QUANTITATIVE ISOLATION OF VICINE FROM FABABEAN PROTEIN CONCENTRATE AND THE INVOLVEMENT OF FREE RADICALS IN THE BIOPHYSIOCHEMICAL EFFECTS OF DIETARY VICINE IN THE CHICKEN

A thesis submitted to

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by

David Stephen Muduuli

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DOCTOR OF PHILOSOPHY

DEDICATED TO MY GRANDMOTHER, Z. KAMPI

ABSTRACT

Experiments were conducted to (1) develop a method for isolating vicine from fababean protein concentrate (FBPC) or dehulled ground fababeans, in such quantities as to permit animal feeding trials; (2) investigate the biophysiochemical effects of dietary vicine in chicks and laying hens; and (3) establish the mode of action of vicine in eliciting its effects in animals.

Crude vicine (vicine + convicine, 5.7:1) was extracted from FBPC with either 10.5 (74:30:1) or 7.5 (25:11.5:1) volumes of acetone/water/3N NaOH mixtures. The extract was concentrated to 0.07-0.08 volumes in a steam-heated cyclone evaporator, and then stored to crystallise at $0^{o}C$. The crystallite was washed successively with water, ethanol and acetone, and then dried to constant weight at $60^{o}C$. Attempts to use dehulled ground beans instead of FBPC were unsuccessful because the sample was difficult to filter, the yield extremely low and the product was contaminated with a sticky substance. Crude vicine was separated into vicine and convicine by recrystallisation, and several properties were determined on these compounds. Crude vicine was added to a normal broiler starter diet at 0, 0.25, 0.5, 1.0 or 2.0% levels, and the diets were fed to 7-day old chicks ad libitum for 2 weeks. In addition crude vicine was added to a chicken breeder diet at 0, 0.5 or 1.0% levels in one experiment; and at 0 or 1.0% level in two other experiments. The diets were fed to laying hens ad libitum or pair-fed. Reduced divicine was prepared from vicine by acid hydrolysis and its spectral, stability and some biochemical properties were investigated. The production of free radicals during the autoxidation of reduced divicine was investigated

utilising the reduction of cytochrome (cyt C) and nitroblue tetrazolium (NBT), or using electron spin resonance spectroscopy.

The physical (e.g. crystalline, melting point), spectral and stability characteristics indicated that the compounds isolated were vicine and convicine. However, the amino acid chromatography method revealed that the "pure" vicine and convicine were 97 and 91% pure, respectively, and each was contaminated by the other. The compound obtained after acid hydrolysis of vicine was confirmed to be reduced divicine from its spectral, stability and reducing properties. Increasing levels of dietary vicine, particularly above 1%, depressed chick growth; this was not due to lowered feed intake. In the laying hen, both 0.5 and 1.0% dietary vicine led to reduced egg and yolk masses ($P<0.05$), due mainly to lower weights rather than production rate.

In the other two experiments where 0 or 1.0% dietary vicine was fed other parameters were investigated. In addition to lowered egg and yolk masses, vicine led to increased yolk membrane fragility and the incidence of yolk blood spots; and reduced fertility and hatchability of the eggs.

Vicine-fed birds had elevated levels of plasma lipids, lipid peroxides and erythrocyte hemolysis in vitro, and depressed hematocrit and the ratio of plasma vitamin E/lipid levels ($P<0.05$). Dietary vicine resulted in lower red blood cell (RBC) hemoglobin but elevated reduced glutathione (GSH) levels and superoxide dismutase (SOD) activity ($P<0.05$). It also led to higher liver weights, and lipid peroxide and GSH levels ($P<0.05$), but the effect on liver lipid levels depended on the assay method; vicine-fed birds had higher ($P<0.05$), but lower ($P>0.05$) liver lipid levels when

wet or lyophilised livers were employed in the assay, respectively. Liver protein levels were slightly depressed (P 0.05) in vicine-fed birds. Activities of liver glutathione peroxidase (GSH-px), catalase and SOD were lower (P 0.05) in the vicine-fed birds. All the above effects were not due to reduced feed consumption. Vitamin E supplementation at levels forty times higher than the NRC recommendations for laying hens, alleviated the vicine-mediated reduction in egg fertility and hatchability, it also reduced liver weight and lipid levels and restored the enzyme activities of liver GSH-px and SOD, but not catalase or the other parameters observed in the laying hen. In addition exogenous glucose in the incubation medium inhibited the spontaneous hemolysis in vitro of RBC from vicine-fed birds.

Reduced divicine slowly autoxidised in acid but rapidly in basic media equilibrated with air but not nitrogen. In solutions equilibrated with air, reduced divicine reacted with GSH to form a product that absorbed strongly at 305 nm. In the presence of GSH, a mixture of cyt C and reduced divicine showed decreased absorbance at 240 and 550 nm, but an increase at 305 nm. Methemoglobin (MetHb) was reduced to hemoglobin (Hb) by reduced divicine, and both MetHb and Hb enhanced the autoxidation of divicine. The autoxidation of divicine was initially inhibited, but finally enhanced by SOD or catalase, while H_2O_2 inhibited the latter and not the initial part of the autoxidation. The effect of H_2O_2 was alleviated by catalase. Reduced divicine reduced both cyt C and NBT. However, unlike NBT, a minor part of the cyt C reduction was susceptible to SOD or catalase inhibition. In addition other proteins like bovine serum albumin (BSA), Hb, MetHb, alcohol

dehydrogenase and ovalbumin at low concentrations inhibited the oxygen-dependent reduction of NBT by divicine.

Considering this data and that in published literature it may be concluded that vicine is hydrolysed to its aglycone, divicine at a site not specifically known (digestive tract, plasma or liver). The increased activity of SOD in the RBC would indicate a high concentration of O_2^- in plasma, implying that most of the autoxidation of divicine occurs in plasma and therefore vicine is either hydrolysed there or before it reaches the circulation. The divicine produced can then condense directly with some biological compounds like GSH, or it autoxidises in the alkaline plasma to produce O_2^-. The O_2^- can them dismutate to H_2O_2 and finally produce $^{\bullet}OH$, which is the most potent oxidant of the known free radicals. The cells would not be able to protect themselves due to the lowered activity and/or levels of the liver enzymes, GSH-px, SOD and catalase. The increased levels of liver and RBC GSH would indicate an inhibition of its utilisation, with little effect and/or increase in its production. Both effects of divicine lead to oxidation of the biological molecules, causing lipid peroxidation and RBC hemolysis. This may cause destruction of the lipid transport proteins or alter the lipids such that they are not transportable to the ovum, but accumulate in plasma. Finally, vitamin E can protect the chicken against some, but not all vicine-mediated effects. Therefore other nutrients may be involved in the action of vicine.

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Sources of the chemicals utilised in the studies

Chemical Name	Chemical Company
Triton X-100 scintillation grade	Amersham Corp.
H_2O_2	Baker Chemical Company
NaCl	" " "
Sodium citrate	" " "
TCA (Trichloroacetic acid)	" " "
$NaH_2PO_4.H_2O$	" " "
Tris buffer	" " "
KH_2PO_4	" " "
$FeCl_3.6H_2O$	" " "
Metaphosphoric acid (glacial)	" " "
$CuSO_4.5H_2O$	BDH Chemical Company
EDTA disodium salt	Fisher Scientific Company
n-propanol	" " "
$FeSO_4.7H_2O$	" " "
Na_2HPO_4	" " "
K_2HPO_4	" " "
Sodium cyanide	" " "
Na_2CO_3	" " "
NaOH	" " "
Glucose (dextrose)	" " "
H_2SO_4	" " "
$KMnO_4$	" " "
Antifoam B	" " "
HCl	" " "

Continued .....

Sources of the chemicals utilised in these studies (Continued)

Chemical Name	Chemical Company
Folin - Ciocalteu (phenol) reagent	Harleco Company
TBA (2-thiobarbituric acid)	ICN Pharmaceuticals Inc.
Sodium azide	Matheson, Coleman & Bell
Methionine	Nutritional Biochem. Corp.
Riboflavin	" " "
GSH (reduced)	Sigma Chemical Company
GSSG (ox) (No. G4501) (grade III)	" " "
5,5^{1} dithiobis-(2-nitrobenzoic acid)	" " "
α, α^{1}-dipyridyl (∂, ∂^{1}-bipyridine)	" " "
d$\ell\alpha$-tocopherol	" " "
BHT (Butylated Hydroxytoluene)	" " "
Nitro Blue Tetrazolium	" " "
NADPH	" " "
Hemoglobin Standard Kit:	" " "
- Hemoglobin Standard	" " "
- Drabkin's Reagent	" " "
- Brij 35 soln.	" " "
Penicillin-G	" " "
NaK tartrate	" " "
Catalase from bovine liver Stock #C-10	" " "
Cytochrome-C, Type VI	" " "

Continued .....

Sources of the chemicals utilised in these studies (Continued)

Chemical Name	Chemical Company
Superoxide Dismutase, Type I from bovine blood	Sigma Chemical Company
Hemoglobin No. H-2500 (from beef blood)	" " "
Methemoglobin No. m-9250 (from beef blood)	" " "
Bovine albumin, fraction V	" " "
Alcohol dehydrogenase (from yeast)	" " "
Ovo albumin, Grade V	" " "
Glutathione, reduced form	" " "
Acetone	Standard Chemical Company
Ethanol	" " "

List of Abbreviations

AA - Ascorbic acid

α - Specific rotation

APH - Acetylphenylhydrazine

DA - Dialuric acid

DETAPAC - Diethylenetriamine pentaacetic acid

DIL (5x) - SAT diluted five times etc.

DMPO - 5,5-dimethyl-1-pyrroline-N-oxide

EDTA - Ethylenediamine tetraacetic acid

e^- - Electron

ε - Molar absorption coefficient

esr - Electron spin resonance

FBPC - Fababean protein concentrate

4-POBN - α-4-pyridy-1-oxide N-tert-butyl nitrone

G-LC - Gas-liquid chromatography

G-6-P - Glucose-6-phosphate

G-6-PD - Glucose-6-phosphate dehydrogenase

GSH - Reduced glutathione

GSSG - Oxidized glutathione

GS* - Free radical of oxidized glutathione

GSSG-red - GSSG-reductase

Hb - Hemoglobin

HCl - Hydrochloric acid

HMDA - Hexamethyldisilazane

λ - Wavelength

Continued .....

List of Abbreviations (Continued)

λ max - Wavelength at which maximum absorption occurs

λ min - Wavelength at which minimum absorption occurs

L-Dopa - L-3,4-dihydroxyphenylalanine

$L^{\cdot}$ - Alkyl radical

$LO^{\cdot}$ - Alkoxy radical

$LOO^{\cdot}$ - Hydroperoxy radical

ME - Metabolisable energy

MetHb - Methemoglobin

NADH, NAD^{+} - Reduced and oxidized Nicotinamide Adenine (diphosphopyridine nucleotide)

NADPH, $NADP^{+}$ - Reduced and oxidized Nicolinamide Adenine Dinucleotide 2^{1}-phosphate

nmr - nuclear magnetic resonance

NSHA - Non spherocytic hemolytic anemia

$^{1}O_{2}$ - Singlet oxygen

O_{2}^{-} - Superoxide anion or radical

O_{3} - Ozone

OD - Absorbance

ΔOD - Change in OD

$^{\cdot}OH$ - Hydroxyl radical

OxyHb - Oxyhemoglobin

PCMB - p-chloromercuric benzoic acid

PUFA - Polyunsaturated fatty acids

PVP - Polyvinylpyrrolidone

Continued .....

List of Abbreviations (Continued)

RBC - Red Blood Cells

RH_2 - Any reduced compound with two hydrogens capable of being oxidized

$RO_2^{\bullet}$ - Peroxy radical

SAT - Saturated solution of divicine or vicine

SH - thiol

6-ADA - 6-aminodopamine

6-OHDA - 6-hydroxydopamine

6-PGD - 6-phosphogluconate dehydrogenase

SOD - Superoxide dismutase

TCA - Trichloroacetic acid

TLC - Thin layer chromatography

TMCS - Trimethylchlorosilane

TMPO - 2,5,5-trimethyl-1-pyrroline-N-oxide

TMS - Trimethylsilyl

TPN - Triphosphopyridine nucleotide

UV - Ultraviolet

INTRODUCTION

Fababeans have been cultivated for more than 3,000 years and have been grown and utilised as a food in many countries, particularly the Near East, Mediterranean and North African states. However, fababeans grown in Europe are used mainly as a livestock feed. In North America, particularly Canada, fababeans are a promising crop both agronomically and economically to alleviate the heavy dependence on imported soybeans as a protein supplement in livestock feeds. Fababeans are also a potential source of protein for man, particularly in developing countries where livestock products are limited.

In spite of their high nutritional qualities (e.g. high protein content (25-35%) with a good balance of amino acids, although deficient in S-amino acids particularly methionine), fababeans contain several antinutritional factors. These antinutritional factors consist of thermolabile and thermostable compounds. The thermolabile factors consist of several polyphenolic compounds, lectins and trypsin and/or general and amylase inhibitors, and are equally distributed between the hull and cotyledon except the lectins which do not occur in the hull. The thermolabile antinutritional factors found in the cotyledon are more specifically associated with the protein fraction. The major thermolabile factors in the hull are the polyphenolic compounds including condensed tannins, which complex with a lot of dietary nutrients, particularly proteins and amino acids rendering them unavailable to the animal. The effects of the heat-labile factors are easily alleviated by heat treatment, in addition dehulling can remove those factors restricted to the hull, while tannins can be selectively bound by PVP or avoided

by feeding tannin-free cultivars which are easily identified by their white flowers.

The thermostable factors are mainly associated with the protein fraction and those so far isolated include vicine, convicine, and L-Dopa and its glucoside. Vicine and convicine are mainly concentrated in the cotyledon, although extremely low levels of both may be found in the hulls of some of the fababean cultivars. On the contrary L-Dopa and its glucoside are mainly found in the green pod, and some in the hull of the seed, but not the cotyledon of fababeans. Vicine and convicine are believed to cause toxicity via their aglycones, divicine and isouramil, respectively. They are suspected in the etiology of favism in man, and have been shown in vitro to lower GSH, ATP and intracellular K^+ levels and to cause hemolysis of human erythrocytes. In addition dietary vicine together with convicine have been shown to depress egg and yolk mass by mainly reducing their weights and to a less degree their production rate. The mechanisms by which the glycosides or their aglycones enlist these effects are not known, although enzyme inhibition and the involvement of free radicals have been proposed, and the former has been demonstrated for some enzymes. Although L-Dopa is widely used to treat Parkinson's disease it has been shown to lower GSH levels in G-6-PD-deficient human erythrocytes, and there is growing evidence that it may act synergistically with vicine and convicine in the etiology of favism.

Detailed studies (particularly feeding trials) on the effects of vicine and convicine on man and animal performance have been hampered by lack of a commercial source of large quantities of these compounds.

Therefore experiments were conducted to (1) develop a method for the quantitative isolation of vicine from FBPC and if possible dehulled ground fababeans; (2) to further investigate the biophysiochemical effects of dietary vicine in chicks and laying hens; and (3) to try and establish the mode of action of vicine in enlisting the observed effects.

CHAPTER 1

LITERATURE REVIEW

1.1. Fababeans in Human and Livestock Diets

A. World Production

Information on the origin of Vicia faba has been reviewed thoroughly by Hawtin and Stewart (1979). The distribution of species related to V. faba put the probable place of origin of V. faba in the near East or the Mediterranean region. Fababeans are the fourth most important pulse crop in the world after dry beans, dry peas, and chick peas. Statistics for 1975-77 (FAO, 1977) inclusive, revealed that the mean annual world dry pulse production was 47,379,000 tons of which 13% were fababeans. China produces about 2/3 of the world fababean crop, while 10-15% are grown in West Asia (Turkey, Iraq, Syria, Cyprus, Lebanon, etc.) and North Africa (Ethiopia, Algeria, Egypt, Morocco, Sudan and Tunisia). Fababeans are a promising alternative to soybeans as a source of protein in Canadian livestock rations. Extensive research is currently underway to evaluate the agronomic, economic and nutritive value of fababeans, (see First National Fababean Conference, Winnipeg, Manitoba February 21-22, 1974; The development of fababeans, Research Report 1977-78, Faculty of Agriculture, University of Manitoba).

B. Nutrient Composition of Fababeans

Fababeans, like most legumes, are higher in protein content than cereals. Improved varieties of fababeans contain 25 to 35% protein on a dry matter basis. The protein content is influenced by type and

variety of the bean, season, location and stage of maturity (Eden 1968; Clarke 1970; Bhatty 1974; Marquardt et al. 1975; Blair 1977). Although the fababean protein is low in the sulphur-amino acids, particularly methionine and cystine (Kakade 1974; Blair 1977) it is high in lysine and has a good balance of the other amino acids (Marquardt et al. 1975).

The carbohydrate content of fababeans was found to vary from 51 to 66% with 28 to 42% starch (White 1966; Pritchard et al. 1973; Bhatty 1974; Cerning et al. 1975). The relative amounts of available and unavailable carbohydrate fractions seem to vary inversely and with season (Prichard et al. 1973). The concentration of glucose-containing polymers was found to be 3.6% in the hull and negligible in the cotyledon (Cerning et al. 1975).

The fat content of fababeans is 1 to 2% (Carpenter and Johnson 1968; Eden 1968; Marquardt et al. 1975) and virtually all (97%) is concentrated in the cotyledon (Blair 1977).

All metabolisable energy (ME) values reported for fababeans exceed 2300 kcal/kg even when corrected for nitrogen retention (Carpenter and Johnson 1968; Waring and Shannon 1969; Edwards and Duthie 1970; Marquardt et al. 1974a) and could be as high as 2700 kcal/kg (Carpenter and Johnson 1968).

The nutrient composition of fababeans as compared to two other protein sources (soybean meal and field pea) and cereals (barley and wheat) is shown in Table 1.1. The relative lysine levels in fababeans are comparable to those of soybean meal and field pea, but considerably higher than those of barley and wheat. Therefore fababeans could be

combined with cereals which are low in lysine but high in methionine to obtain a proper balance of amino acids. All the four grains contain a similar amount of crude fat. However, the linoleic acid concentration in fababeans is 0.7% compared to 0.4% and 0.9% in soybean meal and barley, respectively (Blair 1977).

