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THE METABOLISM OF DESFERRIOXAMINE B AND FERRIOXAMINE B

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Abstract—In incubation experiments desferrioxamine B was metabolized by plasma and slices of pancreas, small intestine, brain, liver, muscle, and spleen from laboratory animals, some large animals, and man. Plasma exerted the most marked metabolizing effect, as could be seen from the fact that DFO lost its ability to bind iron. Neither plasma nor liver slices caused any change in ferrioxamine B. The DFO-metabolizing enzyme in plasma exerts its maximum activity at pH 7·3, its Michaelis constant K_M is 2·5 mM. and it is located in the α_2-globulin fraction of the serum proteins. Plasma retains its metabolizing ability following lyophilization, but loses it linearly within 2 yr when stored in a refrigerator. Dogs given prolonged treatment with subcutaneous doses of DFO displayed only a slight degree of induction of DFO-metabolizing enzyme.

AMONG the sideramines—brownish-red metabolites of Actinomyces which act as bacterial growth factors and contain complexed iron[1]—ferrioxamine B (FO) is of practical interest because the iron-free compound, desferrioxamine B (DFO) can be used as an iron-complexing agent in the treatment of iron storage diseases in man.

Studies on the distribution of DFO in nephrectomized dogs revealed that this substance was metabolized.[2] This finding prompted a more detailed investigation of the metabolic fate of DFO. The present paper describes experiments conducted with a view to studying the metabolic activity of blood plasma and animal tissues on DFO, as well as the properties of the enzyme responsible.

MATERIALS AND METHODS

Preparation of animal tissue and plasma

To obtain tissue slices for incubation experiments, the animals used were bled to death. Their organs were quickly removed, washed in ice-cold Krebs–Ringer bicarbonate solution,[3] and sliced in the cold with a Stadie–Riggs microtome.[4] The small intestine and pancreas were cut up into small pieces with scissors. All tissue slices were weighed moist, after first being dabbed with absorbent paper. For blood sampling purposes, the smaller animals were anesthetized with ether, while the larger animals were not given any anaesthetic. Venous blood was removed from the animals with heparinized syringes and centrifuged immediately so as to provide plasma. Liquemin for intravenous administration, which does not contain any phenol or cresol, was used in 1:5 dilution as heparin solution.

Incubation of DFO and FO with tissues and plasma

DFO was used in the form of the methane sulphonate. For assay purposes it was converted with trivalent iron into the brownish-red complex FO and measured in a spectrophotometer. That proportion of the DFO employed which could no longer be

determined as an iron complex following incubation was considered to have been metabolized.

The experimental conditions for the incubations with DFO are indicated in Tables 1 and 4. The same conditions were used in the FO experiments. Data on the experiments conducted with inactivated tissue are shown in Table 3.

Determination of DFO in tissue-slice and plasma incubation solutions

One millilitre of the incubation solution was brought to pH 2 with 0·1 N HCl in a centrifuging tube, mixed with 0·15 ml of a solution of 600 mg ferric chloride in 100 ml water, allowed to stand for 5 min to let the iron complex form, neutralized with 2 N NaOH, and made up with distilled water to a standard volume of 5·0 ml. After 1·5 g of solid sodium chloride had been added, the solution was vigorously shaken with 5·0 ml benzyl alcohol and centrifuged to separate the phases. Of the benzyl alcohol extract 4 ml was made up to 5·0 ml by adding 96% ethanol in a measuring flask, whereupon the extinction was read off at 430 mμ against a blank reagent sample in a spectrophotometer.

The calibration curve plotted displayed a linear pattern. The smallest amount that could be determined with certainty was 0·005 mg DFO per ml; the standard deviation from the mean was ±10% for 10 determinations. At a concentration of 0·25 mg DFO per ml the standard deviation from the mean was 0·3% for 5 determinations.

In the case of the plasma incubations, the 1 ml sample was deproteinized with 1 ml of 20% trichloroacetic acid. The ferric chloride solution was added to 1 ml of the centrifugation supernatant and the subsequent procedure was the same as described above.

