt had not been inhibited (which would be one explanation for the methaemoglobinaemia), the production of $^{14}CO_2$ in erythrocytes was measured. The cells were incubated in plasma obtained from healthy male adults before or during administration of 4,4′-diaminodiphenylsulphone. The result is shown in Table 2. It is evident that the shunt was not inhibited; on the contrary, it seems to be stimulated.

TABLE 2. $^{14}CO_2$ PRODUCTION ON INCUBATING ERYTHROCYTES IN PLASMA OBTAINED BEFORE AND AFTER THE ADMINISTRATION OF 4,4′-DIAMINODIPHENYLSULPHONE

1 ml concentrated erythrocytes (blood group O) incubated for 2 hr in 2 ml heparinized plasma containing 1 μC of 1-$C^{14}$-glucose. Final glucose concentration 10 mM. pH of the plasma adjusted with M $NaH_2PO_4$ to pH 7·2 Plasma was obtained from three healthy male students (1–3).

| | 1 | 2 | 3 |
|---|---|---|---|
| Plasma before taking the drug $^{14}CO_2$, c.p.m. | 5525 | 5436 | 5587 |
| Plasma after taking the drug $^{14}CO_2$, c.p.m. | 6369 | 6390 | 7019 |
| Increase in $^{14}CO_2$ production, % | 15 | 18 | 26 |
| Concentration of 4,4′-diaminodiphenylsulphone in donor's plasma after taking the drug, mg/100 ml | 0·49 | 0·76 | 0·37 |
| Methaemoglobin in donor's erythrocytes after taking the drug, % of total Hb | 0·4 | 0·5 | 5·0 |

4. *Chromatographic separation of intermediates from 6-$^{14}C$-glucose in erythrocytes incubated in plasma obtained during treatment with 4,4′-diaminodiphenylsulphone.* The result of these incubation experiments is shown in Fig. 4. There is no conclusive evidence of serious disturbances in the breakdown of glucose.

5. *The reduction of methaemoglobin incubated in plasma obtained during administration of 4,4′-diaminodiphenylsulphone or in buffered saline containing nitrosobenzene.* It is shown in Fig. 5 that the reduction of methaemoglobin in haemolysates obtained by gel filtration (Sephadex G-25) and with nitrosobenzene added in different concentrations was slower than in the absence of this substance. The same effect was obtained if the haemolysate was incubated in plasma taken from a healthy male adult during administration of 4,4′-diaminodiphenylsulphone.
The concentration of the drug was $4 \times 10^{-5}$ M in the plasma, but the drug itself cannot have been responsible as in another experiment plasma taken before the administration of the drug and with 4,4′-diaminodiphenylsulphone added to the same concentration gave an almost negligible difference in reduction between the sample and the control. The difference in absorbancy change with plasma obtained during adminstration of the drug was $3 \times 10^{-3}$ per minute. This corresponds to the effect of about 0·4 M nitroso benzene.

6. *Incubation of the erythrocytes in a system with rat liver microsomes and 4,4′-diaminodiphenylsulphone or aniline.* It is evident from Fig. 6 that there was marked formation of methaemoglobin in erythrocytes incubated with 4,4′-diaminodiphenylsulphone and rat liver microsomes. It is also evident that this methaemoglobin formation

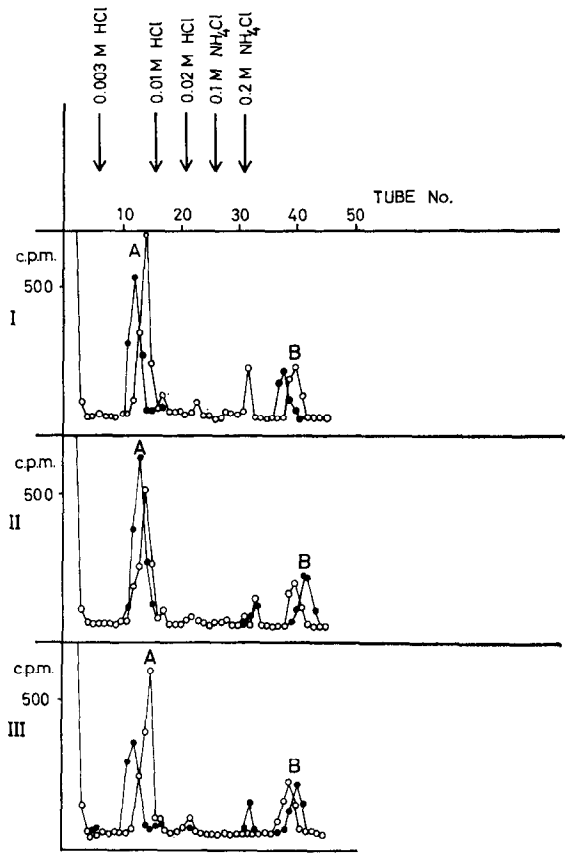



FIG. 4. Elution curves obtained by ion-exchange chromatography of PCA-extract of erythrocytes incubated in media containing 6-$C^{14}$-glucose. o, experiments in which the cells were suspended in plasma obtained before administration of 4,4′-diaminodiphenylsulphone, ●, experiments with plasma obtained after administration of the drug (A = lactate, B = 2,3-diPglycerate).