Table 1.1. Nutrient composition of fababean compared to the field pea, soybean, barley and wheat.

Nutrient	Faba-bean	Soybean meal	Field pea	Barley	Wheat	Ref.
Dry matter (%)	86.0	86.0	-	86.0	-	1
	-	-	89.0	-	-	2
	-	-	-	89.0	89.0	3
	85.0					4
Crude fat (%)	1.3	0.8		1.8	-	1
	2.0	18.0[a]	1.8	-	-	2
				2.1	1.9	3
	1.0	1.0	-	2.0	-	4[b]
	1.0	-	-	-	-	5
Crude fibre (%)	7.0	6.0	-	5.0	-	1
	7.8	4.8[a]	5.5	-	-	2
	-	-	-	5.6	3.4	3
	8.0	6.0	-	5.0	-	4[b]
	7.7	-	-	-	-	5
Crude protein (%)	27.0	45.0	-	11.0	-	1
	24.0	38.0[a]	23.0	-	-	2
	-	-	-	13.0	14.3	3
	27.0	45.0	-	11.0	-	4[b]
	32.4	-	-	-	-	5
Digestibility of crude protein (%)	80-85	80-95	-	77-80	-	1
Lysine (%)	1.5	3.3	-	0.4	-	1
	-	-	-	0.6	0.51	3
	1.6	3.3	-	0.6	-	4[c]
(mg/gN)	350	400[a]	420	-	-	2
	425[d]	-	-	-	-	5
	394[d]	-	-	-	-	6

.....Continued

Table 1.1 (Continued)

Nutrient	Faba-bean	Soybean meal	Field pea	Barley	Wheat	Ref.
Methionine (%)	-	-	-	.20	.20	3
	.21	.67	-	.20	-	4[c]
(mg/gN)	30	80	70	-	-	2
	56.3	-	-	-	-	5
	37.5	-	-	-	-	6
Cystine (%)	-	-	-	.20	.20	3
(mg/gN)	40	110[a]	85	-	-	2
	81.25[d]	-	-	-	-	5
	37.5[d]	-	-	-	-	6
Total sulphur amino acids (%)	0.5	1.6	-	0.2		1
(mg/gN)	70	190[a]	155	-	-	2
Ash (%)	3.5	5.9	-	2.9	-	1
	3.4	4.7[a]	2.6	-	-	2
	-	-	-	2.7	1.8	3
	4.0	6.0	-	2.0	-	4[b]
Calcium (%)	.15	.37	-	.08	-	1
	.09	.21[a]	.06	-	-	2
	-	-	-	.09	.06	3
	.10	-	-	-	-	5
Phosphorus (%)	.5	.67	-	.35	-	1
	-	-	-	.47	.41	3
	.6	-	-	-	-	5
Iron (mg/100 g)	-	-	-	10.0	10.0	3
	3.6	6.5[a]	4.8	-	-	2
N-free extract (%)	48.0	28.7	-	66.4	-	1
	47.0	30.0	-	68.0	-	4[b]
ME (kcal/kg, poultry)	2470	2200	-	2600	-	4
Vit. A (IU/100 g)	100	140[a]	100	-	-	1
Thiamine (mg/kg)	5.4	10.3[a]	7.2	-	-	2
	-	-	-	5.7	5.5	3

.....Continued

Table 1.1 (Continued)

Nutrient	Faba-bean	Soybean meal	Field pea	Barley	Wheat	Ref.
Riboflavin (mg/kg)	2.9	3.0[a]	1.5	2.2	1.3	3
Niacin (mg/kg)	23	21[a]	24	-	-	2
	-	-	-	65	64	3

Key to Table 1.1

All data other than dry matter content and where specified are expressed on a moisture-free basis.

[a]Values were for whole seeds and not defatted meal.

[b]Data were obtained from Canada Grains Council Bulletin and values were corrected to 87% dry matter.

[c]Data was expressed on as fed basis.

[d]Calculations were based on the assumption that protein contained 16% nitrogen.

References: 1. Presber (1972)
2. Aykroyd and Doughty (1964)
3. NAS-NRC Publ. 1232 (1974)
4. Marquardt et al. (1974a)
5. Marquardt et al. (1975)
6. Waring and Shannon (1969)

The crude fiber in whole fababeans is higher than that of soybean meal, field peas, barley and more than double that of wheat. Fababean hulls comprise about 13% of the whole bean, and are 50% fiber and 7% protein (Marquardt et al. 1974a). Conversely, the dehulled bean has greatly reduced fiber content (3%) and a slightly increased protein content (31%) as compared to the whole bean. These observations would suggest that dehulling would increase the available energy and protein levels of fababeans.

A number of antinutritional factors have been detected in the common varieties of fababean, and probably more will be identified in

the future. Those known so far include the trypsin, chymotrypsin and/or general protease inhibitors, amylase inhibitors, hemagglutinins (lectins), tannins, 3, 4-dihydroxyphenylalanine (L-Dopa) and its glucoside, vicine, convicine and S-methyl-L-cysteine. Literature on these factors is reviewed in section 1.1 (e).

C. Fababeans in human diets

(i) General overview

Fababeans have been part of man's diet since Roman times. The early Romans called the bean "faba", it was added to the wheat or millet flour to increase the weight of the loaves intended for sale and it was used to make porridge (see review by Hawtin and Stewart 1979). Fababeans are an important source of dietary protein particularly throughout Egypt, Northern Sudan, the highland areas of Ethiopia and the West Asia and Mediterranean countries (Cyprus, Iraq, Jordan, Lebanon, Syria and Turkey). In these regions beans are eaten in many forms (Belsey 1973). Fresh green beans may be consumed raw or cooked, with or without hulls, and sometimes with the pod if it is sufficiently tender. The dried beans are generally soaked overnight in water and may then be eaten raw or after cooking. Stewed dried beans are a common weanling food in Egypt and many infants are exposed to the bean before the age of two years. In these regions processing of the bean is limited to only milling into flour. Therefore most of the toxic factors present are ingested.

Only small quantities of fababeans are consumed in the Western world (mainly Europe). In spite of this limited use, fababeans have

been found to be a valuable protein supplement in bread-making (McConnell et al. 1974; Patel and Johnson 1975; Fleming and Sosulski 1977), in snack foods and as a meat extender (Vaisey and Tassos 1974); also, in Canada there are currently some companies that process and can fababeans. However, the ingestion of fababeans is associated with a host of undesirable effects in some individuals. Even the Romans considered the beans to have "dulling effect on the senses and to cause sleeplessness" (see Hawtin and Stewart 1979). Fababeans have long been known to cause a disorder in some individuals which is typified by hemolysis. This disorder was given the term "favism" by the Italian physician Montano in 1814 (Sansone et al. 1958a; Mager et al. 1969).

(ii) Favism

Favism has been observed in many different population groups. However, it is most common in Italy (Sansone et al. 1958b) and Greece (Kattamis et al. 1969), though cases have been documented in English (Davies 1962; Holt and Sladden 1965), Polish (Sroczynska and Sychlowy 1973), Portuguese (Kahn et al. 1976), Thai (Panich and Na Nakorn 1973), Sephardic Jewish (Szeinberg et al. 1957a, b; Szeinberg and Bitron 1957), Algerian (Messerschmitt et al. 1967) and Chinese (Du 1952) subjects.

According to Luisada (1941), the first symptoms of favism may appear within a few seconds after inhalation of pollen to, 5 to 24 hours after ingestion of fababeans. The symptoms consist of malaise, headache, dizziness, nausea, vomiting, chills, pallor, lumbar pain, and fever. Hemoglobinuria appears within 5 to 30 hours following exposure to the beans, and a few hours later jaundice is observed (Luisada 1941). Fever

and marked leukocytosis may be present. Sometimes the red cells in the peripheral blood are distorted in shape and the hemoglobin may appear to be contracted away from the membrane (Dacie 1967). It has been claimed that the levels of red cell lipids decline during hemolysis (Khalil et al. 1975). Studies, started soon after incidental intake of fababeans by sensitive G-6-PD-deficient subjects (Gaetani et al. 1979) demonstrated a decrease in both the $NADPH/NADP^+$ ratio and the concentration of reduced glutathione. A typical favic attack lasts from 2 to 6 days and fatalities usually occur within the first 2 days and rarely during the third day (Luisada 1941). Although favism may be quite mild (Sartori 1971) in many cases the anemia is severe; the blood hemoglobin concentration may fall below 1 g/100 ml (Kattamis et al. 1969). This is important because severe anemia might occur even in individuals with normal G-6-PD enzyme. This is plausible given that favic hemolysis resembles premarquine-induced hemolytic anemia (Crosby 1956). Thus the mode of attack on RBC by the favic factor may not distinguish between normal and abnormal cells, but the lack or low activity of G-6-PD may aggrevate the situation. Acute renal failure is common in favic adults and fatalities in adults were usual before the days of blood transfusions (Luisada 1941).

Predisposition to favism depends on the age, sex and genotype of the subject, and the stage of maturity of the ingested bean. The disorder is more frequent and severe among children between 1 and 5 years of age (Kattamis et al. 1969; Sartori 1971). In some studies (Kattamis et al. 1969), but not others (Sartori 1971) favism was observed more frequently in males. All patients with favism or a history of favism

are deficient in G-6-PD, but many individuals with a G-6-PD deficiency can ingest fababeans without experiencing hemolytic anemia. The G-6-PD deficiency gene is located on the x-chromosome; thus the deficiency is fully expressed in the hemizygous ($\bar{x}y$) male because the mutant gene $\bar{x}$ is not counteracted by the normal allele (x) as in the female. This may account for a higher incidence of favism among males in certain areas. Full expression is rare in the female because the gene is recessive and therefore would require a homozygous mutant genotype to show up.

Since not all G-6-PD deficient individuals exhibit favism on ingesting fababeans, other factors may be involved. It has been suggested that another factor inherited as an autosomal gene may be involved (Stamatoyannopoulos et al. 1966), but its mode of action is not yet known. A defect in glucuronide formation is also suspected because decreased excretion of D-glucaric acid (Cassimos et al. 1974), and impaired formation of salicylamide glucuronide (Cassimos et al. 1973; Cutillo et al. 1976) have been observed in individuals prone to favism. It was earlier suspected that sensitivity to favism might have an immunological basis (Luisada 1941; Roth and Frumin 1960), but this has not been borne out by modern immunological techniques (Fiorelli et al. 1974). The feeding habits of the people relative to the incidence of favism have not been investigated. The intake of certain nutrients (Vitamin E, selenium, zinc, riboflavin, niacin, sulfur amino acids, the types of fat and general antioxidants) might be important in the etiology of favism. This may partly account for the sporadic incidence of favism and the difference between American Negroes with better feeding habits compared to other Negroes. It has also been observed that congenital

deficiency of GSSG-reductase (GSSG-red) leads to drug sensitivity (see Mager et al. 1969), therefore other enzyme deficiencies may be involved in the etiology of favism.

Most cases of favism result from ingestion of fresh beans and particularly at mid-length of the pods (Kattamis et al. 1969; Jamalian et al. 1977b), but favism also occurs when dry beans are eaten. Favism has been observed in nursing infants whose mothers have ingested faba-beans (Emanuel and Schoenfeld 1961; Kattamis et al. 1969; Kattamis 1971) and in a child who ingested milk of a goat which had eaten the beans (Luisada 1941). The quantity of the favic factor required to cause favism may be very small (Luisada 1941).

The mode of action of the favic principle in causing hemolysis and the other symptoms of favism is not completely known. It is generally accepted that a lack and/or depletion of reducing components, particularly GSH may be part of the explanation. Levels of GSH and the stability of RBC are diminished in patients with a history of favism (Sansone and Segni 1956; Szeinberg et al. 1957a, b; Szeinberg and Bitron 1957; Sansone et al. 1958b; Gaetani et al. 1979). Therefore individuals lacking G-6-PD would not be able to generate enough NADPH needed to regenerate GSH from GSSG. In addition the GSSG generated during the oxidation of GSH, inhibits a number of enzymes (see review by Mager et al. 1969), including hexokinase which is important for both the pentose phosphate and glycolytic pathways. However, the above do not explain how the RBC membrane is disrupted to lead to cell lysis. Since small amounts of fababean are adequate to cause favism, it seems that the toxic factor(s) is (are) a cyclic catalyst and/or initiates a chain reaction in the cells.

Several factors in fababeans have been considered as possible hemolytic principles. These include vicine, convicine (Mager et al. 1965; Razin et al. 1968), and L-Dopa and its glucoside (Kosower and Kosower 1967). The circumstantial evidence is given in Section 1.1 (e). The possibility that free radicals may be involved was raised by Flohe et al. (1971), but no direct evidence has yet been provided. If the latter is true, then one would have to study several enzymes such as superoxide dismutase, catalase, peroxidases and GSSG-reductase and the dietary nutrients that may affect these enzymes. This possibility would further complicate the already complex picture concerning favism.

(d) Fababeans in livestock rations

The nutrient composition of fababeans makes them a potential alternative to soybean or fish meal in livestock rations. The utilisation of fababeans by various classes of livestock is discussed in the subsequent sections.

(i) Ruminants

Information on the use of fababeans in growing and lactating dairy cattle rations is limited. The potential for fababeans as a concentrate and silage in cattle nutrition has been discussed by Ingalls et al. (1974, 1979) and McKnight and Macleod (1977). Preliminary data indicate that the use of fababeans at levels of 30% and 24% of starter (0-7 weeks) and grower (7-20 weeks) rations, respectively as a replacement for soybean meal and barley, resulted in no significant differences ($P>.05$) in feed intake, growth rate and feed efficiency. Dairy cattle performance was also similar when they were fed isonitrogenous rations

containing either ground fababean-hay (35:65) or soybean-hay mixtures. However, milk protein levels seem to be depressed ($P<.05$) and there are indications that reproduction may be affected when dairy cattle are fed fababean-containing diets (Ingalls, personal communication). Pilot studies with steer calves (Ingalls et al. 1974) indicated that fababeans compared to soybeans as a protein supplement led to lower weight gains, and poor feed efficiency; similar results were obtained with sheep. In the latter case pelleting improved both feed efficiency and growth rate.

Whole fababeans make good silage that has a much higher protein level than cereal silages and is highly palatable. It is nutritionally equivalent to other conventional silages (from corn, grass and grass-legume mixtures) (Ingalls and Devlin 1978; McKnight and Macleod 1977) for dairy cows.

It may be concluded that more work is needed in the area of beef cattle and sheep nutrition and to establish the cause of depression in milk protein when dairy cows are fed fababeans. Additional research is also needed in the area of fababean silage.

(ii) Swine and poultry

(a) Swine

Preliminary work by Stothers (1978) indicates that fababeans compare favourably with soybean meal in dry matter, protein, lysine and methionine digestibility and availability. Fababeans can replace soybean meal and fish meal if methionine is supplemented in the diet at a level of 0.05% for prestarter-starter pig rations. Rations with no soybean or fish meal, but supplemented with 10% or 15% fababean bean protein concentrate and vitamins gave best results in feed efficiency.

Work with growing-finishing pigs (80 lbs and over) (Stothers 1974) again demonstrated that fababeans can effectively replace soybean meal as a total supplemental protein source. Heat treatment and methionine supplementation may further improve the performance of growing-finishing pigs on fababean diets. These results are similar to those obtained at the University of Alberta (Aherne 1975; Bowland et al. 1975; Onaghise and Bowland 1977). Aherne (1975), however, did not observe any improvement in pig performance after heating the fababeans and cautioned against too much heat. Aherne (1975) and Onaghise and Bowland (1977) recommend that not more than 20% fababeans should be present in pig diets; over that, there is a reduction in average daily feed intake, average daily weight gain and feed conversion efficiency. The addition of lysine and methionine improves such diets, but not to control levels. Therefore some toxic factor(s) and not deficiency of certain nutrients may be responsible for these effects.

(b) Poultry

(ii) Growing chickens

A number of experiments have shown that fababeans (Vicia faba L.), particularly at low levels (less than 20%), when supplemented with methionine are a suitable protein supplement for growing chickens and turkeys (Blair and Bolton 1968; Blair et al. 1970; Wilson and McNab 1972; Marquardt and Campbell 1973; Kadirvel and Clandinin 1974; Marquardt et al. 1974b). However, feeding diets containing unprocessed ground fababeans, particularly at high levels (over 20%) has been shown to result in lower growth rate and feed efficiency, decrease or increase

in liver size, increase in pancreas size, poor feathering, increased incidence of perosis and a decrease in yellow pigment in skin, beak and shanks of the chicks. The effects, and the levels of fababeans in the diet that enlist them are variable; Kadirvel and Clandinin (1974) observed no adverse effects when they fed levels as high as 20% raw ground fababeans to broiler type chickens or turkey poults, 0-4 weeks old. Bletner et al. (1963) reported similar results with 30 and 60% fababeans in the diet, but observed poor feathering, increased incidence of perosis and a decrease in yellow pigmentation of the skin, beak and shanks of the chicks with the 60% level. Blair and Bolton (1968) noted no adverse effects on live weight gain or feed conversion efficiency during the period 4-9 weeks when broiler chicks were fed rations containing up to 30% fababeans. On the contrary, Blair et al. (1970), found that feeding broiler chicks diets containing 15, 30 and 40% fababeans for 0-4 and 0-8 weeks, resulted in significantly ($P<.05$) poorer feed efficiencies and weight gains compared to those fed diets without fababeans.

The poor productive performance observed with fababean diets was attributed to poor digestibility and hence availability of nutrients, particularly the limiting sulfur amino acids (Wilson and McNab 1972). Thus Marquardt and Campbell (1975) found that when a diet containing 90% raw ground fababeans was fed to broiler chicks, optimal performance (weight gain and feed efficiency) was obtained with birds consuming diets that contained 0.24% added methionine (total dietary methionine = 0.46%). Potassium sulphate (0.14 or 0.28%) gave slight improvement in performance, and the effect of cystine depended on the levels of methionine in the diet. Addition of 0.08% cystine to a diet containing

0.05% added methionine led to decreases in weight gain, but increases were observed with 0.10 or 0.15% added methionine. Similarly, the addition of cystine to a low methionine diet did not affect feed efficiency, whereas its addition to diets containing higher levels of methionine resulted in improved feed efficiency. In these experiments the performance of fababean fed birds under optimal conditions was lower than that for soybean-wheat fed birds. However, the improvement in metabolisable energy values following dehulling (Edwards and Duthie 1973) was so dramatic that it could not be accounted for completely by improved digestibility and availability of nutrients considering that the hull comprises 13% of the whole bean. Marquardt et al. (1976) found that a growth-depressing factor was associated with both the protein and the hull but not the starch portion of the bean. The factor in the hull was later isolated and found to be a condensed tannin and it was found only in the hull portion of the bean (Marquardt et al. 1977; Ward et al. 1977; Marquardt and Ward 1979). This factor accounted for over 50% of the growth depression due to dietary fababeans and its effects were partially ameliorated by autoclaving (121^{o}C) for 30 minutes and completely by autoclaving and binding the factor to polyvinyl-pyrrolidone (PVP). However, the fababean hull contains L-dopa and its glucoside (Nagasawa et al. 1961; Pridham and Saltmarsh 1963) which may be deleterious to the animal. These later factors are not so sensitive to heat.