Determination of FO in tissue-slice and plasma incubations

FO was determined in the same way as DFO, except that the addition of ferric chloride was omitted.

Serum protein fractionation by means of continuous paper electrophoresis

Of a rat serum which had been dialysed for 40 hr against a Veronal buffer at pH 8·4, 5 ml was lightly coloured with bromphenol blue and then subjected to continuous paper electrophoresis for 5 hr at 1200 V and 250 mA in an "Elphor VaP" electrophoresis apparatus (Dr. Bender and Dr. Hobein, Zurich/Munich/Karlsruhe). Veronal buffer, (0·025 M, pH 8·4) was employed as a solvent, and the injection rate was 1 ml serum/hr.

Identification of the serum proteins by means of paper electrophoresis

Following dialysis and lyophilization, the samples of the serum protein fractionation were dissolved in 1/7 M Veronal-sodium acetate buffer of pH 8·6 and subjected to electrophoresis for 18 hr at 200 V, and with a distance of 28 cm between electrodes on Schleicher Schüll paper No. 2043 impregnated with the same buffer. After drying, the paper was coloured for 10 min in a bath of 1 g amido black 10 B in 200 ml methanol–glacial acetic acid (9:1) and washed in methanol–glacial acetic acid.

RESULTS

Metabolization of desferrioxamine B by plasma and tissue

The tissues in which DFO is metabolized were ascertained in incubation experiments with blood plasma and organ slices from various species of animal and with blood plasma from man. Table 1 shows that in all species DFO was metabolized to

TABLE 1. METABOLIZATION OF DFO BY PLASMA AND ORGAN SLICES IN PERCENTAGE OF DOSE

Volume or weight	Plasma or organ slices	Species								
		Rat	Rabbit	Mouse	Guinea-pig	Cat	Dog	Bovine	Pig	Man
0·5 ml	Plasma	56 44–64 (6)	40 31–49 (2)	60 — (2)	29·5 24–35 (2)	48 37–59 (2)	23 10–34 (4)	38 36–40 (3)	42 39–46 (3)	14 10–18 (2)
500 mg	Liver	11·6 10–13 (6)	12 8–16 (2)	18·6 16–23 (3)	13·3 9–18 (4)	8·8 — (2)	4·8 3– 7 (4)	15 14–16 (2)	10·5 5–16 (2)	
500 mg	Pancreas	14·5 7–31 (9)	16 10–22 (2)	18 17–19 (2)	23·7 20–27 (4)	25 24–26 (2)	17 8–33 (3)			
500 mg	Small intestine	19 16–23 (5)	14·5 3–26 (2)	16·5 16–17 (2)	22 20–25 (4)	20·7 12–30 (3)	13·4 6–34 (4)			
500 mg	Brain	11·4 4–19 (6)	12·5 11–14 (2)	16·5 15–18 (2)	13·6 12–14 (4)	14·5 13–16 (2)	11 — (1)			
500 mg	Muscle	4·4 2– 7 (4)	11·5 6–17 (2)	3·5 2– 5 (2)	15 8–22 (2)	20 — (2)	9 2–16 (2)			
500 mg	Kidney	1 0– 2 (6)	0 0– 1 (2)	1 0– 2 (2)	0 0– 1 (3)	0 0– 1 (2)	0 — (2)			
500 mg	Spleen	3 — (2)	3 — (2)	8 7– 9 (2)	3 — (2)	6 3–10 (3)	3 — (1)			
500 mg	Lung	1 0– 2 (2)	1 1– 2 (2)	1 1– 2 (2)	2 — (2)	1 0– 2 (2)	1 — (1)			

Experimental conditions: 2·5 mg DFO + 10·0 ml Krebs–Ringer bicarbonate solution + 500 mg organ slices,
or: 2·5 mg DFO + 9·5 ml Krebs–Ringer bicarbonate solution + 0·5 ml plasma.
Shaker thermostat, 37°, gassing with a mixture of 95% O_2 and 5% CO_2, duration of experiment 3 hr.
The table indicates the mean values, range, and, in brackets, the number of animals used (pooled groups in the cases of mice).

