both free and conjugated nomifensine concentrations are required, plasma samples must be deep frozen immediately after collection to prevent hydrolysis. Failure to observe these rigorous sample handling conditions will render any plasma drug concentration data invalid.

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Molecules related to Tris and some derivatives that protect haemoglobin during freeze-drying

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Freeze-drying is a classic stabilizing process for preserving fragile compounds. However, it cannot be used with haemoglobin without precautions, because during the desiccation a large amount of the ferrous iron of the heme oxidizes, forming methaemoglobin. Drying becomes possible if one or several adjuvant substances are added to the solution.

We have demonstrated a protection of haemoglobin by Tris [tris (hydroxymethyl) aminomethane, or 'THAM'] (Labrude et al 1976). This effect led us to seek other 'active' agents among related compounds. Seven molecules related to Tris were tested in 1976. We have now enlarged that study in order to demonstrate the chemical groups that are responsible for the protection Tris gives, in the hope of finding the most simple active molecule and also of understanding the mechanism of the protection which is, at now, unknown.

Materials and methods. Haemoglobin solutions, prepared as for transfusion, were obtained by haemolysis of washed red blood cells, two centrifugations and decantations to eliminate the stromata, dialysis against distilled water, adjustment of the haemoglobin concentration to 75 $\pm$ 3 g litre^{-1}, and storage at +4 °C for no more than 3 to 4 days. The 27 compounds studied are shown in Table 1, classified according to their similarity to Tris. The following modifications to this amine buffer were studied: replacement or removal of the alcohol functions (compounds 2 to 6); substitution (compounds 7 to 11), removal (compounds 12 to 14), or replacement (compound 15) of the amine function; or modification of both alcohol and amine functions (compounds 16 to 18). Finally, compounds not closely allied to Tris were studied as checks on the hypothesis about the specificity of action: compounds without an amine function (compounds 19 to 22), with a preponderant amine function (compounds 23 to 25), or containing heteroatoms (compounds 26 to 28).

Our nomenclature for these molecules does not conform to the usual norms because we have chosen to consider them all as derived from 1,3-propanediol: thus Tris is 2-amino-2-hydroxymethylpropane-1,3-diol. Among these compounds, several are used as amine buffers in biology: 2-amino-2-methylpropane-1,3-diol (AMPD), 2-amino-2-methylpropanol, mono-Tris, bis-Tris, Tricine, bis-Tris-propane, bis-AMP, Bicine, and triethanolamine.

The compounds were made up in 0·3 M stock solutions. We had already noticed in the previous study (Labrude et al 1976) that Tris is more effective in these conditions than at higher concentrations. Five ml of haemoglobin solution was mixed with 5 ml of an aqueous solution of the molecule being studied, oxygenated by bubbling with oxygen, and frozen at −40 °C under oxygen. A control was performed by adding 5 ml of demineralized water to the haemoglobin solution. The results are the means of 30 measurements for the control, 9 for the Tris, 3 for each of the other compounds studied.

Before each solution was frozen, its oxyhaemoglobin saturation and its pH were measured (with a Radiometer Hemoximeter OSM2 and a Beckman Phasar II pH meter, respectively), See Table 1.

The samples were freeze-dried in a Chaix-Meca apparatus (Nancy, France) in the following conditions: freezing to −40 °C; primary desiccation for 16 h up to −10 °C; secondary desiccation for 8 h up to +5 °C. Then the apparatus was opened to the atmosphere. The flasks, immediately stoppered, were kept under air at 4 °C for the several hours that preceded the analyses.

The freeze-dried materials were redissolved in 10 ml of demineralized water at room temperature (20 °C), and then for each sample the amount of methaemoglobin (method of Evelyn & Malloy 1938), the oxyhaemoglobin saturation, the pH, the appearance of the freeze-dried material and of the solution, and the time required for redissolution were noted.

Results (Table 1). Since Tris was taken as the model, the activities of the other compounds are reported relative to its activity. Three classes can be distinguished:

* Correspondence.