The growth-depressing factor(s) associated with the protein fraction of the bean is not clearly known. Trypsin inhibitors (Wilson et al. 1972a, b; Marquardt et al. 1975), and hemagglutinins (Marquardt

and Campbell 1974; Marquardt et al. 1974b, 1975a) are present in the protein fraction of the bean. Marquardt et al. (1975) found that all hemagglutinin activity was associated with the cotyledon, but the trypsin inhibitor content per unit of dry matter was more than twice in the hull compared to the cotyledon. In addition there were tremendous cultivar differences in the levels of these factors in fababeans.

A number of processing methods have variable effects on the productive performance of chicks fed fababean rations. Autoclaving has been widely used and shown to improve weight gain, feed efficiency, increase or decrease liver weight, and decrease pancreas size (Marquardt and Campbell 1973, 1974; Marquardt et al. 1974a, b, 1976; Edwards and Duthie 1973) particularly in diets containing high levels but not low levels of fababeans.

Marquardt et al. (1974a, b) found that autoclaving (121^{o}C for 15 min) had the most pronounced effect in diets of high (85%) but not low (57 or 28%) fababean content. Heat treatment resulted in lower pancreas size, feed/gain ratio and improved growth response in chicks fed the diet containing 85% fababean. Weight gain in chicks, however, was not significantly ($P>0.05$) affected with diets containing 57 or 28% fababeans and was the same as that for chicks fed the 85% heated fababean diets. In this study (Marquardt et al. 1974a), chicks given a commercial starter diet (21% protein) had weight gains similar to those given diets containing 85% raw fababean but lower than those given the other diets.

The effect of varying heat treatment periods (autoclaving for 0, 15 or 30 min at 121^{o}C) on the productive performance of chicks and

rats was investigated by Marquardt and Coworkers (1974a, b). All diets contained 90% fababeans and were supplemented with methionine. Autoclaved (average of the 15 and 30 min. autoclaving times) as compared to raw fababeans, in diets of chicks and rats resulted in significant ($P<0.05$) improvements in the respective weight gains (9 and 7%) and feed/gain ratios (14 and 5%). Heat treatment of fababeans also reduced ($P<0.01$) feed intake (6%) and pancreas (16%) and liver (4%) sizes in chicks. Autoclaving for 30 instead of 15 minutes tended to give greater improvements in growth response. The differential response of chicks and rats to autoclaving of fababeans suggest a difference between chicks and rats in their sensitivity to the thermolabile factor(s) in raw fababeans. Hemagglutinin levels were markedly reduced and trypsin inhibitor levels were reduced by approximately 50% in fababeans that had been autoclaved for 15 or 30 minutes. In comparison to raw soybeans the level of trypsin inhibitor in raw fababeans is about 20 fold lower, but the hemagglutinin activity of raw fababeans is higher than that of raw soybeans and contains some activity that is not denatured by autoclaving (121^{o}C) for 15 minutes.

In another experiment (Marquardt et al. 1976) fababeans were autoclaved for 0, 3, 10, 20, 30 or 40 minutes at 121^{o}C and added to diets at a level of 85%; all diets were supplemented with methionine and then fed to chicks. It was found that weight gain and feed efficiency were improved ($P<0.05$) after heating for 20 minutes; there was further improvement with the 30-minute autoclaving time, but no additional improvement thereafter. Conversely pancreas weight was reduced ($P<0.05$) after only 10 minutes of autoclaving and was similar to the 20-minute

autoclaving time; both the 30- and 40-minute autoclaving times had similar effects and caused slightly greater depression in pancreas size.

Since all soybean hemagglutinin activity is destroyed by autoclaving for 15 minutes it is suggested that the lectins in the two beans are different or fababeans contain some heat-resistant lectins. It may be concluded that autoclaving (121°C) for 15 minutes virtually eliminates the low level of growth depression that may be caused by trypsin inhibitors and hemagglutinins. The cause of the additional and major portion of the heat sensitive growth depression has not been established. Perhaps some factor more resistant to heat than trypsin inhibitors and hemagglutinins is responsible.

Marquardt et al. (1976) investigated the effect of autoclaving (121°C for 20 min), microwave heating (for 20 or 30 min) and effect of moisture content of fababeans, steam pelleting (at 70°C) or extrusion at 130 or 152° on the productive performance of chicks. Pelleting and microwave treatment of fababeans that contained 10% moisture resulted in maximal improvements in chick growth rates and feed efficiency of only 6% and 8%, respectively. Autoclaving extrusion (130° or 152°C) or microwave treatment of fababeans containing 18% added water resulted in maximal improvements of 16% in growth rates and 19% in feed/gain ratios. Microwave treatment (20 or 30 min) reduced hemagglutinin activity by only 50% but destroyed nearly all of the trypsin inhibitor activity; pelleting had no effect on trypsin inhibitor or hemagglutinin activities. Conversely, the other treatments destroyed most of the activities of both trypsin inhibitors and hemagglutinins, and resulted in better chick performance. This latter result does not mean that the better chick per-

formance was due to destruction of lectins or trypsin inhibitors as has already been disproved. However, moist heat is more effective than dry heat in denaturing the growth inhibitor(s).

Grey et al. (1972) reported that pelleted rations containing 45% fababeans resulted in heavier birds compared to the control diet. Infrared radiation has also been shown to improve the ME value of fababeans when fed to broiler chicks (Edwards and Duthie 1973). Dehulling removes not only the fibrous component of the seed but also condensed tannins. These procedures increase the digestibility and availability of nutrients particularly amino acids and may be benificial for rapidly growing chicks.

Overall it may be concluded that fababeans are deficient in methionine and that supplementation of fababean-containing diets results in markedly improved utilisation by the growing chicken. Fababeans also appear to contain two types of heat sensitive antinutritional factors. One of these factors is a condensed tannin which is located in the testae of the bean. The second heat sensitive factor which does not seem to be a hemagglutinin or trypsin inhibitor is located in the cotyledon portion of the bean. Prolonged heat treatment (e.g. autoclaving at 121^{o}C for 30 minutes) destroys the major antinutritional factor located in the testa. In addition to the heat-sensitive antinutritional factors there may also be heat stable antinutritional factors.

(ii) Laying hens

Low levels of fababeans (less than 20%) properly supplemented with the sulfur amino acids, particularly methionine are considered

safe in laying hens (Vogt 1972; Wilson and Teague 1974; Davidson 1973; Robblee et al. 1977). High levels of dietary fababeans have been shown to depress the rate of lay and egg weight (Marquardt et al. 1974a; Robblee et al. 1977). Decreased feed efficiency (Vogt 1972; Robblee et al. 1977) and increased mortality (Robblee et al. 1977) have also been reported. Results with laying hens like those of growing chickens are variable, and again this could be due to effects of cultivar, growth and storage environment on the content of the toxic factors (Pitz 1979) and/or the variability of certain dietary ingredients.

Vogt (1972) observed that while 10% fababeans in laying rations supplemented with S-amino acids had no adverse effects, the use of 20% fababeans resulted in decreased feed conversion efficiency and egg weight. Similarly Wilson and Teague (1974) reported that 20% fababeans in laying rations significantly ($P<.05$) decreased egg weight, but had no effect on egg production or feed intake. Davidson (1973) concluded that fababeans supplemented with methionine could be a useful source of dietary protein, but stipulated that not more than 15% fababeans should be used in laying rations. Marquardt et al. (1974a) indicated that 25% raw dietary fababeans lowered egg prodcution rate and depressed egg weight even more. Feed consumption was not affected. Methionine supplementation (0.13%) resulted in normal egg production rate but did not improve egg weight. In two experiments Robblee et al. (1977) investigated the effect of various levels of raw dietary fababeans (0, 5, 10, 15, 20 or 30%) and supplemental methionine and cystine on the performance of laying hens. The rations were designed to be isocaloric, isonitrogenous and to have equal levels of methionine

plus cystine. The results obtained indicated that levels up to and including 20% fababeans in rations supplemented with methionine (0.28% in diet) had no adverse effect on mortality, egg production rate, feed conversion efficiency or body weight. A level of 30% fababeans resulted in increased mortality, and decreased egg production rate and feed conversion in one experiment. Egg size tended to decrease with the increase in the level of fababeans in the ration. There was, however, an increase in Haugh units as the level of dietary fababeans was increased, but specific gravity of the eggs was not affected by dietary fababeans. The cause of egg depression in fababeans was not known. Olaboro (1978) investigated the effects of feeding high levels (40%) of autoclaved (121^{o}C for 10 min) fababeans, various levels (15 or 30%) of whole and dehulled fababeans which represented different energy levels, and methionine supplemented fababeans (25%) to laying hens on their productive performance. Egg production rate was not affected ($P>0.05$) by the fababean content of the diet, but egg weight was depressed. Egg depression could not be corrected by autoclaving, but was improved ($P<0.01$) by methionine supplementation at levels over and above the NRC (1977) recommendations for the laying hen. The extent of egg size depression was proportional to the level of whole and dehulled fababeans added to the diet.

Feed intake and feed conversion were not responsible for the depression in egg weight although body weight gain which was not ($P>0.05$) affected, tended to increase with increases in the level of methionine in the fababean containing diets. Mortality was normal. In an attempt to facilitate the identification of the causitive agent(s)

(the egg size depressing factor), short term (32 days in length or less) trials were conducted and a standard bioassay test procedure was developed. This test procedure consisted of groups of individually caged hens, with one group designated as the control group and the other(s) as treatment. Control diets were fed to the control group at all times whereas fababean diets were fed to the treatment group(s) during the test periods which were separated by a control period during which the control diet was refed. Eggs were collected and weighed daily. The effects of the treatments were evaluated by representing the data graphically and by statistical analysis which involved a comparison between groups of the percent change in egg weight during a test period in relation to a preceeding control period.

Energy or protein level in the diet was shown not to be the major cause of egg weight depression. Addition of 12% raw or autoclaved (121^{o}C for 10 min) fababean protein concentrate to a laying hen diet depressed ($P<0.01$) egg weight. Fababean hulls (10% of the diet) or fababean starch (26% of the diet) did not ($P>0.05$) depress egg weight. These results, thus precluded the trypsin inhibitors, hemagglutinins or condensed tannins from being involved in the egg weight depression.

An ethanol-water (3:2 or 1:1) extract of raw or autoclaved FBPC depressed ($P<0.01$) egg weight. The magnitude of the egg weight depression was dependent on the concentration of the causative agent. Fractionation of the ethanol-water extract of autoclaved FBPC by lowering pH with hydrochloric acid (HCl) produced fractions which did not ($P>0.05$) depress egg weight. However, acetone fractionation of the extract produced a supernatant that depressed ($P<0.05$) egg weight.

This supernatant was composed of 84% of a very water-soluble fraction and 11.5% of a relatively water-insoluble fraction. Both fractions depressed ($P<0.01$) egg weight, although the concentration of the active component was higher in the insoluble fraction. The insoluble fraction contained 80 to 90% total vicine and was recrystallised in water to produce crystals which, by determination of various physical properties, were identified as vicine crystals, containing 96% vicine. Convicine was detected in the residue of crystallisation. Chemical analysis of faba-bean fractions showed that the starch and hull fractions that did not ($P<0.05$) depress egg weight contained little or no vicine activity while the potency relative to egg weight depression of the other fractions was proportional to vicine activity. It was concluded that vicine and convicine were the egg weight depressing factors in faba-beans with vicine being the major principle.

In these studies Olaboro (1978) also observed that these compounds caused a lowering in yolk weight, liver lipid content, and to a lesser degree, liver weight. L-Dopa, and a number of amino acids that were also present in low concentrations in the isolates obtained by acetone fractionation of the ethanol-water extract did not cause egg weight depression, even at concentrations more than twice that in the control diet. Since dietary energy levels were without effect and were not limiting it was concluded that vicine interfered with lipid synthesis, which resulted in the formation of a small yolk; a small egg is, therefore formed as other egg components are laid down in proportion to the yolk size. There also is a possibility that vicine may interfere with synthesis of proteins involved in lipid transport into the yolk. The

lipids could therefore accumulate in plasma and be unavailable for maximal rate of yolk formation. The reduction in milk protein (Ingalls et al. 1979) in dairy cows fed fababeans may lend support to this hypothesis. Alternatively, the vicine or its metabolic products may attack the lipoproteins in plasma en route to the yolk, producing forms that are either unstable or cannot be transported across membranes to the ovum to form the yolk. It is important,
however, to establish the mode(s) of action of vicine in causing egg weight depression in the hen as this could become an animal model for studying favism in man.

(e) Antinutritional factors in fababeans

Fababeans contain several antinutritional factors some of which are heat sensitive and others that are relatively insensitive to heat destruction. The thermolabile factors seem to be equally distributed between the hull and cotyledon. That of the cotyledon is more specifically associated with the protein fraction. The heat labile factors in the hull consist mainly of several polyphenolic compounds including condensed tannins (Marquardt et al. 1977; Ward et al. 1977; Cansfield et al. 1980), and trypsin inhibitors (Marquardt et al. 1975) but the hull does not possess hemagglutinin activity. Condensed tannins complex with a lot of dietary nutrients particularly proteins and amino acids rendering them unavailable to the animal. The effect of condensed tannins can be prevented by heat treatment, dehulling or selective binding with PVP or feeding tannin-free cultivars. Trypsin inhibitors are generally easily denatured by heat treatment.

The thermolabile factors in the cotyledon consist of lectins (hemagglutinins), trypsin, chymotrypsin and amylase inhibitors. However, these seem to have a minor effect on animal performance and are readily denatured by autoclaving for 10-20 minutes. There seems to be a relatively heat insensitive factor that is destroyed only after autoclaving for at least 30 minutes. It does not seem that this effect is due to general protein denaturation and therefore increased digestibility (Marquardt, personal communication).

The heat resistant factors are mainly associated with the protein portion of the cotyledon, and consist mainly of pyrimidines and pyrimidine glucosides of which vicine and convicine have been identified. Very low levels of vicine and convicine are found in the hulls of some but not all fababean cultivars. L-Dopa and its glucoside are also heat resistant factors that are mainly concentrated in the green valve of green pods, and some in the hull but not the cotyledon of fababeans. The biological effects of these factors are discussed in detail in the subsequent sections.

(i) Trypsin, chymotrypsin and/or other protease inhibitors

Trypsin inhibitors are found in varying concentrations in most legumes. Soybeans seem to have the highest activity, while fababean extracts exhibit 2.9% to 20% of the activity exhibited by similar extracts from soybeans (Wilson et al. 1972a; Marquardt et al. 1974a; Bhatty 1975). Some of the trypsin inhibitors isolated from fababeans seem to be general proteases inhibitors (Warsy et al. 1974). Indirect studies (Nitsan 1971) point to the existance of an amylase inhibitor(s) in fababeans. These enzyme inhibitors are a potential threat to nutrient

utilisation in both man and farm livestock but the concentrations in fababeans do not cause a practical threat.

The concentration of the trypsin inhibitors in fababeans is higher in the hull than the cotyledon (Wilson et al. 1972a; Marquardt et al. 1975), however, the hull is only 13% by weight of the whole bean, and therefore based on data of Marquardt and associates (1975) would contribute only 29% to the total trypsin inhibitor activity in the seed. The air classification process of fababeans produces protein and starch fractions which are high (8.4 units/g) and low (0.4 units/g) in trypsin inhibitor activity, respectively (Marquardt et al. 1975).

Heat labile and thermostable trypsin inhibitors have been recognised in the fababean and soybean (Nitsan 1971; Wilson et al. 1972a; Marquardt et al. 1974a; Warsy et al. 1974; Bhatty 1975). Since stability of the trypsin inhibitors is influenced by the pH of the solvent (Warsy et al. 1974), while the yield of the inhibitor depends on both the pH and the type(s) of salt in the extraction solvent (Bhatty 1975), comparisons among data from different laboratories using different reagents may be futile. Trypsin inhibitors in the diet have also been implicated in decreased feed efficiency and hypertrophy of the pancreas in the chick and weanling rat (Wilson et al. 1972a, b). Thus it can be concluded that the enzyme inhibitors seem to be in low concentrations in fababeans and therefore may not affect animal performance; and in case they do, can be easily denatured by heat treatment.

(ii) Hemagglutinins

Hemagglutinins are proteins and/or glycoproteins that can

clump (agglutinate) red blood cells. They have been found mostly in plants. A small number of similarly acting substances have been isolated from snails and fish eggs, slugs, sponges and others. These are sometimes called protectins. However, hemagglutininins of plant origin (also known as agglutinins, phytohemagglutinins, phytagglutinins) are present in greater concentration and have been studied in greater detail (see review by Jaffe 1973) than those obtained from other sources. Therefore the rest of the literature deals with these plant agglutinins, and the popularly accepted term, lectins will be employed instead of hemagglutinins.

Lectins differ widely from each other and may exhibit considerable specificity in regard to blood groups. Some lectins have been referred to as being non-specific (see review by Jaffe 1969, Sharon and Lis 1972) because they can agglutinate cells of any human blood group. However, these same lectins show characteristic differences in sugar specificity, and when in contact with RBC from different animal species. In fact a small fraction of the known lectins exhibit blood group specificity.

Most lectins are heat labile and normal cooking destroys their specific action. Many of them, however, are remarkably stable in presence of a number of proteolytic enzymes. This might indicate that there are some non-protein lectins or that their conformation protects them from proteolysis.