the greatest extent by blood plasma. A considerable metabolizing effect was exerted by pancreas, small intestine, brain, liver, and muscle—the order varying according to the species involved. The spleen also produced a slight effect, but kidney and lung, and, as additional tests showed, fatty tissue as well, did not give rise to any changes in the DFO. The differences between the various species were particularly marked in the metabolic activity of the plasma. Plasma from mice acted most rapidly, while plasma from rats, cats, pigs, rabbits, bovines, and guinea-pigs—in that order—was slower to take effect. A striking feature was the low activity of human or dog plasma.

Examination of whole blood from man and from rats revealed that metabolizing ability stemmed solely from the plasma (Table 2). Intact or haemolysed erythrocytes were inactive.

TABLE 2. METABOLIZATION OF DFO BY BLOOD CONSTITUENTS IN PERCENTAGE OF DOSE

1 ml blood or constituents from 1 ml blood	Rat	Man
Whole blood	46·5 (4)	—
Plasma	45·1 (4)	—
Erythrocytes	0 (3)	0 (1)

Experimental data as in Table 1.

As Table 1 shows, the DFO-metabolizing principle is widespread in the organism; remarkably enough, it also occurs in the brain. The incubation experiments with inactivated tissue slices and plasma show that the decrease in the DFO concentration in the incubation solution cannot be due to adsorption of DFO on to the tissue slices or plasma proteins (Table 3).

TABLE 3. METABOLIZATION OF DFO BY NATURAL AND INACTIVATED PLASMA AND ORGAN SLICES IN PERCENTAGE OF DOSE (RAT)

Plasma and organ slices were inactivated by heating them to 60°C for 60 min in Krebs–Ringer solution. Other data as in Table 1.

Volume or weight	Plasma or organ slices	Natural material	Inactivated material
2 ml	Plasma	84·2 (24)	2·6 (4)
500 mg	Pancreas	14·5 (9)	3·5 (2)
500 mg	Small intestine	18·9 (5)	1·3 (2)
500 mg	Liver	11·6 (6)	1·3 (4)
500 mg	Brain	11·4 (6)	1·3 (2)

Metabolization of ferrioxamine B by plasma and tissue

Incubation experiments with rat plasma and rat liver slices showed that no metabolic changes in FO were detectable, not even when incubation with plasma was continued for 22 hr. Hence, it is safe to assume that FO is not metabolized in the animal organism; this confirms previous findings.[2]

Properties of the DFO-metabolizing enzyme

The DFO-metabolizing principle is not dialysable. It must be an enzyme because incubation with plasma or organ slices at 0°, or incubation at 37° with plasma or organ slices inactivated by heating for 1 hr to 60°, failed to cause any metabolization of DFO.

In order to characterize the DFO-metabolizing enzyme, rat plasma was selected as an enzyme source. Following incubation in a tris-maleate buffer (pH 7·4; 37°) the metabolic rate of DFO decreased slowly with time. After 180 min, 83 per cent of the DFO employed had been metabolized (Fig. 1). Similar patterns were obtained following incubation in other buffer solutions at pH 7·4, such as phosphate buffer (Sörensen), Veronal-acetate buffer, and Krebs–Ringer bicarbonate solution (gassing with 95% O_2 and 5% CO_2).