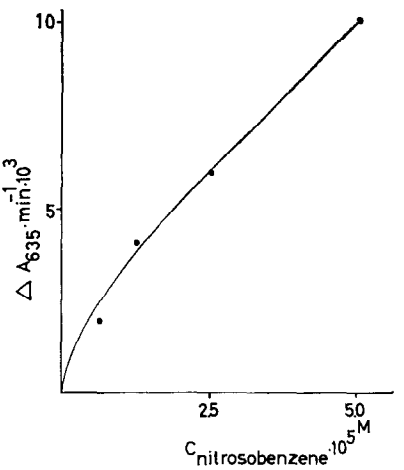



FIG. 5. Relation between nitrosobenzene concentration and fall in methaemoglobin reduction rate in a haemolysate system.

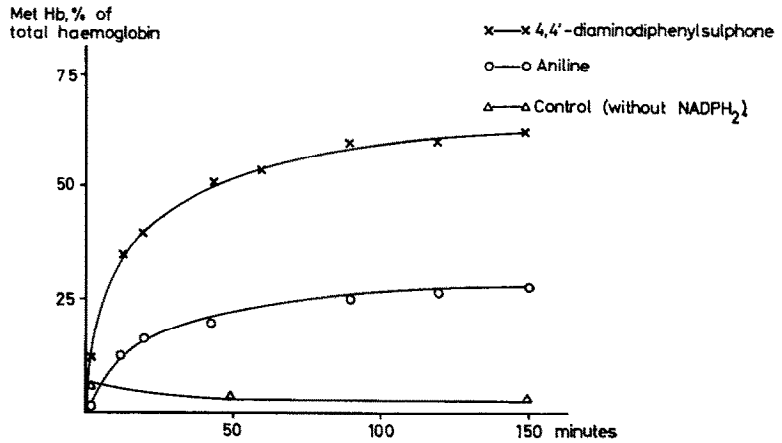


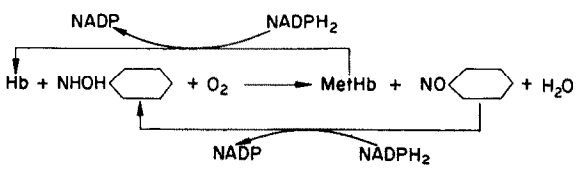

FIG. 6. Methaemoglobin formation in an *in vitro* system consisting of aromatic amine, rat liver microsomes, human erythrocytes, and a $NADPH_2$-regenerating system. In this experiment the aromatic amines, aniline, and 4,4'-diaminodiphenylsulphone were compared.

was dependent on the presence of $NADPH_2$. About twice as much methaemoglobin was formed with 4,4'-diaminodiphenylsulphone added as with aniline-HCl.

## DISCUSSION

The findings confirm the many reports on methaemoglobinaemia, Heinz-body formation, and haemolysis attributed by 4'4'-diaminodiphenylsulphone, and it also seems clear that these haematological side effects can be induced in healthy individuals as well as in patients with dermatitis herpetiformis or leprosy.

The mechanism of these disturbances is not known, but the investigation indicates that a metabolite of the drug is responsible for the haematological findings. Kiese *et al.*[21, 22] have made detailed studies of the methaemoglobinaemia induced by aromatic amines *in vivo* in dogs, rats, and other animals. They have shown that $NADPH_2$-dependent enzymes present in microsomes from the liver and some other tissues of these animals are capable of oxidizing the amino group of aniline and substituted aniline derivatives. The amino group is converted to a nitroso group, and the nitroso compound can permeate the intact erythrocytes. In the cells the nitroso group is first reduced to a hydroxyl-amino group with $NADPH_2$ generated in the pentose phosphate shunt. In a "coupled oxidation" the phenylhydroxylamino-metabolite and haemoglobin are oxidized to a nitroso-metabolite and methaemoglobin in accordance with the following scheme,




As long as the corpuscular pentose phosphate shunt is intact, the nitroso-metabolite generated in the liver has a catalytic effect, and only small amounts of the metabolite are necessary for converting considerable amounts of haemoglobin to methaemoglobin. The methaemoglobin formed is continuously reduced with the aid of $NADH_2$

and $NADPH_2$ generated by the breakdown of glucose. The net effect will be a change in the quotient haemoglobin/methaemoglobin.

The results of the incubation experiments with plasma obtained during administration of the drug and with microsomes and 4,4′-diaminodiphenylsulphone indicate a similar mechanism for the methaemoglobinaemia induced by 4,4′-diaminodiphenylsulphone. The effect of plasma obtained during administration of the drug on the production of $^{14}CO_2$ and on the reduction of methaemoglobin in a gel-filtered haemolysate also supports this view.

No metabolic changes other than methaemoglobin formation in the erythrocytes are known at present. Preliminary investigations, however, suggest that there are other changes which directly or indirectly may be responsible for the haemolysis caused by the drug.[14] These changes may be induced by the nitroso-metabolite and/or 4,4′-diaminodiphenylsulphone, since it has been reported that several sulphones and aromatic substances are inhibitors of erythrocytic enzymes.[23, 24] The relation between these isolated observations *in vitro* and the haemolysis caused by 4,4′-diaminodiphenylsulphone *in vivo* is obscure. It is also important to take into account the permeability of the drug and the age distribution of the red cells, the older cells probably being more susceptible to haemolysis.[11]

The precise nature of the methaemoglobin inducing metabolite is not known at present. Though a nitroso-derivative seems to be most likely there are theoretically also other possibilities, e.g. a phenolic configuration of the metabolite.[25] Further investigations may clarify this problem.

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