When comparing lectin activities of different plant species the source of RBC used has to be the same because of the problem of specificity and the diverse numbers and nature of the lectins (see review by Jaffe 1969; Marquardt et al. 1975). There could be variety

differences within the same plant and/or animal species. In addition, the capacity to cause agglutination depends on the concentration of the lectins; this concentration is not standard for all lectins.

The biological effects of isolates of lectins are influenced by the animal species, the route of administration, and the source and concentration of lectins in the medium (Jaffe 1973; Pusztai et al. 1975; Marquardt et al. 1975). Thus lectins isolated from soybean protein were found lethal when injected into rats, while comparable or higher dietary levels (Jaffe 1973) only depressed growth rate. However, death resulted from ingestion of lectins from other legumes (Jaffe 1973). In phaseolus species lectins are heat stable and are extremely toxic (Jaffe 1973; Pusztai et al. 1975) when ingested. In contrast lectins in other legumes and cereals seem to be relatively harmless when ingested in the diet. These results would suggest that lectins are harmful to the animal following their ingestion only if they remain stable in the animal gastrointestinal tract and are absorbed intact.

In fababeans hemagglutinin activity is restricted to the cotyledon (Marquardt et al. 1975). Earlier studies with the chick attributed the reduction in growth rate and feed efficiency to lectins in raw dietary fababeans (Livingstone et al. 1970; Wilson et al. 1972a,b; Marquardt et al. 1975). However, Marquardt and Coworkers (1976) have presented data which show that the lectins in fababeans are heat sensitive and readily denatured by autoclaving (121^{o}C) for 10-15 minutes, and yet this results in minimal improvement in chick performance. In addition, autoclaving for 30 minutes, further improves chick performance,

and over 50% of the growth depression due to fababeans was associated with the hull portion of the bean which is devoid of lectins. These observations would suggest that fababean lectins are relatively unimportant as an antinutritional factor.

(iii) Tannins

Any plant polyphenolic substance with a molecular weight exceeding 500 can be considered to be a tannin (Singleton and Kratzer 1973). Tannins are categorised into hydrolysable and condensed forms, based on their resistance towards hydrolytic agents, particularly acids (Haslam 1966). The hydrolysable tannins which have a polyester structure are readily hydrolysed by acids or enzymes. Condensed tannins are quite stable under physiological conditions and hydrolyse only under drastic conditions.

Although both groups exhibit protein-binding and leather-forming properties, they differ in their botanical distribution, breakdown products and other properties. Hydrolysis of the hydrolysable tannins yields tannic acid, which is readily hydrolysed spontaneously or enzymatically to a sugar or related polyhydric alcohol and a phenol carboxylic acid (Fig. 1.1). The hydrolysable tannins can be subdivided into gallotannins and ellagitannins depending on the nature of the phenol carboxylic acid produced on hydrolysis. Thus on hydrolysis the gallotannins release gallic acid while the ellagitannins yield hexahydroxydiphenic acid (isolated normally as its stable dilactone ellagic acid), or acids which can be considered to be derived by simple chemical transformations of hexahydroxydiphenic acid such as oxidation, reduction and ring fission.

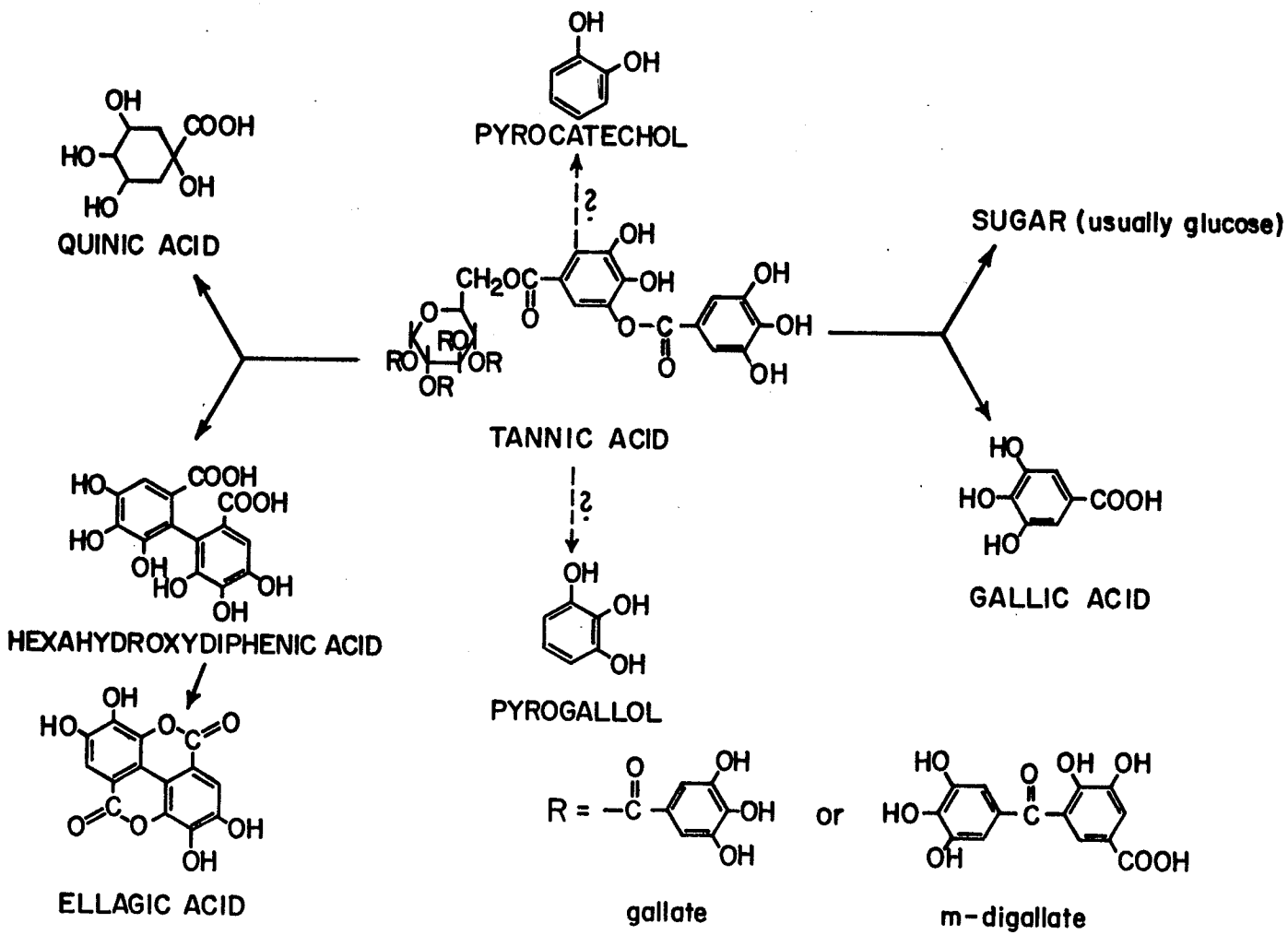

Fig. 1.1 Tannic Acid and its common hydrolysis products.

The condensed tannins (flavolans) are polymeric flavonoids which are synthesized mainly from leucoanthocyanidin (leucoanthocyanin or flavan-3, 4-diol) units linked carbon to carbon from the 4-position of one unit to the 6- or 8-position of the next (Fig. 1.2). When hydrolysis occurs, condensed tannins produce either the less soluble polymeric "phlobaphenes" or flavonoid monomers, particularly catechins and anthocyanidins.

Tannins have been isolated from a variety of plants either in the leaves, bark and/or seeds (Haslam 1966; Singleton and Kratzer 1973; Strumeyer and Malin 1975; Marquardt et al. 1977). Marquardt and Co-workers (1977) reported that the condensed tannins isolated from fababeans had properties similar to those isolated from sorghum grains (Strumeyer and Malin 1975) and that the tannins were essentially restricted to the testa portion of the seed. Several common cultivars of fababeans (Vicia faba L.) grown under similar environmental conditions were found to have similar concentrations of condensed tannin; the average percent tannins in the testae of eight cultivars was 4.3± 0.2 SEM (Marquardt et al. 1978a). However, cultivars from different locations within Canada were found to vary in their tannin content. This was attributed to environmental differences during growth and/or storage of the beans; these workers found that storage of beans at 23°C for 14 months resulted in an average decrease in tannin content of the testae of 11 percent. The reason for the decrease is not known, but it is possible that the tannins are slowly oxidized and/or interact with the protein portion of the bean. The effect of the decrease in condensed tannin content with storage time and/or conditions, on the

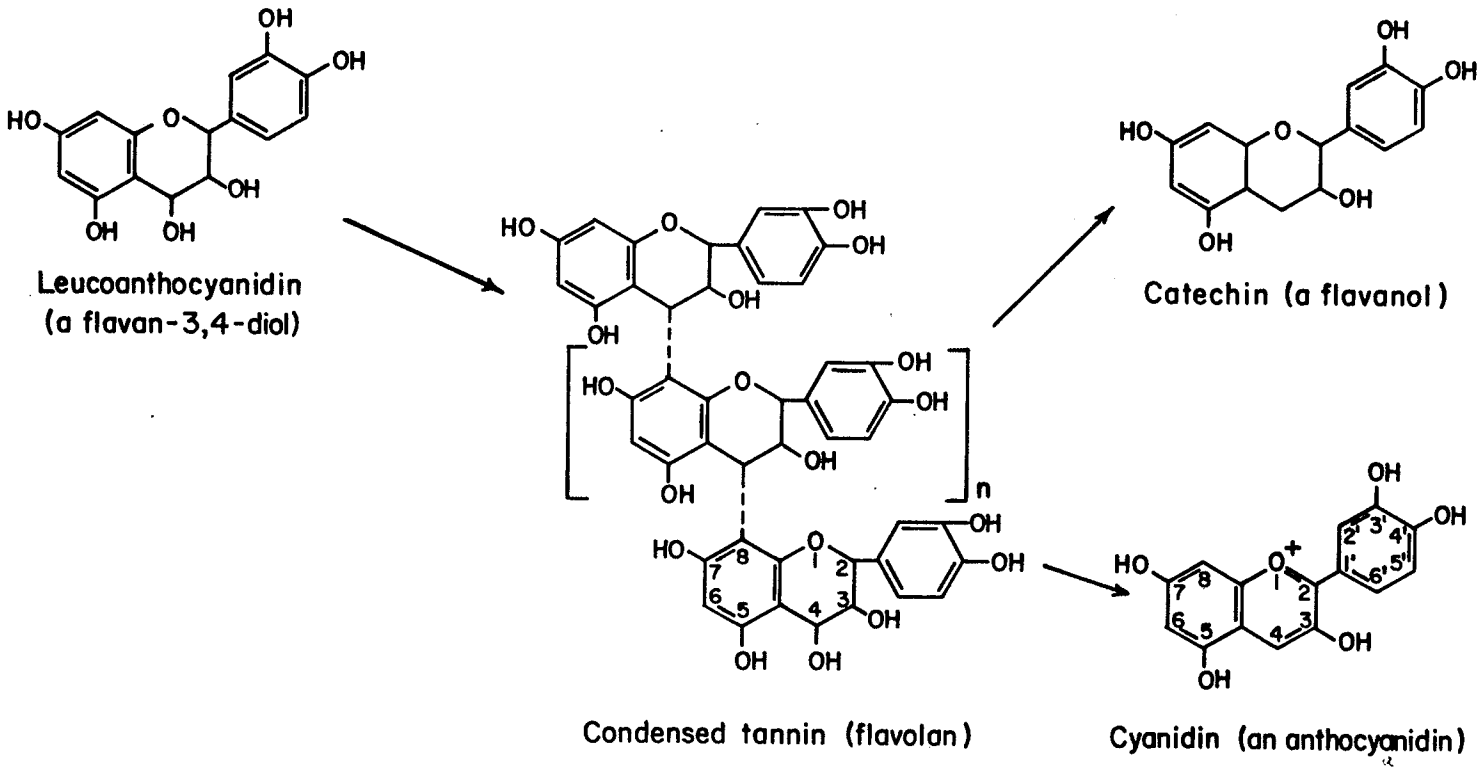

Fig. I.2 Condensed tannins and some of their common hydrolysis products.

nutritional quality of the fababeans is not known. The analysis for condensed tannins levels in a diverse selection of fababean cultivars resulted in the identification of three cultivars of Vicia faba that were tannin-free (Marquardt et al. 1978a). These cultivars, Kodrin, Tripple white and Fidrim were all obtained from Europe (Holland). The tannin-free cultivars are characterised by flowers, testae and hilum that are white (Bond 1976; Marquardt et al. 1978a) and are deficient in those compounds responsible for the formation of a dark-coloured polyjeric complex when exposed to oxygen (Marquardt et al. 1978a), Marquardt and coworkers (1978a) identified these compounds as being responsible for darkening that occurred during their migration on a sephadex LH-20 column.

The biological and biochemical effects of tannins are diverse; this would be expected because of the diverse nature of types and products of tannins. Tannins from different plant species have been shown to have undesirable effects on man, rats, chickens and other animal species (Singleton and Kratzer 1969; 1973). Their effects vary depending on the type(s) of tannins, their concentration(s), the animal species and their physiological state, the level(s) and type(s) of dietary amino acids, and the route of administration of the tannins.

Dietary tannins retard growth rate in chicks (Chang and Fuller 1964; Rostagno et al. 1973a; Marquardt et al. 1977; Ward et al. 1977) at levels as low as 0.5% in the diet (Vohra et al. 1966; Rayudu et al. 1970). Tannic acid and other tannins at 5% level in diet caused mortality among chicks (Vohra et al. 1966); condensed tannins were less detrimental than tannic acid, as was expected (Singleton and Kratzer

1969). The hydrolysis products of tannic acid (particularly pyrocatechol and pyrogallol) are more growth-depressing and toxic to rats and chicks than either tannic acid or the condensed tannins (Vohra et al. 1966; Joslyn and Glick 1969; Rayudu et al. 1970). Destruction of the intestinal epithelium following oral tannic acid ingestion is more severe in newborn than older rats. Similarly effects of repeated tannic enemas were found to be more severe in juvenile patients. The severity was even greater if the juvenile patients had pre-existing ulcers or inflammation (Boyd et al. 1965) which could cause severe liver damage and result in death (Janower et al. 1967; Singleton and Kratzer 1969). The ulcers supposedly facilitate the absorption of large tannin-protein complexes from the gastrointestinal tract. The ability of newborn animals to absorb large milk proteins and hence tannin-protein complexes may also account for their greater susceptibility. Rectal administration of tannins to patients was found more severe than the oral route. Possibly the protein concentration in the digesta is lower and hence complexing with tissue protein is more extensive. Further a small injection of tannic acid to rats causes recognisable cellular and subcellular changes within an hour in the kidney and liver. These effects include reduced liver glycogen, proteinuria, altered mitochondria and fragmentation of polyribosomes associated with strong inhibition of protein and RNA synthesis in the liver. Carcinogenicity seems to be a definite component in tannin toxicity in both rats and man (Kirby 1960; Korpassy 1961; Morton 1970). Tannins seem to interfere with the transport of colour pigment from the digestive tract to the egg yolk in chicken; mottled and green egg yolks are typical of chickens after

ingestion of tannins at levels as low as 1% of diet (Potter et al. 1967; Weber 1970; Fry et al. 1972; Hughes 1973). Pyrocatechol and pyrogallol are somewhat similar to divicine and isouramil except that the former do not contain nitrogen. Hence the observation that tannic acid and other hydrolysable tannins cause, among other things, anaemia and decreased hemoglobin levels in rats (Lease and Mitchell 1940; Singleton and Kratzer 1969) is intriguing in relation to favism.

The mechanism(s) by which tannins lead to the above abberations are not clearly understood. However, effort has been made towards understanding these mechanisms. Grain sorghums and fababeans (Vicia faba L.) high in tannins are less digestible than those varieties low in tannins (Rostagno et al. 1973b; Bond 1976; Marquardt et al. 1978b). Bond (1976) found that the in vitro dry matter digestibility of the whole bean (Vicia faba L) was 4.7% higher in tannin-free compared to tannin-containing varieties; the corresponding values for dry matter digestibility in the whole bean and the testae portion of the bean were 56.4% and 17.2% respectively for the tannin-containing cultivars. The differences between varieties in acid-pepsin solubility and protein content of the testae were much smaller and not associated with the presence of tannins. It was concluded that some factor in addition to tannins influenced digestibility. This factor seemed to be independent of seed size and proportion of testa. Marquardt et al. (1978b) found significant ($P<0.01$) fraction and cultivar differences, and cultivar x fraction interaction in the in vitro digestibilities of tannin-free and tannin-containing cultivars of fababeans. The fraction differences were attributed to the much higher digestibility of the cotyledon (88%) as compared to the

testa portion of the bean (18.4%). The differences among cultivars was primarily due to differences in the in vitro digestibility of the testa rather than of the cotyledon portion of the bean. Thus in one trial the digestibilities of the cotyledon fractions varied only between 87 and 91%, while those of the testae were between 8.3 and 34.4%. In all cases the in vitro digestibilities of the testae from tannin-containing cultivars (9.2-11.2%) were lower than those of the tannin-free cultivars (26.3-27.7%). Thus the cultivar x fraction interaction was also attributed to a rather uniform digestibility of the cotyledon but a variable digestibility of the testa portion of the various cultivars.

In addition to differences in tannin content the testae of tannin-free and tannin-containing cultivars also had different ($P<0.01$) levels of lignin, acid detergent fibre and cellulose (Marquardt et al. 1978b). Remarkable is the fact that tannin-containing cultivars had higher lignin levels than tannin-free cultivars, and negative correlations were obtained between the in vitro dry matter digestibility and the amount of either condensed tannin ($r = -0.86$, $P<0.01$) or lignin ($r = -0.89$, $P<0.01$). When expressed on a similar basis there was no correlation between levels of cellulose or acid detergent fibre and in vitro dry matter digestibility. Thus in part complementing the results of Bond (1976).