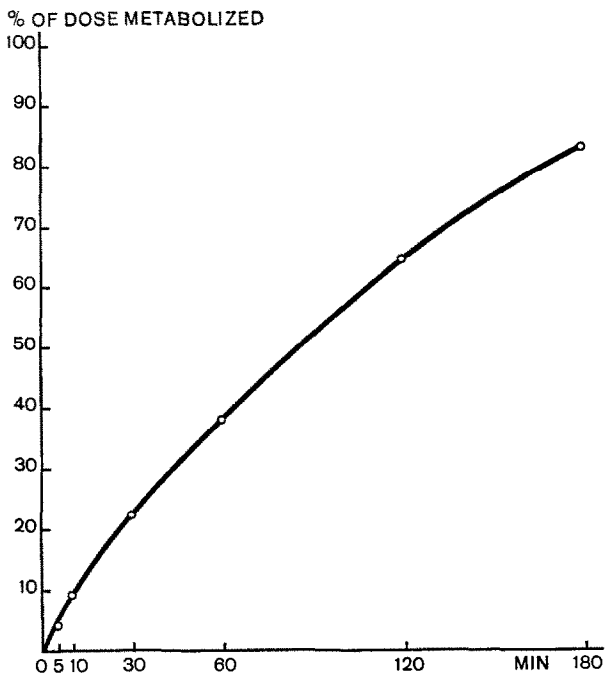


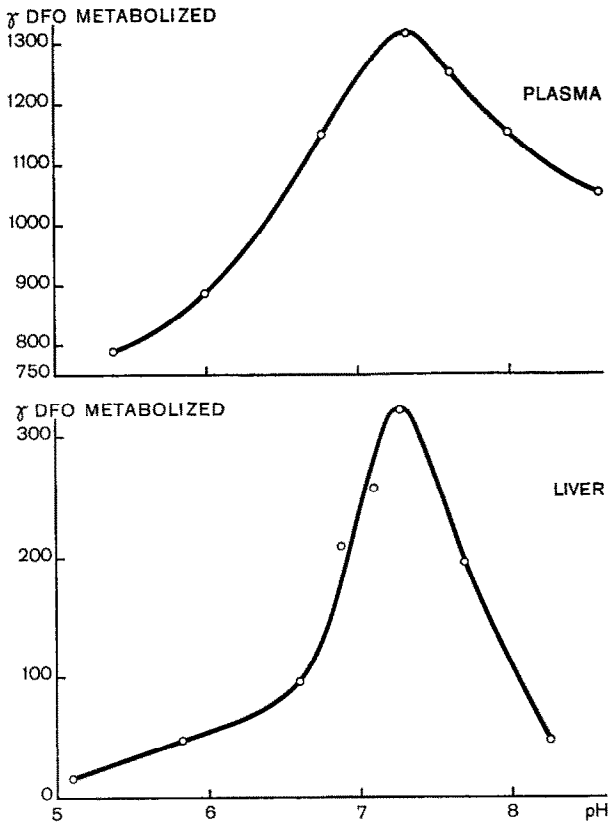
FIG. 1. Chronological pattern of the metabolization of DFO by plasma. 2 ml rat plasma + 8 ml tris-maleate buffer (pH 7·4) + 2·5 mg DFO, 37°.

The pH dependence of the enzymatic activity was ascertained by incubating rat plasma and rat liver slices at various pH values in tris-maleate buffer. Maximum activity was attained at pH 7·3 (Figs. 2 and 3). Maximum activity was also found to occur at the same pH in rat brain and small intestine slices.

Incubations with various amounts of rat plasma and a constant concentration of DFO produced a linear pattern of metabolism (Fig. 4).

With the aid of Lineweaver-Burk plots, the Michaelis constant for the DFO-metabolizing enzyme was ascertained by incubating increasing concentrations of DFO, ranging from 0·152 to 1·22 mM/l, with an unvarying amount of 2 ml rat plasma at pH 7·4 (Fig. 5). The Michaelis constant K_M was found to be 2·5 mM.