Very effective molecules. Four amines were strongly protective-mono-Tris (7), bis-Tris (8), bis-Tris-propane (10) (which are all richer in hydroxymethyl groups than is Tris, by 4, 5, and 6 groups, respectively), and bis-AMP (11). The levels of methaemoglobin were lower than with Tris, while the oxyhaemoglobin saturation was almost 100% of that before freeze-drying. Bis-Tris-propane was the best product, followed by mono-Tris, then bis-Tris, then bis-AMP. The pH values of the preparations were unremarkable, being found practically unchanged after regeneration of the freeze-dried materials, which was rapid and total, giving solutions with a good appearance.

Moderately effective molecules. Few compounds fell into this category: 2-amino-2-methylpropane-1,3-diol (2); its slightly less effective ethyl homologue 2-amino-2-ethyl-propane-1,3-diol (3); 1-amino-2-propane-1,3-diol, or serinol (4); and trihydroxymethylphosphine oxide (28). These four molecules gave results less satisfactory than Tris in the amount of methaemoglobin produced and in the oxyhaemoglobin saturation.

Ineffective compounds. All the other compounds, whether or not structurally close to Tris, were ineffective. The extent of oxidation to methaemoglobin was even higher than in the control haemoglobin samples. With some of these compounds, the totals of methaemoglobin plus oxyhaemoglobin accounted for less than 100% of the initial amount of haemoglobin, suggesting that the denaturation of haemoglobin also formed other products than those we measured. The strongly alkaline solutions (pH >10) gave bad results, probably because of immediate denaturation of the haemoglobin on con-

Table 1. The compounds compared with Tris in haemoglobin lyophilization. pK of some buffer-amines are indicated; pH of assays have been measured before desiccation. Methaemoglobin and oxyhaemoglobin levels show the effect of polyhydroxy-compounds 7,8,10 and 11.

No.	Compound	pK (20 °C)	pH (±0·1)	Methaemoglobin %	Oxyhaemoglobin %
	Reference			49 ± 10	50 ± 10
1	Tris Merck	8·06	8·9	9	85
2	2-Amino-2-methyl propane-1,3-diol (AMPD) Prolabo	8·79	9·4	11·7	80
3	2-Amino-2-ethyl propane-1,3 diol Aldrich		9·5	12·5	76·5
4	2-Amino propane-1,3 diol (serinol) Sigma		6·2	16·1	83
5	2-Amino 2-methyl propanol Prolabo	9·69	10·5	47·3	63
6	2-Amino propanol (alaninol) Fluka		10·2	34·5	26
7	2(2-Hydroxyethylamino) 2-hydroxymethyl propane-1,3 diol (mono Tris) Serva	7·8	8·6	2·7	95
8	2[bis(2-Hydroxyethyl)amino] 2-hydroxymethyl propane-1,3 diol (bis Tris) Serva	6·5	7·4	3·8	97
9	2-Carboxymethylamino 2-hydroxymethyl propane-1,3 diol (Tricine) Aldrich	8·15	5·5	38	64
10	1,3-bis(Trihydroxymethyl methyl amino) propane (bis Trispropane) Calbiochem	9 6·8	9·9	1·9	97
11	2[bis(2-Hydroxyethyl)amino] 2-methyl propanol (bis AMP) Sigma		6·2	7·4	92
12	2-Hydroxymethyl 2-methyl propane-1,3 diol (Tris-ethane) Aldrich		5·6	50	48
13	2-Hydroxymethyl 2-ethyl propane-1,3 diol (Tris-propane) Ega		6·0	56	53
14	2,2-bis-Hydroxymethyl propane-1,3 diol (pentaerythritol) UCB		6·2	60	56
15	2-Hydroxymethyl 2-nitro propane-1,3 diol Fluka		5·8	51	54
16	1-Amino propane-2,3 diol UCB		9·8	21·5	55
17	3-Amino propanol Aldrich			turbid, unworkable	
18	1-Amino 2-propanol Aldrich		10·3	47	26
19	2,2-Dimethyl propane-1,3 diol Aldrich		6·1	44	52
20	1,2,3-Propanetriol glycerol Prolabo		6·1	29	80
21	1,2-Propanediol Prolabo		6·2	52	58
22	2-Propanol = isopropanol Prolabo		6·2	55	60
23	1,3-Diamino propane Aldrich		11·2	turbid unworkable	16
24	1,3-Diamino 2-propanol Aldrich		10·5	100	0
25	3-amino propane Prolabo		10·9	turbid unworkable	21
26	NN Hydroxymethylglycine (Bicine) Merck	8·35	5·6	50	60
27	Triethanolamine (T.E.A.) Prolabo		8·7	28	45
28	Trihydroxymethyl-phosphine oxyde Aldrich		6	22	84

tact. Three compounds prevented the satisfactory redissolution of the freeze-dried haemoglobin.