Fababean condensed tannins also affect chick performance. When Marquardt and coworkers (1977) fed chicks a diet containing 3.9% of purified condensed tannins from fababeans they observed negative weight gains and feed:gain ratios. In addition feed intake (69%), and dry matter (22%), amino acid (25%) and crude fibre (175%)

retentions were markedly reduced ($P<0.01$). Fat retention in contrast, was increased by 27% ($P<0.01$). Further, autoclaving (121°C for 30 min) the diet with condensed tannins did not completely alleviate the growth depression or the high feed:gain ratios compared to chicks fed tannin-free diets; but autoclaving and PVP (a tannin-binding agent) together, completely alleviated the effects. Nelson et al. (1975) fed chicks hybrid sorghums of varying tannin content. They obtained significant ($P<0.05$) negative correlations between tannin content and ME ($r = -0.62$), percentage of gross energy utilised ($r = -0.62$), dry matter digestion (-0.63) and amino acid availabilities (-0.82). However, the gross energy content of these hybrids were not correlated ($r = 0.1$, $P>.05$) with tannin content.

Phenols combine with proteins reversibly by hydrogen bonding and irreversibly by oxidation followed by covalent condensation (Loomis and Battaile 1966). If tannins and their hydrolysis products managed to enter the blood circulation, tannin-protein precipitates would result which would be lethal to the animal. Further, dietary tannins would react with proteins, other complexing agents and even secreted enzymes in the digesta. It is not surprising therefore that dietary tannins preferentially depress protein digestibility and availability, and in some cases have been shown to particularly lower availability of methylating amino acids like methionine (Loomis and Battaile 1966). Conversely, supplementing a diet containing tannins with higher than normal levels of protein or the amino acids, methionine, arginine (or, ornithine) alleviates the tannin effects depending on the levels of the latter in the diet (Fuller et al. 1967; Rayudu et al. 1970; Rostagno

et al. 1973a). Iron and calcium salts, PVP and other compounds that can precipitate, oxidize or bind tannins do aid in preventing toxicity if added to the diet (Singleton and Kratzer 1969; Marquardt et al. 1977).

Finally, another compound that may interfere with the availability of methionine in fababean diets and yet react as methionine in the cyanogen bromide cleavage assay system is S-methyl-L-cysteine. It can also be assayed as a sulfur amino acid. No information is available concerning the distribution of S-methyl-L-cysteine in different cultivars of fababeans. However, it has been detected in some varieties of vicia faba L. minor and major (Evans and Boulter 1975). Though the levels are low in fababeans (0.035-0.041 g/16 g N), these levels are about four times those found in soybean.

(iv) Vicine, convicine and L-Dopa

(a) Vicine and convicine

(i) Properties of vicine and convicine

Vicine and convicine were first isolated from seeds of Vicia sativa (Ritthausen and Kreusler 1870; Ritthausen 1881) and later in Vicia faba (Johnson 1914; Fisher and Johnson 1932). Since then vicine has been detected in a number of plants including beet juice and peas (Bendich and Clements 1953; Jamalian et al. 1977a) and in Lupinus albus (Pompei and Lucisano 1976). It is interesting that Lupinus albus can contain up to 6.55% total glycoside (Pompei and Lucisano 1976) compared to 2% in fababeans (Brown and Roberts 1972). Vicine and convicine are pyrimidine glycosides and their structural formulae (Fig. 1.3) have been established (Bendich 1952; Bendich and Clements 1953; Bien et al. 1968). Both glycosides are crystalline in

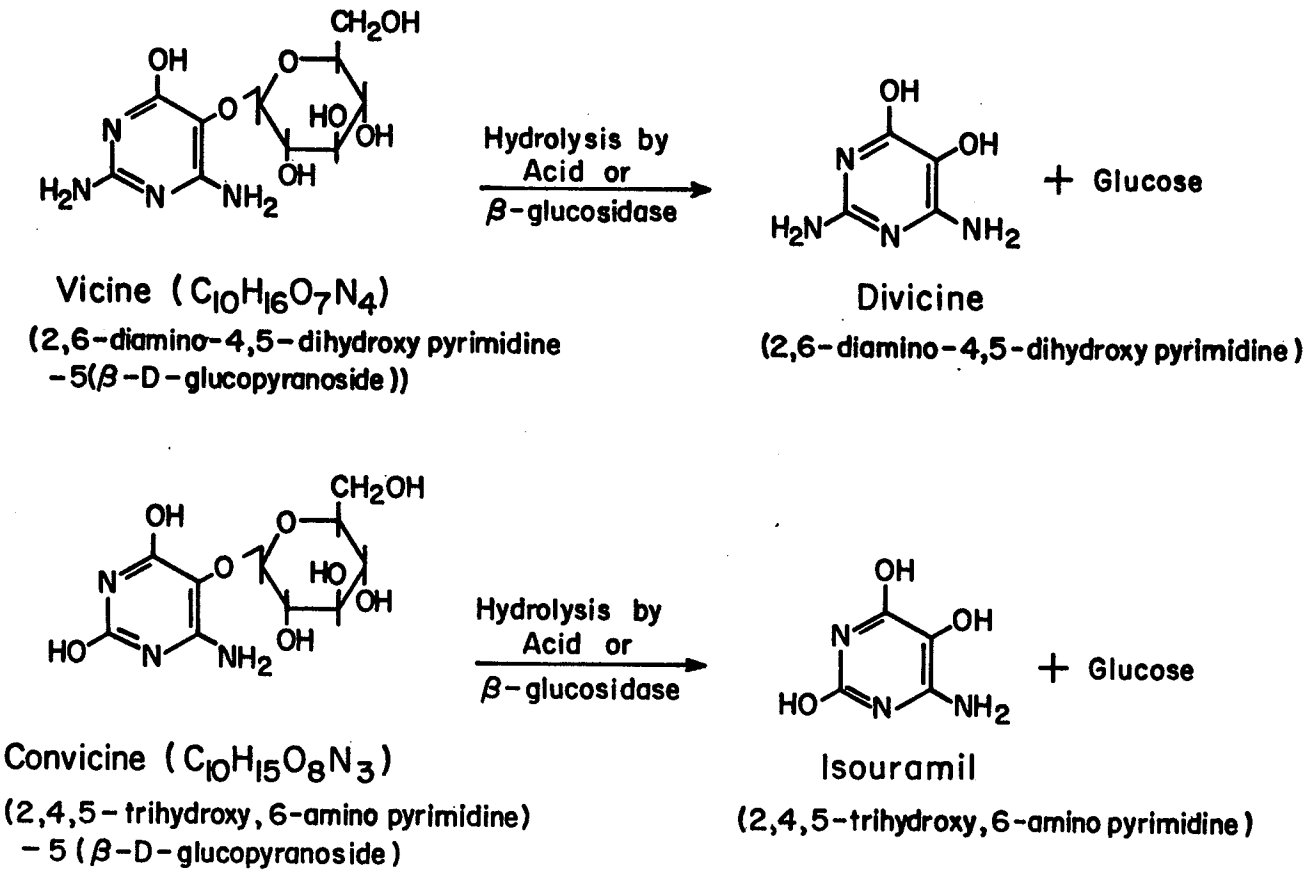

Fig. 1.3 Structure of Vicine, Convicine and their aglycones.

nature and decompose without melting, though at different temperatures; vicine at 239-243°C (Levene 1914; Gmelin and Hasenmaier 1957; Lin and Ling 1962a; Bendich and Clements 1953) and convicine at 287-288°C (Fisher and Johnson 1932; Bien et al. 1968).

Vicine is soluble in dilute acids and alkali, slightly soluble in water (1 g/100 ml), less soluble in alcohol and virtually insoluble in non-polar organic solvents such as ether, acetone and chloroform. The solubility properties of convicine are similar to those of vicine, except that convicine is not readily soluble in dilute acids. The ultraviolet (UV) absorption spectra of vicine show a maxima at 275 and a minima at 248 nm in aqueous solution (Lin and Ling 1962a); the maxima shifts to 274 nm in 0.1 HCl, and in alkaline medium the maxima and minima occur at 269 and 248 nm, respectively. Vicine has a molar absorptivity (ε) of 16.4×10^3 in 0.1N HCl solution (Bendich and Clements 1953); its specific rotation has been reported as $(\alpha)_D^{20°C} = -11.7$ in 0.2N NaOH (C = 3.9) (Bendich and Clements 1953) or $(\alpha)_D^{15°} = -12.1$ (C = 6.7) (Winterstein 1919). Convicine UV spectra show maxima and minima, respectively at 273 and 245 nm in alkaline solution, 271 and 245 in neutral solution, and two maxima (at 271 and 220 nm) and minima (at 242 and 214 nm) in acid solution; the molar absorptivity at pH 1 is 17.3×10^3 (Bien et al. 1968).

Solutions of vicine in water or N NaOH show no perceptible change in 5 days at 37°C but in 2N HCl vicine is almost completely hydrolysed after 4 days (Bendich and Clements 1953). Due to the structural similarity, convicine is likely to react in a like manner. On treatment with β-glucosidase or mineral acids vicine and convicine liberate glucose and their respective aglycones, divicine and isouramil (Herissey and

Cheymol 1931; Bendich and Clements 1953; Mager et al. 1965, 1969).

The solubility properties of divicine (1 g/350 ml H_2O) are similar to those of vicine and at temperatures above 280°C it decomposes without melting (Lin and Ling 1962a). Divicine UV absorption spectrum differs from that of vicine in acid solution (0.25N HCl) in that it shows a maximum at 280 nm. However, the UV spectrum in aqueous solution is similar but the maxima is reduced on prolonged exposure to UV light (Bendich and Clements 1953; Lin and Ling 1962a). The spectral properties of isouramil are similar to those of divicine.

Unlike vicine and convicine that are stable as solids and in most aqueous solutions, the aglycones are highly unstable in presence of oxygen. The aglycones are more stable as solids than in solution. Their rate of breakdown is most rapid at alkaline pH and falls with decreasing pH. At room temperature, the half-life of divicine in neutral solution has been estimated at 30-40 minutes. The breakdown is accelerated by traces of copper (II) and other heavy metals, but is inhibited in a nitrogen or carbon dioxide atmosphere. Unlike the glycosides, the aglycones in solution are almost instantaneously destroyed by boiling (Bendich and Clements 1953; Mager et al. 1969).

The presence of a free phenolic hydroxyl group at carbon 5 which is substituted in the glycosides, makes the aglycones very reactive compared to the unreactive glycosides; the former are strong reducing agents in their reduced form (Fig. 1.4) (Bendich and Clements 1953) whereas the latter compounds are not. Divicine and isouramil are similar to dialuric acid and ascorbic acid in some of their properties such as the strong reducing nature, spectral characteristics and molecular instability in oxygen (Bendich and Clements 1953; Mager et al.

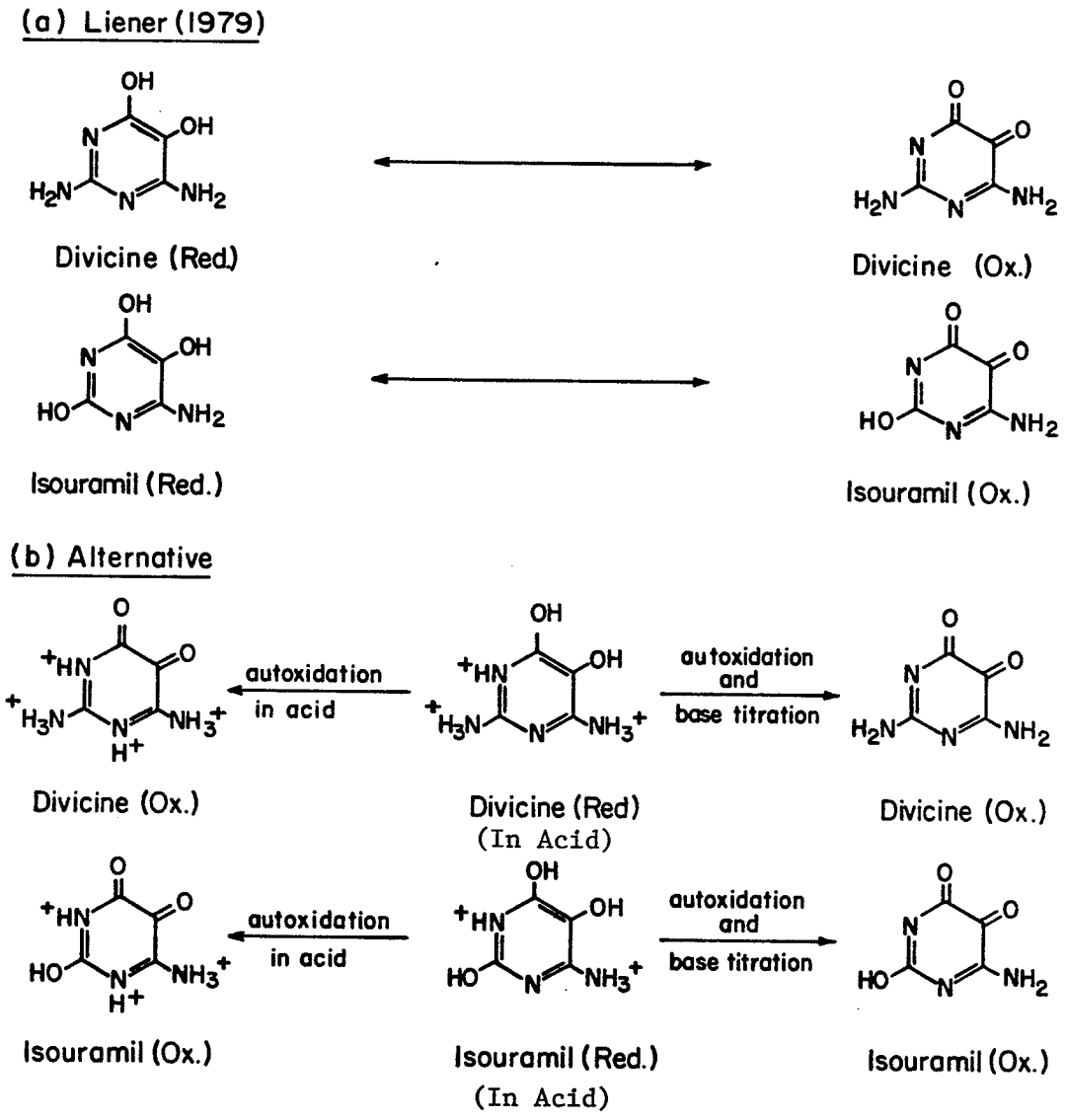

Fig. I.4 Reduced and oxidized forms of Divicine and Isouramil.

1969). This similarity is due in part to the carboxyl-conjugated aminoenol and enediol rings of these compounds. Although dry fababeans are devoid of ascorbic acid the fresh beans contain about 22 mg/100 gm wet weight (Collier 1976) and therefore might interact synergistically with the aglycones to enhance their deleterious effects.

(ii) Synthesis and distribution of vicine and convicine in fababeans (Vicia faba L.)

The glycoside content of fababeans depends on the stage of maturity, genotype and growth environment. The formation of vicine and convicine has been studied in germinating seeds and young fababean seedlings using ^{14}C-labelled precursors (Brown and Roberts 1972). It was found that the glycosides were synthesised within the pod and not translocated into them. Their data indicated that the orotic acid pathway may be involved in the formation of the pyrimidine ring of the glycosides. Synthesis of either of the glycosides was not observed in the seeds or pods of young pods up to 10 cm in length. However, in pods 13-15 cm long both glycosides were readily detected in the seeds, but not in the pods. Jamalian and Bassiri (1978) confirmed the absence of vicine in fababean pods, but contrary to the findings of Brown and Roberts (1972) young seeds from pods 1-2 cm long were found to contain 1.14% vicine-reactive material. This increased to about 2.46% when pods were 5-6 cm long and then decreased to about 0.9% at pod maturity. The pattern of variation in concentration was similar for whole seeds, cotyledons and seed coats. This pattern of variation is in accord with earlier reports (Jamalian et al. 1977b) that a higher degree of toxicity is associated with immature seeds. The discrepancy might be due to the

manner in which materials were handled, the growth environment, and/or assay procedures.

In fresh seeds vicine is concentrated in the cotyledons and the seed coat contains less than half that found in cotyledons (Jamalian et al. 1977a; Jamalian and Bassiri 1978). In mature seeds too, the seed coat contains proportionately less vicine (Jamalian 1978). In addition Pitz (1979) observed that while convicine seems to be uniformly distributed throughout the seed, vicine is localised in the plumule-half of the seed. Examination of fababean fractions obtained by pin-milling and air classification has revealed that the glycosides are associated with the protein and not the starch fraction (Collier 1976; Olsen and Andersen 1978).

Several investigators have indicated that there are varietal differences in vicine, convicine and total glycoside content of fababeans. Engel (1970) reported that the vicine concentration in 64 broad bean cultivars collected mainly from Iran was between 0.07% and 0.68%. Conversely, Collier (1976) reported that the 14 cultivars he examined had a mean ± SE glycoside content of 0.83±0.6% and none of the individual values differed significantly from the mean. Jamalian (1978) examined the vicine content of 58 fababean cultivars, 25 from Iran and 33 from various areas; seed coats contained 0.0-0.07% vicine, and the cotyledons 0.23-0.61%. The reports of Engel (1970) and Jamalian (1978), however, may be confounded by stage of maturity and growth environment. Olsen and Andersen (1978) found that 5 cultivars grown at a similar location contained an average of 0.73% and 0.30% vicine and convicine respectively, with no significant differences among means. Pitz (1979)

investigated, extensively, the effect of genotype and growth environment on the vicine and convicine concentration in 36 fababean cultivars. Large cultivar differences in convicine, vicine and total glycoside were found within each location for two crop years. Convicine values at one location ranged from 0.20 to 0.68% while vicine and total glycoside contents ranged from 0.52 to 0.85%, and 0.86 to 1.31%, respectively. The cultivar differences were consistent between replicates at each location. Significant ($P<.05$) cultivar x year and cultivar x location interactions were observed. Growth environment was also found to affect glycoside levels as indicated by the significant differences ($P = .01$) between replicates at one location and lack of differences at another location. Location differences within a given year and between years were, however, consistent.

(b) L-Dopa

Fababeans are an important commercial source of 3, 4-dihydroxyphenylalanine (L-Dopa) (Beutler 1970). L-Dopa is present in fababeans mainly in free state and partly as its 3β-glycosidic derivative (Nagasawa et al. 1961; Pridham and Saltmarsh 1963; Andrews and Pridham 1965). Reports of L-Dopa concentration in fababeans range from 0.01% to 0.25% by weight (Razin et al 1968; Longo et al. 1974; Collier 1976). Longo et al. (1974) reported that L-Dopa and tyrosine levels were highest in fababean pods during the fruiting period (0.56% and 0.044%, respectively), and lowest in the seed (0.075% and 0.01%). L-Dopa can be generated by hydrolysis of L-Dopa glucoside with acid or β-glucosidase, or from tyrosine by tyrosinase hydroxylation and dehydrogenation (Fig. 1.5). Tyrosinase further converts L-Dopa to dopaquinone.