To discover which serum protein fraction the enzyme belongs to, rat serum was subjected to continuous paper electrophoresis. The protein content of the twenty-seven fractions obtained was determined by Lowry's method.[5] The results are illustrated in Fig. 6. The individual fractions were tested for their ability to metabolize DFO. Only fractions 16 and 17 were found to be active. Following dialysis and lyophilization, fractions 15–18 were subjected individually to paper electrophoresis and



FIGS. 2 and 3. pH dependence of the activity of the DFO-metabolizing enzyme.
2 ml plasma (rat) + 8 ml tris-maleate buffer + 2·5 mg DFO, 37°, 60 min.
500 mg liver slices (rat) + 10 ml tris-maleate buffer + 2·5 mg DFO, 37°, 180 min.

compared with whole rat serum in order to identify the proteins. As Fig. 7 shows, the enzymatically active fractions 16 and 17 have only the α_2-globulin in common, which means that the enzyme is located in the α_2-globulin fraction.

Dried plasma as an enzyme preparation

Whereas lyophilization does not affect the metabolizing ability of fresh plasma, both liquid and dried plasma loses its activity upon storage. Dried human plasma from a commercial source (reconstituted 14 months after its preparation) did not metabolize

DFO at all. A liquid standard human serum (ampoules, Schweizerhall AG) metabolized 12 per cent of the DFO used. The ability of lyophilized plasma preparations to metabolize DFO is reduced even when they are stored in a refrigerator at —2°. After being kept for 1 yr under these conditions, a preparation of this type was found to possess only half of its original activity. After 2 yr, no activity at all could be detected.

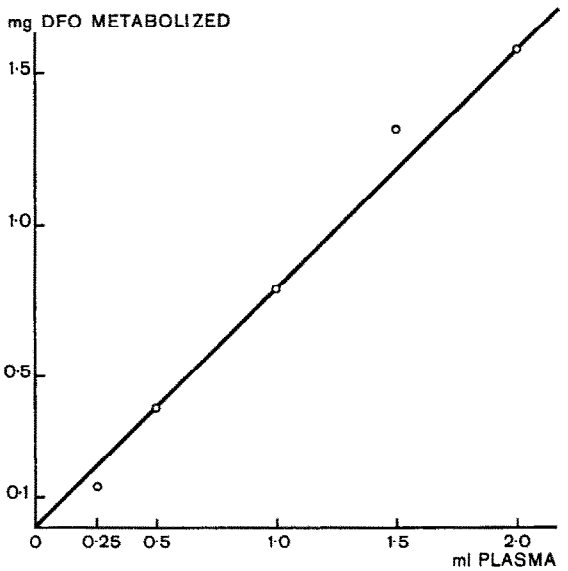


FIG. 4. Dependence of enzymatic activity on amount of enzyme present. 12·5 mg DFO + plasma (rat) + Krebs–Ringer bicarbonate solution made up to 10·0 ml, 37°, gassing with 95% O_2 and 5% CO_2, 60 min.

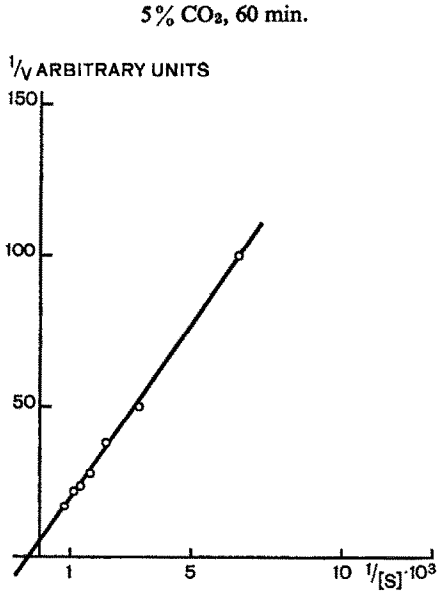


FIG. 5. Determination of the Michaelis constant in rat plasma. Lineweaver–Burk plot. Metabolization of DFO (0·152–1·22 mM/l.) within 10 min.