Four compounds with more alcohol functions than Tris were also more active while the simple replacement of one -OH in Tris with an -H, making AMPD, increased the amount of methaemoglobin formed. This result was confirmed with the ethyl and demethyl homologues (3 and 4). One alcohol function is even less effective, as 2-amino-2-methyl propanol (5) and alaninol (6) show. It seems that the simplest effective structure is that of 2-amino-2-methylpropane-1,3-diol (2), which may have a dose-effect relationship different from that of Tris, while the protective effect may be a function of the physical properties of aqueous solutions of the active compounds.

If the protection given is of chemical origin, and the result of one or more well-defined haemoglobin-amine interactions, the compounds allow an evaluation of the importance of several structural elements.

The presence of at least 2 primary alcohol functions is necessary. If 2-amino-2-methylpropane-1,3-diol (2) is considered the simplest active molecule, all the compounds richer in alcohol functions are more active, even if the functions are differently distributed, as in bis-AMP. Addition of more alcohol functions leads to the most effective compounds, mono-Tris, bis-Tris, and bis-Tris-propane. This observation is true for trialcohols containing nitrogen (triethanolamine) or phosphorus (trishydroxymethyl phosphine oxide), which are not totally devoid of protective effect.

The presence of a quaternary carbon atom with several alcohol functions and one amine function seems to be necessary. This function directly bound to the carbon atom bearing the alcohol functions, constitutes the third necessary condition. The need for this function is demonstrated by the ineffectiveness of pentaerythritol (14) and of dimethylpropanediol (19). Its position is, however, critical, as the bad results with the diamines and Bicine show. The amine nitrogen atom may carry one or two hydroxyethyl ($-CH_2CH_2OH$) substituents, or may even join two Tris moieties, which increase the protective effect, but it must not carry a carboxymethyl ($-CH_2$ COOH) substituent, as with Tricine. Certain compounds of theoretical and no doubt practical interest, in which this amine function is replaced by $-CH_3$ $-CH_2OH$, or is separated from the carbon at the core of Tris or AMPD by $-CH_2-$, are not yet commercially available and could not be studied. The same is true for 2,2-diaminopropane and for 2,2-diaminopropane-1,3-diol.

Why such structures prevent the oxidation of haemoglobin remains unexplained. Several explanations of the denaturation (the presence of free radicals, the protection of the iron, the blockage of the heme pocket, etc.), have been put forward. The protection of haemoglobin did not seem to depend on the pH of the medium being freeze-dried. The solutions had pH values, ranging from about 5·5 to 10·5, while the pK values for some of the amine buffers are also very widely separated.

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LETTERS TO THE EDITOR

Gastrointestinal erosions and the lack of inflammatory response

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As part of an investigation into the pathogenesis of drug induced gastric erosions (Robins 1978) it was noted that when aspirin and indomethacin cause erosions in the mucosa of the rat stomach, remarkably they fail to provoke an inflammatory cell response.

This observation confirms that of Muir & Cossar (1961) who gave soluble aspirin to patients about to undergo gastrectomy and of Brodie et al (1970) who saw a similar lack of cellular response to aspirin in the rat intestine. Smith & Butler (1974) also noted that mechanical trauma to the full thickness of the mucosa of the mouse colon elicited no inflammatory cell response. In each case this lack of response was recorded while describing the lesion and its significance was not discussed. My own findings in rats indicated that, in the stomach, inflammation proceeds normally if the submucosa is damaged, but if it is not and damage is confined to cytolysis of the mucosa and its microvasculature, resolution occurs after 4–12 h without the appearance of the polymorphonuclear and mononuclear cells usually associated with an episode of necrosis.

There is no easily apparent reason why the inflammatory process is not provoked in the gastrointestinal tract. The mucosa is mainly epithelial but also contains a reticulin network, a vascular system and it is innervated. It contains therefore the features essential to the recognition of a necrotic episode. The products of necrosis are not immediately diluted into the lumen of