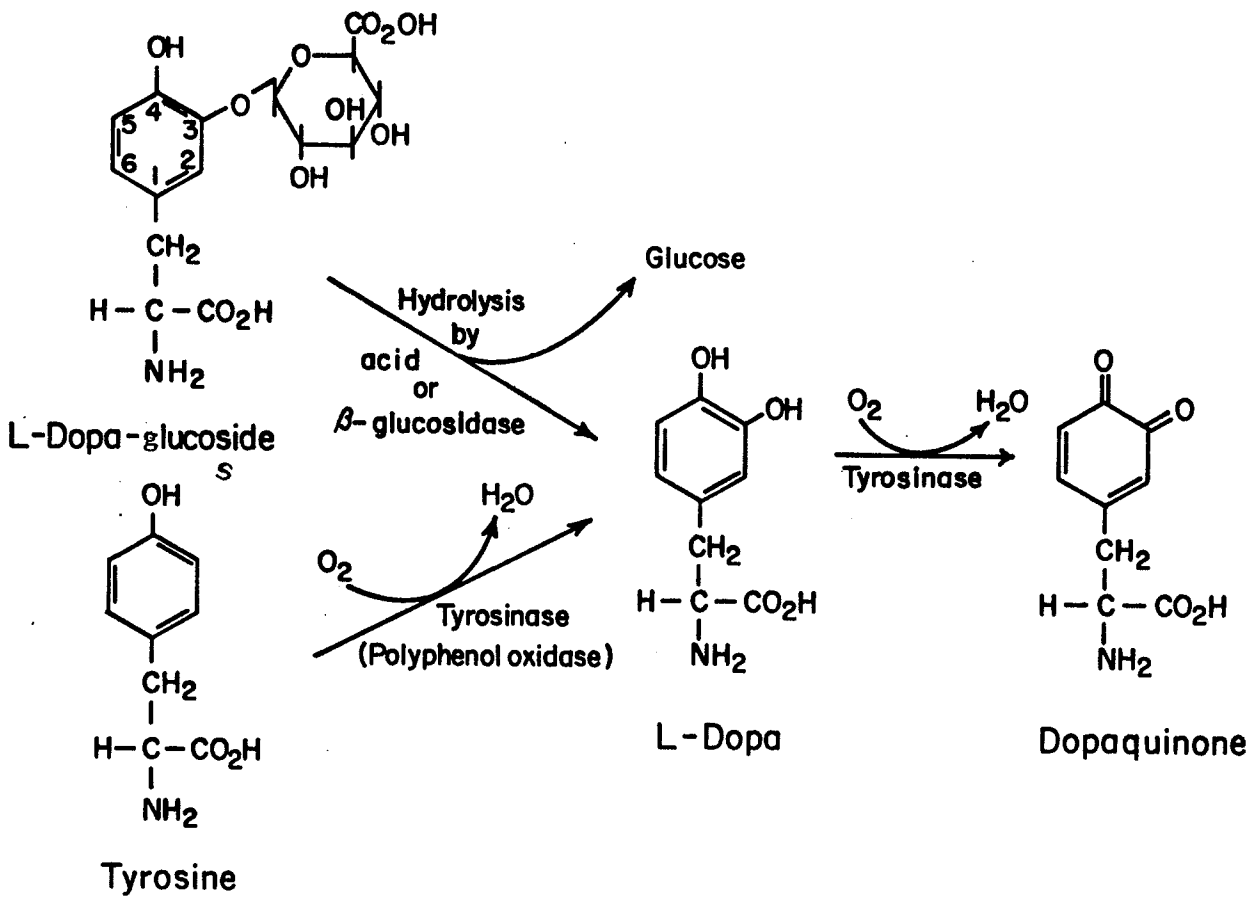

Fig. I.5 L-Dopa, its precursors and oxidation product, Dopaquinone.

(c) Biophysiochemical effects of divicine, isouramil and L-Dopa

Research with these compounds has been biased towards favism due to earlier observations by Mager and coworkers (1969). The latter presumed that the favic agent(s) in fababeans had the capacity to oxidize GSH in G-6-PD-deficient red blood cells (RBC) but not in normal cells, when incubated in the presence of glucose. Some of the isolates from fababeans were sparingly soluble in water, and in neutral solutions they lost the ability to oxidize GSH (Lin 1963; Mager et al. 1965). The fractions were stable under nitrogen or carbon dioxide, but underwent spontaneous oxidation in air. These properties are similar to those of the aglycones divicine and isouramil (Bendich and Clements 1953; Lin and Ling 1962a).

Incubation of GSH (in 0.1 M phosphate buffer, pH 7.4) with divicine leads to a lowering in the GSH content and the appearance of a new maximum at 305 nm and minimum at 265 nm (Lin 1963). The maximum at 305 nm was greater and the rate of formation was faster when the GSH concentration was far in excess of that of divicine. The divicine solution had to be used within 5 minutes of preparation, otherwise the peak at 305 nm was reduced or abolished. A saturated solution of sodium chloride inhibited the formation of a peak at 305 nm. Attempts to isolate and characterise the compound(s) responsible for the peak at 305 nm were unsuccessful due to the instability of the compound(s); it was suggested that the peak at 305 was a complex between divicine and GSH (Lin 1963). Divicine reacted with cysteine too, forming initially two maxima and minima at 245 and 285, and 230 and 265 nm, respectively. Divicine had

no effect on some proteins like bovine hemoglobin and serum albumin even after the proteins were denatured with urea. Liener (1979) suggested that one molecule of oxidized divicine or isouramil reacts with two molecules of GSH to produce the reduced aglycones and one molecule each of oxidized glutathione (GSSG). This scheme does not give the fate of reduced divicine thereafter, and it does not give an intermediate step to account for the peak at 305 nm observed by Lin (1963). This is important since neither oxidized nor reduced divicine or GSSG alone would be responsible for that peak. The non-stoichiometry of the reaction between GSH and divicine would suggest that divicine plays a catalytic role (Lin 1963), but also that a chain oxido-reduction reaction may be involved. Unlike acetylphenylhydrazine and primarquine, divicine and isouramil can oxidize GSH in pure solution in the absence of RBC or hemoglobin (Beutler et al. 1957; Mager et al. 1965).

Vicine ($5x10^{-3}M$) but not divicine, inhibited G-6-PD (38%) and 6-phosphogluconate dehydrogenase (6-PGD) (27%) in RBC hemolysates from normal individuals (Lin and Ling 1962b). At constant NADP (TPN) concentration ($4x10^{-7}M$) vicine inhibited G-6-PD competitively but competitive inhibition was not observed with increasing levels of NADP; the inhibition of 6-PGD under these conditions was non competitive. Vicine was found to be a more potent inhibitor of G-6-PD than acetylphenylhydrazine sulfanilamide and β-napthol, but less than p-chloromercuric benzoic acid (PCMB); the latter is one of the thiol (SH) blocking agents. Vicine was found to be a competitive inhibitor with glucose-6-phosphate (G-6-P) but not NADP. The inhibition of G-6-PD by PCMB or heavy metals has not been reported to be hemolytic. Thus the inhibition of the

enzymes of the glycolytic and pentose phosphate pathways may predispose one to hemolysis or accentuate but not initiate it.

Mager et al. (1965) incubated for 3 hours at 37°C, washed suspen sions of RBC from normal or G-6-PD-deficient individuals with divicine, isouramil or the other hemolytic drugs, acetylphenylhydrazine (APH), ascorbic acid (AA) and dialuric acid (DA). Divicine and isouramil were prepared from their respective glycosides, in addition synthetic isouramil was tested. All the compounds tested except the glycosides (vicine and convicine) and DA caused a decline in GSH and ATP levels. The activity of the aglycones, divicine and isouramil, was 10 or 20 times higher, on a molar basis, than APH or AA, respectively. Inosine (0.01) M) prevented the fall in ATP but not GSH; normal RBC were protected by addition of glucose. Dialuric acid had no effect on GSH content because its oxidized derivative (Alloxan) forms an addition compound with GSH (Patterson et al. 1949) rather than acting as a hydrogen acceptor. The oxidation of GSH by divicine and isouramil was enhanced by shaking in air and completely suppressed in an atmosphere of pure nitrogen. It was further shown in this study (Mager et al. 1965) that GSSG inhibits hexokinase and therefore impairs production of energy and cellular reducing components like NADPH. Considering the reactions and conditions of the interaction between GSH and the aglycones, Mager et al. (1965) suggested that the reaction mechanism was a catalytic oxido-reduction similar to the GSH-ascorbic redox system (Borsook et al. 1937). The inertness of convicine and vicine in which the readily oxidizable 5-hydroxy group is blocked by the β-glycosidic linkage supports the above; the fundamental

similarity in the chemical properties of the fava pyrimidines and ascorbic acid therefore, is due to the structural resemblance of the carboxyl-conjugated aminoenol and enediol rings of these compounds.

Flohe et al. (1971) reported that under aerobic conditions in vitro, high concentrations of divicine and DA caused a rapid fall in human RBC GSH content and the formation of lipid peroxides; after considerable delay K^+ loss was observed and consequently hemolysis occurred. Oxidised glutathione could be biologically reduced after treatment with diamide (N, N, N^1, N^1 - tetramethylazoformamid), while efforts to regenerate GSH after treatment with divicine or DA were unsuccessful. A previous oxidation of intracellular GSH by diamide did not cause a significant effect on the degree of lipid peroxidation. Correspondingly, the characteristic effects of divicine and DA on the cells were not seen with vicine or 2, 4-diamino-6-hydroxy pyrimidine. Vicine, however, was hydrolysed in the presence of serum, therefore the comparatively low K^+ loss observed with vicine was attributed to the divicine generated hence. Divicine (3.5 nM) incubated with normal human RBC caused formation of heinz bodies and changed the shape of the RBC from biconcave to an irregular one, and there was increased formation of methemoglobin (Lin et al. 1977). Flohe et al. (1971) suggested that the effects of divicine and DA on human RBC could be explained on the basis of generation of free radicals during the autoxidation of the 0-dihydroxy pyrimidine derivatives. However, no direct evidence for free radical production has been presented to date. The failure to regenerate GSH after incubation with divicine and DA indicates that GSSG was not the product of GSH oxidation by divicine or DA. This does not necessarily support Lin's idea of a complex between divicine

and GSH (Lin 1963), but an assay for GSSG in the system would have helped; free radicals of GS* might be the final product.

The study of favism and its biochemical basis has been hampered by the lack of a suitable animal model. Thus D'Aquino et al. (1979) addressed themselves to this task. They investigated the response to isouramil of RBC from rats deprived of protein, riboflavin and vitamin E. The rats were fed the diets for 30 days and then blood GSH-reductase (GSH-red) activity was assayed every week until it was less than 10% of the rats on control diet. Erythrocytes from rats fed a protein-free diet had lower GSH content but the GSH-red activity was similar to that of the control group. The GSH content and in vitro hemolysis of RBC from rats fed a riboflavin-free diet were similar to those from rats fed the control diet, but the GSH-red activity of RBC from the former were less than 10% of those from the latter group. The low GSH-red activity in rats fed riboflavin-deficient diet was expected since riboflavin is a coenzyme for the GSH-red enzyme. Conversely, RBC's from rats fed a vitamin E-free diet showed no change in GSH content or GSH-red activity; however they showed very marked hemolysis in vitro. In the second part of the study whole blood was diluted 1:1 with phosphate-saline buffer and then incubated in absence or presence of increasing amounts of isouramil. Isouramil led to a decrease in GSH content in RBC from the control, tocopherol-, protein- and riboflavin-free rats with increasing effect; riboflavin-free RBC were drastically affected at concentrations of 0.75 mM, while the dose curves for protein-free and control were parallel with the latter above the former. Thus the initial GSH concentration was less in treated rats except for those fed vitamin E supplemented

diets. Thus they proposed that RBC from riboflavin-deficient rats could be used to screen for favic principles (D'Aquino et al. 1979). However, GSH destruction occurred in all groups; in addition hemolysis is important in favism, therefore the lack of hemolysis in riboflavin-deprived rats and yet marked in vitamin E deficient rats might indicate the role played by some specific dietary constituents like vitamin E that are part of or help to preserve the cellular membranes. Therefore the lack of GSH and/or GSH-red would appear to be predisposing and not initiating.

Whole animal experiments with vicine or convicine have been limited by the lack of a commercial source of large amounts of the compounds. Lee (1950) reported that dietary divicine (1% w/w) fed to rats produced no adverse effects other than growth retardation. However, in this study, most of the divicine might have been destroyed before leaving the gut. The results nevertheless are in agreement with those of Lin and Ling (1962c). The latter fed a diet containing vicine (0.6% w/w) to 6 male and 6 female rats weighing initially 57 to 107 grams. No acute toxic symptoms were observed but growth rates were lowered in both sexes compared to the controls. The inhibition in growth rate was higher in the males (40%) than females (24%). This was associated in the males with decreased feed intake; the food consumption however, gradually increased after the 3rd week though not to normal levels. Female food consumption did not change. After 3 weeks, the male rats exhibited initial toxic symptoms such as hair loss and at the end of the 6th week areas with thinned fur could be seen near the neck, but this symptom gradually disappeared and the animals had recovered by the end of the experiment

(6 wks). Female rats on the vicine-containing diet showed slight hair loss, but there were no thinned out areas. It should be noted that the level of vicine used in the diet was within the range that occurs naturally in fababeans; it would therefore be in a physiological range particularly where fababeans may make up most or the entire meal. The initial weights of the rats were quite variable, particularly those of the male test group were much higher than the rest. It is possible that the response was modified by the weight differences particularly if these reflected differences in fat content. In the second part of the study Lin and Ling (1962c) fed vicine (0.2 gm/kg body wt) through a stomach tube in 50 ml of water to 4 male and 4 female one month old dogs (0.8-1.5 kg wt). The dogs were put in metal cages and urine was collected every three hours until six samples were obtained. The colour of the urine (yellow) did not change after treatment. The pH changed from 7.0 to 5.8, but this was transient and was normal by the third collection. In addition hemoglobinuria was observed in the first urine fraction, was less in the second and was not detected in the third collection. No change in RBC count was observed in the urine. Thus RBC lysis seems to occur quite fast. In guinea pigs (Anderson et al. 1952) subcutaneous injections of divicine were shown to cause severe bone deformities and in particular the medulla developed a reddish colour.

In 1945 Horvath reported that vetch seeds had a toxic compound that caused loss of weight in hens; this toxic factor was thought to be divicine. Later Arscott and Harper (1963) fed a diet containing divicine (1%) to chicks and they observed no adverse effects other than growth

retardation.

Recent studies (Olaboro 1978) have shown that dietary vicine and/or convicine depressed egg and yolk weights in laying hens; liver lipid content was lowered and liver weight was slightly depressed. Various amino acids including L-Dopa that are found in fababeans did not cause the above effects. Dietary energy levels were without effect. Methionine improved egg weight but not to control levels even at concentrations in the diet at which maximum effect was obtained (0.48% dietary methionine). It is postulated from these results that vicine and/or convicine and their aglycones, lower the yolk weight by interfering with the synthesis of lipids which make up a substantial fraction of the yolk material. Since the rest of the egg components are laid down in proportion to the egg yolk, reduced yolk size would lead to a small egg. However, destruction of lipids during their transfer to the ovum and/or interference with synthesis of lipid-transfer proteins, or the destruction of membranes involved in lipid transport might all alter the availability of lipids to the ovum. Thus more research must be carried out to establish the mode(s) of action of vicine and/or convicine and their aglycones.

L-Dopa is widely used in the treatment of Parkinson's disease (Beutler 1970; Longo et al. 1974). Since no signs of anemia are observed in these patients, it is generally believed that L-Dopa per se is not hemolytic. However, recent studies seem to indicate that there are clinical disorders (Bianchine et al 1971) and hemolysis may occur following L-Dopa therapy (Borg et al. 1978). Kosower and Kosower (1967) reported that L-Dopa at concentrations as low as 0.75 μmoles/ml

of a glucose incubation mixture caused significant losses of GSH in G-6-PD-deficient RBC. Alkaline-treated Dopa was found more effective as an oxidant of GSH than native Dopa (Beutler 1970). Conversely, Razin et al. (1968) did not find any effect even when 10 µmoles of Dopa/ml of incubation mixture were used. There is increasing evidence that L-Dopa divicine, isouramil, ascorbic acid, and other pyrimidine or benzene aglycones may act synergistically in lowering the GSH levels in G-6-PD-deficient RBC and perhaps leading to hemolysis in vivo (Mager et al. 1969; Patwardham and White 1973). This is supported by reports that autoxidation of ascorbic acid can be enhanced by other more rapidly autoxidizing compounds like 6-hydroxydopamine, 6-aminodopamine or dialuric acid (Borg et al. 1978). L-Dopa, like divicine and isouramil, is a reducing compound and therefore to oxidize GSH it must be in its oxidized state or just catalyse the oxidation immediately after conversion to the oxidized state. The higher capacity to oxidize GSH in alkaline medium, and some reports that tyrosinase was necessary for L-Dopa to cause a decrease in RBC GSH levels (Beutler 1970) seem to support the theory that the oxidised form (Dopaquinone) is the active form of Dopa.