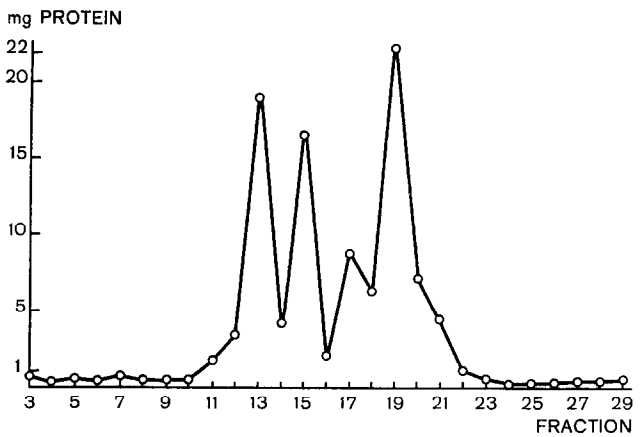


FIG. 6. Continuous paper electrophoresis of rat serum. Separation of the serum proteins (only fractions 16 and 17 displayed ability to metabolize DFO.)

Enzyme induction

The question whether prolonged administration of DFO induces formation of the metabolizing enzyme or enzymes was examined in dogs. Six dogs were given high daily doses of DFO s.c., two of them receiving 150–200 mg/kg for 50 days and the other four 150 mg/kg for 27 days. Six other dogs were left untreated and served as

TABLE 4. DFO-METABOLIZING ABILITY OF PLASMA FROM PRETREATED AND NON-PRETREATED DOGS

2 ml dog plasma + 8 ml Krebs–Ringer bicarbonate solution + 2·5 mg DFO; 37·2°C, 180 min, gassing with 95% O_2 and 5% CO_2.

	Metabolization	
	Pretreated dogs (n = 6) γ	Control dogs (n = 6) γ
Individual values	362	345
	345	214
	395	395
	460	198
	395	345
	428	198
Total	2,385	1,695
$\bar{X} + S\bar{x}$	397 ± 17	282 ± 36

The difference between the mean values is statistically significant (P = 0·01).

controls. The metabolizing ability of the plasma was tested at the end of the period of treatment (Table 4).

There was no difference between the dogs pretreated for 50 days and those pretreated for 27 days. As the metabolizing ability of the plasma from pretreated dogs

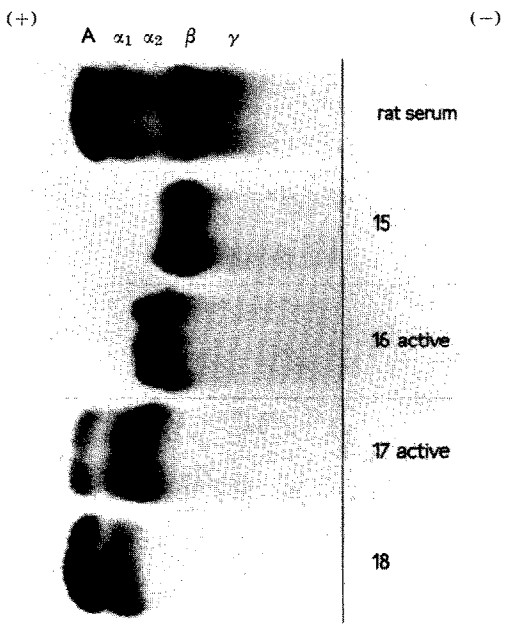


FIG. 7. Identification of the separated rat serum proteins (cf. Fig. 6) of fractions 15–18 by means of electrophoresis. Paper coloured with amido black 10B (fractions 16 and 17 have only α_2-globulin in common).

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differs relatively little from that found in the controls, it may be assumed that, in dogs, only a slight induction of plasma enzyme occurs.

DISCUSSION

Incubation experiments with organ slices and plasma have shown that DFO is metabolised by an enzyme which occurs widely in the organisms of man and various animals and which is localized mainly in the plasma. Some properties of the metabolizing enzyme in rat plasma have been established, but no indication has yet been found as to the type of the biochemical reactions catalysed by this enzyme.

It is still not known whether one or several enzymes are involved in the metabolism of DFO, whether the same biotransformations occur in all species, and whether the metabolic processes are identical in all organs. Experiments with tritiated DFO are now in progress and should help to shed light on these points.

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