1.2. Quantitative isolation of vicine and convicine and the preparation of their aglycones

A Isolation of vicine and convicine

The methods for isolating these pyrimidine glycosides can be divided into two groups viz. those that exploit the solubility properties of the glycosides in pure or mixtures of different solvents, and those that exploit their ionic properties. The ionic methods are slow and yield small quantities and are therefore less employed except in

analytical work.

i Vicine

(a) Methods based on solubility

The principle of the method(s) is that the glycoside is extracted in some solvent in which it is stable. Removal of protein, amino acids or other potential contaminants may be performed before, during or after the extraction step. Further purification then involves precipitating the glycoside from the extract with a salt, removal of the salt and then concentration leading to crystallization. Precipitation might be omitted if the removal of protein and amino acids also eliminates most of the potential contaminants that can co-precipitate during crystallisation of the glycoside, or if several recrystallisations can achieve the desired purity. The methods that have been used (Levene 1914; Levene and Senior 1916; Bendich and Clements 1953; Lin and Ling 1962a) are based on the procedure originally developed by Ritthausen and Kreusler (1870) to isolate vicine from common vetch. Essentially the ground beans are extracted with dilute sulphuric acid, the extract is filtered or centrifuged and then neutralised. Mercuric sulphate is used to precipitate the vicine, which is subsequently washed in water and freed from the mercury using hydrogen sulphide. Alcohol is added, followed by concentration in vacuo and then crystallisation. However, 65% ethanol (in water) instead of sulfuric acid, and lead acetate in alkaline solution instead of mercuric sulphate combined with ether washing to remove lipids have been used successfully (Lin and Ling 1962a). Denaturation of protein with acetone and a series of extractions

with ethanol-water mixture combined with filtration and/or centrifugation, concentration *in vacuo* and crystallisation (with pH and temperature manipulation) was the method employed during the study in which vicine was obtained and implicated (perhaps with convicine) in the egg weight depression in laying hens (Olaboro 1978). Yields reported range from 0.14% (Bendich and Clements 1953) to 0.5% (Lin and Ling 1962a), but the quality may vary.

(b) Methods based on ionic properties

The use of ion exchange chromatography was first employed by Gmelin and Hasenmaier (1957). Ground seeds of *Vicia sativa* or *Vicia faba* were extracted with 50% methanol (in water) and the alcohol evaporated *in vacuo*. The residue was passed through a column of Lewatit S 100 (H^+-form) and eluted with dilute ammonium hydroxide. Following concentration under vacuum, vicine crystallised out. This method has been used by Flohe and coworkers (1971), and other resins both cation (Jamalian *et al*. 1976) and anion (Brown and Roberts 1972) have been used; thin layer chromatography can be used to further purify the vicine (Brown and Roberts 1972). However, some properties like melting points of the preparation (Jamalian *et al*. 1976) were much higher than those accepted for vicine, which might indicate contamination by convicine.

ii Convicine

(a) Methods based on solubility

The method described by Bien et al. (1968) was initially employed by Ritthausen (1896). Fresh ground beans are extracted with

95% ethanol (Ritthausen used 80%) for 3 days. After filtration, the solution is extracted with ether and the aqueous layer concentrated *in vacuo* at 40°C. Crystallisation of convicine starts when the solution is kept in the refrigerator. The yield of convicine was 0.06% (Bien *et al.* 1968). Fisher and Johnson (1932) have described a method where ground beans are initially extracted with dilute sulfuric acid. Following neutralisation, filter paper clippings are added to the mixture and the resultant paste squeezed under a hydraulic press. The juice is then made slightly acid and filtered through pulp. Convicine is precipitated from the filtrate with mercuric sulphate, and the mercury removed later with hydrogen sulphide. Mercuric sulphide precipitate is removed and alcohol added to the solution, followed by concentration *in vacuo* and crystallisation. The yield was 0.04%.

(b) Methods based on ionic properties

Brown and Roberts (1972) homogenised fababeans in 50% methanol (in water) in a chilled motor and pestle. The homogenates were boiled for 5 minutes and filtered through millipore membrane. The filtrates were evaporated to dryness *in vacuo* at 37°C, redissolved in water and the pH adjusted to slightly alkaline with dilute ammonia and then applied to an anion exchange resin (Dowex 1, for mate x 8, 200-400 mesh). The column was washed with water and the washings combined with the non-exchangeable fraction. The solution was evaporated to dryness as before and then redissolved in a suitable small volume of water. Vicine and convicine were separated by paper chromatography.

B Preparation of divicine and isouramil from their glycosides

Both divicine (Bendich and Clements 1953) and isouramil (Davoll and Laney 1956; Bien et al. 1968) can be chemically synthesized. However, the methods are complex, tedious and may be expensive. Divicine and isouramil can be prepared from their glycosides by hydrolysis with acid or the enzyme, β-glucosidase. Enzyme hydrolysis is performed at slightly alkaline pH and the aglycones are highly unstable at this pH in air. Therefore it must be performed in complete absence of oxygen, i.e. under an atmosphere of nitrogen or carbon dioxide, it is also advisable to exclude oxygen under conditions of acid hydrolysis. Failure to do so led to highly variable yields of divicine during the early trials (Levene 1914; Bendich and Clements 1953).

i Acid hydrolysis

(a) Divicine

Divicine can be prepared by the method(s) described by Lin and Ling (1962a) and Flohe et al. (1971). Flohe et al. (1971) described their method precisely and it will be presented here briefly. Dry vicine is dissolved in 10 volumes of 2N H_2SO_4 solution that has been preheated to 100°C. Heating is continued at 100°C for 15 minutes under nitrogen. Allow to cool, and add 25 volumes (based on vicine weight) of absolute ethanol. Divicine precipitates out as divicine sulphate. This may be recrystallised in acid and then washed with acetone, chloroform or ethanol. Bendich and Clements (1953) observed that maximum sugar yield was attained after 7 minutes of heating in acid, and that further heating in air destroyed

the divicine so produced. Therefore the timing might be subjective depending on the conditions.

(b) Isouramil

Preparation of isouramil by acid hydrolysis has not been tried under the same conditions as indicated above for divicine. Ritthausen (1896) observed that hydrolytic cleavage of the convicine under acidic conditions did not yield the aglycone, but some other products. Johnson (1914) and Fisher and Johnson (1932) suggested that isouramil was the initial product but alloxan and dialuric acid were secondary products. It seems that isouramil can be prepared by acid hydrolysis under nitrogen but no such report has been made.

ii β-glucosidase hydrolysis

This method is convenient when the aglycone is to be used directly without need for the solid form. However, with this procedure glucose is not removed from the preparation. Therefore proper controls are required to assure that the observed effects are attributable to aglycone and not glucose.

Several researchers have shown that vicine is converted to divicine in presence of β-glucosidase (Mager et al. 1965) but a routine method has not been established. The method standardized for convicine (D'Aquino et al. 1979) could be applied to divicine. In this method, convicine is dissolved at 60-70°C in 0.1 M saline-phosphate buffer (pH 7.4 at a concentration of 3.3 mg/µl. The incubation with

β-glucosidase (50 µg/mg convicine) is performed at 37°C for 20 minutes under nitrogen.

C Estimation of vicine and convicine in fababeans

Analytical methods for determining the concentration of vicine and convicine fall into two broad categories, viz. those that measure total glycoside content (vicine + convicine together) and those that determine vicine and convicine separately. The latter category also includes methods that were developed to measure both and/or either one of the glycosides.

i Methods that measure total glycoside content

(a) Higazi and Read (1974)

The Higazi-Read method was the first method that was developed for quantitation of vicine and convicine and has been widely used. The method was developed to determine the concentration of the glycosides in small quantities of plant material or plasma. The principle of the method is that vicine and convicine, and many other compounds (proteins, amino acids and phenolics) react with the Folin-Ciocalteu phenol reagent and produce a blue colour in sodium carbonate solution. Therefore removal of all reacting compounds except vicine and convicine allows the estimation of the content of the latter in the test material. A brief description of the method is outlined below. Finely ground fababean samples are extracted with trichloroacetic acid (TCA) and then centrifuged. The supernatant is washed with ether to remove TCA and lipid material, and then mixed with copper carbonate and aluminum oxide to remove amino acids and other interfering compounds.

An aliquot is then reacted with dilute phenol reagent and sodium carbonate solution, and the absorbance of the solution determined at 650 nm. The total glycoside content is determined from a vicine standard curve. Higazi and Read (1974) estimated the error of the assay to be 5%, and a recovery of 96±1%. The authors feel that since samples may have variable water content and the application of heat in moisture may destroy some of the vicine, the vicine content should be expressed in terms of nitrogen content rather than dry weight of material. Since autoclaving of fababeans (121^{o}C for 30 minutes) in presence of moisture does not destroy vicine in fababeans (Marquardt et al. 1974a) it is unlikely that heating to constant weight would be destructive. If necessary, heat can be avoided by lyophilisation of the sample.

(b) Collier (1976)

Both vicine and convicine absorb strongly in the UV region, particularly between 270 and 280 nm. Therefore after precipitating out the protein and some of the other compounds that absorb in this region, the absorbance of the extract would be mainly due to vicine and convicine. Thus Collier suspended 10 g of fababeans in 100 ml of 4% (W/V) metaphosphoric acid (HPO_3) solution, chopped in a Waring blender, homogenised in a virtis homogeniser and centrifuged. The protein-free extract was diluted in 0.1N HCl and the UV spectrum scanned. Using the λ max (273.5nm) and ε (16.3×10^3) values of the pure vicine, the apparent vicine and convicine concentration of the extracts was calculated. Chromatography of a protein-free bean extract on a cation exchange resin revealed two UV-absorbing constituents apparently

convicine and vicine in the ratio 1:2. Thus the method can be coupled to an ion exchange column to estimate vicine and convicine separately. This might not be necessary since the ε value for convicine (17.3×10^3) in acid (pH 1) is close enough to that of vicine. However, the λ max for convicine in acid medium is 271 nm and therefore its contribution to the total glycoside content is underestimated at 273.5 nm. Dialysis and gel-exclusion chromatography of the bean extract indicated that 5-15% of the UV-absorbing material was not free vicine or convicine. This accounted for the consistently higher glycoside values obtained by the Collier method compared to that of Higazi and Read (1974). Recoveries in the Collier method were essentially quantitative.

ii Methods that measure vicine and convicine separately

(a) Paper chromatography

Brown and Roberts (1972) combined the isolation and quantitation of vicine and convicine in one process. Their method has been described under section 1.2 (i) b. After extraction and separation as described, vicine and convicine were estimated spectrophotometrically. The ε max values used were those given by Bendich and Clements (1953) and Bien et al. (1968) for vicine and convicine, respectively. Recovery of added vicine and convicine was 87-93%.

(b) Thin layer chromatography (TLC)

(i) Engel (1970). Engel seems to have been the pioneer of this method. Finely ground fababean seeds were extracted in a dilute solution of TCA. Excess TCA and lipids were removed with ether, and

the aqueous solution passed through a cation exchange resin (Dowex 50W x4, H^+-form). Vicine was sluted from the column with a dilute solution of ammonia, and the eluent concentrated *in vacuo*. An aliquot was then spotted on a cellulose TLC plate. Similar spots of vicine from solutions of known concentrations were made, and the chromatogram developed in a mixture of methanol-water-HCl (60:5:35). The plate was sprayed with a 0.2% solution of ultraphor W.T. and the spots examined under UV light. The error with this method was about 15%.

(ii) Jamalian *et al*. (1977a). The method is essentially the same as the Engel (1970) method. However, the extract is passed through the ion exchanger without prior washing with ether. In addition the proportions of methanol-water-HCl (70:10:20) are different. The spots are eluted with 0.1N NaOH and quantitated spectrophotometrically using the ε value given by Bendich and Clements (1953). The methods of Engel (1970) and Jamalian *et al*. (1977a) estimate only vicine.

(iii) Olsen and Andersen (1978). This TLC method can estimate both vicine and convicine. In their trial, Olsen and Andersen (1978) boiled 10 gm of ground fababeans for 5 mins in 200 ml of ethanol-water (3:1) and filtered the suspension through a glass filter. The extraction procedure was performed three times. The combined filtrates were evaporated *in vacuo* at 50°C to 3-5 ml, followed by dilution to 25 ml with ethanol-water (1:1). If UV-absorbing polyphenols were found interfering at the subsequent separation on TLC, then the extract was passed through aluminium oxide (Fluka) to which polyphenols are bound. This step was

not found necessary for dehulled beans. Fifty micro litres of the evaporated and rediluted extract were separated by two dimensional TLC on cellulose 300 MN. Solvents used were ethanol, followed by methanol-25% ammonia-water (14:1:5) in the same direction. Finally the plate was developed with methanol- 0.02M phosphate buffer pH 5.8 (7:3) at right angles to the previous runs. The absorbing dark spots - the glucosides - were visible in UV-light (250-300 nm) against a weakly flourescent background. The spots were scraped and the vicine and convicine extracted with 0.1M phosphate buffer, pH 6.8 for 10 minutes, centrifuged at 10°C, and the supernatants measured spectrophotometrically at 275 nm (vicine) and 271 nm (convicine), using the ε values of Bendich and Clements (1953) and Bien et al. (1968), respectively. The coefficients of variation, excluding the ethanolic extraction step, were estimated at 4% and 6% for vicine and convicine, respectively; corresponding values, including the ethanolic extraction step were 7% and 15%.

(c) Amino acid analyser method

This method is still being developed in Marquardt's laboratory (personal communication), but it is quite promising. Preliminary trials with the method (Olaboro 1978) gave results similar to those of Higazi-Read (1974) and Collier (1976) methods. One gram of autoclaved or raw fababean protein concentrate, starch or hulls were added to 10 ml of water and homogenised. One milliliter of 0.67N H_2SO_4 and 1 ml of 10% sodium tungstate ($Na_2WO_4.2H_2O$) were added to 2 ml of the solution and mixed. The solution was centrifuged (25,000x g for 20 min) and the supernatant was decanted and re-centrifuged.

The pH of the solution was lowered to 3.25 with citrate buffer just prior to application in case of hydrolysis at this pH. It was later found that samples were stable at this pH for over 20 hours. An aliquot of the supernatant was then applied to a column containing a cation exchange resin (Beckman W-1) in a Beckman model 119C automatic amino acid analyser. Authentic vicine and convicine samples are applied in 0.2N sodium citrate buffer (pH 3.25). Elution was accomplished with 0.2N sodium citrate (pH 3.25) followed at 100 minutes by 0.2N citrate buffer (pH 4.12). Column temperature was 50°C. The compounds that were eluted from the column were detected at 280 nm in an ISCO type-6 optical unit and monitored in a model UA-5 absorbance unit. Preliminary studies had demonstrated a direct proportionality between amount of vicine/convicine sample(s) applied to column and peak area; this area was constant for a given amount of sample and the recovery of vicine added to crude extracts was quantitative. Thus the vicine, convicine and total glycoside content of a test sample can be calculated from the peak areas of the standards and test material.

(d) Gas-liquid chromatography (G-LC)

In this method developed by Pitz (1979) finely ground beans are oven-dried overnight at 60°C. Ten grams of the sample are weighed into an Erlenmeyer flask and 100 ml of 0.1N NaOH added. The flask contents are homogenised for 1 minute with a polytron homogeniser and filtered through a Whatman (#4) filter paper. Ten millilitres of the filtrate are transferred to a test tube and 10 ml of 0.1N HCl added to bring the pH of the mixture to 4.2. After shaking, the mixture

is filtered as above. Five milliliters of the filtrate are then transferred to a second test tube containing 1 ml of freshly prepared internal standard solution (aqueous solution of p-nitrophenyl-β-D-glucopyranoside, 1 ml = 1 mg), for a sample to internal standard ratio of 250:1. The mixture is shaken and a 1 ml aliquot transferred to a small teflon-lined screw cap vial and lyophilised. Trimethylsilyl (TMS) derivatives of the dry sample are then prepared by adding 1 ml of Tris-sil (Pyridine:HMDS:TMCS, 9:3:1 V/V) and incubating overnight at 60°C. Following incubation, the sample is shaken and centrifuged, and 2 µl of the supernatant injected into a G-LC column. Standard mixtures containing a constant weight (0.25 mg) of internal standard (p-nitrophenyl-β-D-glucopyranoside) and concentrations of convicine and vicine varying from 0.1 to 2.0 mg were processed for the calibration curve. Injection port and detector oven temperatures were maintained at 280°C and the column oven operating temperature was isothermal at 230°C. Carrier gas (helium) flow rate was 60 cc per minute. It is imperative to centrifuge the sample to remove the ammonium chloride prior to column application. Ammonium chloride is formed during the overnight incubation and it introduces extraneous peaks. In addition the derivatives of convicine and vicine were found susceptible to decomposition by moisture, therefore anhydrous conditions must be maintained in the preparation and storage of the derivatives. This method gave estimated coefficients of variation of 2.60% and 2.96% for vicine and convicine, respectively. This method like many others requires authentic samples of vicine and convicine for the calibration curve.

1.3. Biochemical basis for the hemolytic activity of certain compounds

A number of drugs and chemicals are known to cause hemolysis in individuals deficient in G-6-PD enzyme. Beutler (1978) thoroughly and critically reviewed the literature on "hemolytic" drugs and chemicals. From his survey it is clear that caution must be used when designating a compound hemolytic; as a number of compounds previously categorised as hemolytic were only indirectly involved in an impending hemolytic situation. Febrile diseases themselves are usually hemolytic due to infection. Therefore proper methods must be employed in evaluating the hemolytic potency of a compound.

This critical review (Beutler 1978) produced two classes of "hemolytic" drugs, viz. those that have clearly been shown to cause clinically significant hemolytic anemia in G-6-D-deficiency, and those that can probably safely be given in normal thereapeutic doses to G-6-PD-deficient subjects without non-spherocytic hemolytic anemia (NSHA). In clinical medicine "hemolytic" drugs include mainly the antimaterials and drugs developed to treat febrile and neurological diseases. The purpose here is not to review the effects of each drug, but to try and examine the mode(s) of action of hemolytic drugs in general with reference to a few specific ones.

A Methods for evaluating the hemolytic potency of a compound

i Field clinical observations: Due to lack of human volunteers with G-6-PD-deficiency or suitable animal models, initial data consisted entirely of sporadic clinical observations

following the administration of the drug(s) to patients. Since one cannot differentiate hemolysis due to infection from that due to the drug, this method is unreliable and not scientific.

ii GSH stability test: A number of investigators have attempted to verify their conclusions that a drug is hemolytic in G-6-PD-deficiency by substituting the drug in question for acetyl-phenylhydrazine (APH) in a modified GSH stability test. Such tests have been carried out with a number of chemicals (Zinkham and Childs 1957; Kimbro et al 1957; Dawson et al. 1958; Szeinberg et al. 1959, 1960; Doyen et al. 1967; Flatz et al. 1970) and fababean extracts (Mager et al. 1965, 1969; D'Aquino et al. 1979). The response of erythrocyte GSH levels in such studies did not always correspond to the known hemolytic effect of the drugs involved; for example sulfanilamide and sulfa-pyridine, both known to produce hemolysis in G-6-PD-deficient cells, did not decrease GSH levels (Szeinberg et al. 1959). Yet ascorbic acid, not hemolytic in ordinary doses caused a fall in the GSH level of primaquine-sensitive cells in initial studies of the GSH stability test (Beutler 1957). Thus the metabolism of a compound and its levels in blood (related to its clearance rate) are important in its oxidative challenge to the cell; the presence of other compounds like metal ions (e.g. iron) or some chelating agents may be synergistic in the hemolytic potency. Therefore substitution of a drug for APH in an in vitro test does not tell much about its hemolytic potency. This is a serious drawback to the animal model suggested by D'Aquino and associates (1979). The method can be improved by examining other parameters associated

with hemolysis (Flohe et al. 1971) like lipid peroxidation and K^+ loss from the cell.

iii Effect of plasma drug metabolites on oxidative metabolism of normal erythrocytes: This technique is based on the assumption that a hemolytic compound is one that stresses the pentose phosphate pathway (PPP) of red cells (Welt et al. 1971; Gaetani et al. 1976). The RBC of G-6-PD-deficient subjects may be handicapped in the capacity to generate NADPH to detoxify the products of drug-induced peroxidation. In normal individuals, a challenge of RBC with a hemolytic drug should stimulate the pentose phosphate pathway. In the human RBC, $^{14}CO_2$ is evolved from glucose-1-^{14}C almost entirely by the pentose phosphate pathway. Thus Welt et al. (1971) incubated RBC from normal individuals with glucose-1-^{14}C before and after ingestion of primaquine. They found that ingestion of primaquine, significantly increased the $^{14}CO_2$ evolution and glucose utilisation, but the degree of stimulation was variable and small. No such effects were observed after the same seven subjects ingested comparable amounts of darapin which does not cause hemolysis in G-6-PD-deficient subjects. However, some hemolytic drugs may inhibit initial enzymes of the PPP, particularly G-6-PD or hexokinase, and hence a reduction in $^{14}CO_2$ evolved from glucose-1-^{14}C may be misinterpreted.

iv Transfusion to normal volunteers of ^{51}Cr-labelled G-6-PD-deficient RBC followed by administration of the putative hemolytic agent: The principle of this test is that the labelled and not the normal cells will be destroyed and this should result in reduced specific

radioactivity after treatment (see Beutler 1978). This method should be more reliable but the problem is one of getting enough donors and volunteer recipients, in addition the protection accorded to the G-6-PD-deficient cells by the host environment is difficult to evaluate under these conditions.

v Production of free radicals: The effects of free radicals on RBC, other cells and whole animals are discussed under section 1.4. It suffices to mention that they have been implicated in a number of pathological states. It is becoming increasingly accepted that any free radical if not removed, is potentially hemolytic. Many "hemolytic" compounds including dialuric acid, acetylphenylhydrazine and menadione have been shown to produce free radicals (particularly O_2^-) in vitro, either during autoxidation or when incubated with hemoglobin or RBC (Fee and Teitelbaum 1972; Goldberg and Stern 1975; Borg et al. 1978; French et al. 1978; Jain and Hochstein 1979; Winterbourn et al. 1979). The major obstacle with this method is to establish a cause-effect relationship between a free radical and hemolysis; in addition, the methods available for detecting free radicals may be too slow to detect unstable radicals, or not specific for free radicals (see section 1.4). Therefore the methods for detecting free radicals must be carefully selected, particularly the indirect methods (see section 1.4). In some studies (Borg et al. 1978) a combination of certain hemolytic compounds (a highly unstable and relatively stable one), was found to facilitate formation of free radicals by the relatively stable compound

and thus facilitate the propagation of the free radical production process. This may in part account for the synergistic oxidant effect observed for various combinations of L-Dopa, divicine, isouramil and ascorbic acid (Mager et al. 1965; 1969).

vi Development of an animal model: In this method an attempt is made to identify animals or birds that may respond to hemolytic drugs when fed a normal diet or a special diet (e.g. deficient in vit. E) that cause the cells to be more susceptible to hemolytic drugs. The putative hemolytic drug may be fed to the animal in the diet and spontaneous hemolysis determined according to Draper and Scallany (1969); or the RBC may be incubated in vitro with the putative hemolytic drug in a manner similar to method 1.3 (A) (ii). The latter alternative has been tried by D'Aquino and associates (1979), but is subject to the criticism filed against the GSH stability test. The feeding trial if coupled with determination of parameters associated with hemolysis (lipid peroxidation, activity levels of certain enzymes, GSH levels etc.) may be the best alternative. Of course the complicating factor has always been how to apply animal data to humans, particularly when you consider differences in enzyme levels and activities among species. In addition feeding trials need large amounts of the chemical and some chemicals are not available commercially like the glucosides, vicine and convicine. A major advantage with animal models is that you can evaluate dietary effects more accurately, a factor almost impossible to achieve in humans.

B Mechanism(s) of action of hemolytic compounds

A wide range of compounds are known to be hemolytic in G-6-PD-deficient individuals, viz. primaquine, phenylhydrazine, dialuric acid and alloxan, ascorbic acid and menadione bisulphite (see Mager et al. 1969; Beutler 1978; Borg et al. 1978). It is therefore difficult to imagine a single uniting feature to explain the hemolytic potency of such a diversity of compounds. A number of hemolytic compounds are unstable in alkaline solutions particularly in presence of oxygen. They autoxidise and produce what might be the hemolytic factors. If the hemolytic action of these compounds has a common basis, then it might help explain and implicate vicine and convicine and their aglycones in precipitating favism (Beutler 1978) and reducing egg weight in laying hens (Olaboro 1978).

It is likely that denaturation of hemoglobin into insoluble precipitates (Heinz bodies) and damage to RBC membrane are the final events preceding the removal of the RBC from the circulation by a perceptive filtering system. However, the sequence of events leading to that are not clearly known. Below is a brief review of the cellular effects of some hemolytic drugs.

i Inhibition of G-6-PD and other enzymes: Some potentially hemolytic drugs have the ability to inhibit G-6-PD activity in vitro (Desforges et al. 1960; Cotton and Sutorius 1971) but the concentrations required to register reasonable inhibition are very high. However, abnormal G-6-PD like the mediterranean type is unstable and therefore may be more prone to drug inhibition.

ii Generation of hydrogen peroxide: Many drugs that cause destruction of the G-6-PD-deficient RBC have been shown to generate hydrogen peroxide (Cohen and Hochstein 1964; Liebowitz and Cohen 1968) possibly through superoxide anion (Carrell et al. 1975; Borg et al. 1978) during the autoxidation or when interacting with hemoglobin. The presence of hydrogen peroxide in intact cells was detected after the addition of several 8-aminoquinolines (e.g. primaquine) but not with the non-hemolytic drugs, 4-amino quinoline, chloroquine; after the addition of the hemolytic agents phenylhydrazine, menadione and hydroquinone-p-quinone redox system, but not after the addition of the non-autoxidizable resorcinol (Cohen and Hochstein 1964; Liebowitz and Cohen 1968) and dialuric acid (Fee and Teitelbaum 1972). GSH detoxifies hydrogen peroxide through the GSH-peroxidase reaction; but G-6-PD-deficient cells cannot maintain GSH levels because of the limited capacity to produce NADPH from $NADP^+$. The H_2O_2 accumulated in cells may produce oxidative denaturation of hemoglobin and membrane components leading to formation of heinz bodies and ultimately to destruction of the red cells. The GSSG formed may itself be damaging by forming mixed disulphides with hemoglobin and inhibiting enzymes like hexokinase (Beutler and Teeple 1969; Mager et al. 1969).

iii Lipid peroxidation: Several investigators found that hemolysis was preceded by lipid peroxidation (see Brownlee et al. 1977), it was therefore generally assumed that hemolysis was a consequence of lipid peroxidation. Although lipid peroxidation is observed in G-6-PD-deficient RBC challenged with hemolytic drugs (Goldstein and

Donagh 1976), malonaldehyde (an indicator of lipid paroxidation) production in G-6-PD-deficient cells treated with H_2O_2 was similar to that of patients with elevated reticulocyte count. Using normal and vit. E-deficient rat RBC, Brownlee et al. (1977) found that H_2O_2 was the primary extracellular source of oxidant stress; experiments with dimethyl sulfoxide showed that intracellular hydroxyl radical generated from H_2O_2 was the hemolytic agent. In addition it was found that lipid paroxidation and hemolysis in RBC from vit. E-deficient rats were concurrent rather than consecutive. This may be difficult to assess since some membrane molecules traverse the whole membrane and they can act as radicals externally and internally even when the electron obstruction is from one side of the membrane.

iv Oxidation of hemoglobin to methemoglobin:

Methemoglobin is hemoglobin in which the iron is present in the trivalent rather than the divalent state. Methemoglobin is formed from oxyhemoglobin during which oxygen is lost as superoxide (Misra and Fridovich 1972a; Wever et al. 1973). The mechanism by which this occurs proposes that in oxyhemoglobin there is a polarisation of an electron from the heme iron to the bound oxygen (Carrel et al. 1975) such that oxyhemoglobin could be called a ferri-superoxy rather than ferro-oxy compound (Fridovich 1978) (Fig. 1.6). Normally this shared electron is returned to the iron when oxygen is released from hemoglobin and the iron retains its ferrous state. This return of the electron to the iron is dependent on the absence of any displacing anions in the heme pocket. The heme pocket is normally hydrophobic, but random fluctuations

in the surrounding globin may allow the entry of small anionic molecules. This results in the displacement of oxygen with an extra electron (0_2^-); thus the heme iron loses an electron resulting in the formation of ferric (Fe^{3+}) methemoglobin.

The role of methemoglobin in the sequence of events leading to hemolysis has been controversial. Many drugs that produce hemolytic

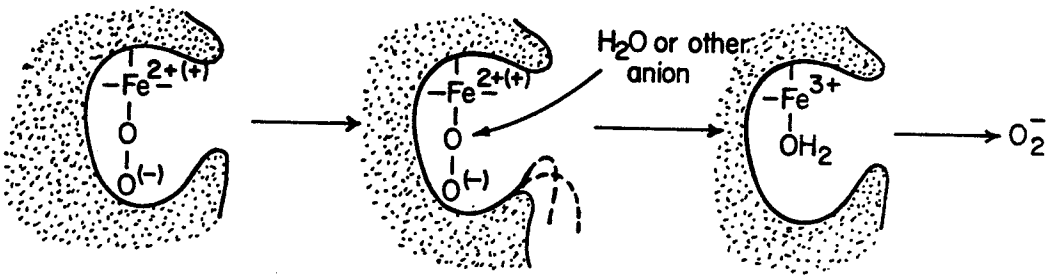

Fig. I.6. Formation of methemoglobin from oxyhemoglobin. The heme pocket of oxyhemoglobin is represented on the left. Small fluctuations of the haem pocket may allow access of water or other anion to the heme (centre). This may result in the formation of methemoglobin and release of O_2^- (right).

anemia in G-6-PD-deficient individuals also have the capacity to induce some degree of methemoglobinemia. Heme iron is much less firmly bound in methemoglobin compared to hemoglobin (Bunn and Jandl 1966) and hem-free globin is particularly vulnerable to denaturation. In addition methemoglobin does not bind oxygen, hence this process may inevitably liberate oxygen as superoxide anion. However, induction of methemoglobinemia with nitrite does not make rats more susceptible to phenylhydrazine-induced hemolysis (Beutler et al. 1962). According to

Jain and Hochstein (1979), phenylhydrazine must participate in the hemoglobin-methemoglobin redox cycle, before the superoxide ion can be produced and this may explain the data of Beutler and coworkers (1962). An inverse relationship appears to exist between the hemolytic potency of drugs and their capacity to form methemoglobin (Finch 1948; Rentsch 1967). This phenomenon has been observed with L-Dopa (see Mager et al. 1969; Beutler 1978). The capacity to convert hemoglobin to methemoglobin depends on the form in which the compound exists, reduced or oxidised, and the redox potentials of the reduced/oxidised forms of hemoglobin/methemoglobin and the test drug. Thus divicine and isouramil are strong reducing agents in their reduced form, but strong oxidizing agents when oxidised (Mager et al. 1965, 1969; Liener 1979). Therefore the time the drug is exposed to air in solution will affect the direction of the results.

Treatment of hemoglobin with phenylhydrazine results in production of denatured globin hemochromogens (Warburg et al. 1931; Rachmilewitz et al. 1974; Peisach et al. 1975). It has also been suggested that phenylhydrazine and its derivatives produce hemochromogens with the haem of hemoglobin (Itano et al. 1974, 1975, 1976, 1977; Itano and Mannen 1976). Such a reaction results in denaturation of hemoglobin. Recently it was shown that phenylhydrazine reacts with hemoglobin to produce the superoxide anion and other free radicals (Goldberg and Stern 1975; Jain and Hochstein 1979).

v Production of free radicals: In addition to phenylhydrazine, numerous commonly known hemolytic compounds including dialuric acid, alloxan, acetylphenylhydrazine, ascorbic acid and

menadione (vit. K) have been shown to produce free radicals during their autoxidation (see Borg et al. 1978) or when incubated with hemoglobin or RBC (Fee and Teitelbaum 1972; Grankvist et al. 1979; Winterbourn et al. 1979). The production of the radicals seems to depend on pH and gas medium, being rapid in alkaline solutions and oxygen-containing medium, and quite slow or absent in acid and/or in a medium containing carbon dioxide or nitrogen gas. The types of free radicals produced vary but O_2^- is the most common; others include the free radical of the drugs themselves and others like the hydroxyl radical ($\dot{O}H$) or singlet oxygen ($^1O_2$) that may result from subsequent reactions (Khan 1970; Singh 1978; Willson 1977a).

Even where free radicals have not been directly demonstrated, their existence has been deduced from the effects of known radical scavengers like the enzyme superoxide dismutase (SOD) ethanol, sodium benzoate and vitamin E in the incubation medium.

The effects on cells observed for some hemolytic drugs particularly premaquine, dialuric and ascorbic acid are similar to those of divicine and isouramil discussed in section 1.1 (e). Thus the speculation that divicine and isouramil may cause favic hemolysis through free radical generation or mechanisms similar to those of the other hemolytic compounds is reasonable. Borg et al. (1978) presented data that, together with what is already known about autoxidation, enabled them to link autoxidation, lipid peroxidation and cytotoxicity. This data will be presented in detail in the discussion section relating to divicine autoxidation. One could postulate that the hemolytic compounds in the alkaline plasma autoxidise to their active forms, which may be

free radicals or they subsequently react with special molecules like hemoglobin to subsequently produce free radicals, and unstable molecules they reacted with (methemoglobin or hemochromogens). Other free radicals may be generated by interconversion. The oxidizing compounds may then peroxidise lipids or important target molecules of the cell membrane. More important, the free radicals will easily abstract hydrogen from polyunsaturated lipids to produce their radicals. Radicals of (poly) unsaturated lipids react rapidly with oxygen to form lipoperoxy radicals and these will react with other lipids to form lipid hydroperoxides. Thus propagating the cyclic production of free radicals. Hydrogen abstraction may occur in lipoproteins or glycoproteins that traverse the cell membrane and therefore facilitate the activity of a free radical a distance from its source. The whole process may end in intramolecular reactions in lipids, yielding products like malondialdehyde, biomolecular reactions to give nonfunctional polymers and involvement of functionally sensitive or target biomolecules and oxidation of cell reducing components like GSH, NADH, NADPH and other protein-SH (Fong et al. 1973; Schaich 1974; Kellog and Fridovich 1975; Hafeman and Hoekstra 1977). It is evident however that the compounds may act at various sites that may involve even inactivation of enzymes and the differences in potency may be related to the redox potentials of the hemolytic compound and/or its products and those of the target molecules.

1.4. Free radicals

Free radicals are compounds which have special bonds between two atoms at one or several points of the molecule, such that one of the

atoms has an unpaired electron. These are the one- and three-electron bonds (Pauling 1931). Pauling was the first to point out that a stable one-electron bond could be formed only when there are two conceivable electronic states of the system with essentially the same energy, the states differing in that for one there is an unpaired electron attached to the second atom. A three electron bond can be formed between two atoms, A and B, with A having an orbital occupied by a pair of electrons and B having an orbital occupied by a single electron or the reverse, if the energies of these two structures are essentially the same. The resonance energy corresponding to the interaction of the two structures is the energy of the three electron bond. This energy has been found to be about 60% of the energy of a shared-electron-pair bond between two atoms (Pauling 1931). This concept was employed by Pauling (1933) to identify such bonds in helium molecule ion, other compounds like KO_2, RbO_2 and CSO_2, and under the guidance of Pauling, Neuman (1934) identified the superoxide anion or radical (see Pauling 1979). It is interesting that recently Fridovich (1978) has suggested that oxyhemoglobin and oxymyoglobin should be viewed as ferri-superoxy rather than ferro-oxy compounds; therefore compounds that produce O_2^- when incubated with hemoglobin just displace the superoxide anion.

Oxygen radicals have been studied in more detail than any other, and seem to be the most important in biology. Based on Pauling's quantaum mechanics, the structure of O_2^- may be presented as in Fig. 1.7. Thus the resonance structure of O_2^- is between the above structures, consisting of a three-electron bond plus a single electron-

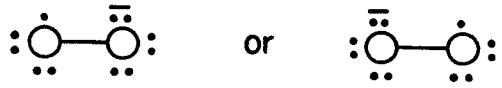

Fig. I.7 The possible structures of the superoxide anion (O_2^-).

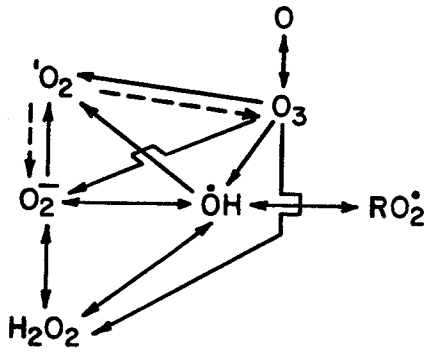

Fig. I.8. Interconversions among oxygen radicals.

$^1O_2$ – singlet oxygen	O_2^- – superoxide radical or anion
O_3 – ozone	$\dot{O}H$ – hydroxyl radical
O – atomic oxygen	H_2O_2 – hydrogen peroxide
	$R\dot{O}_2$ – peroxy radical.

pair bond. The superoxide anion can be converted to other species. Singh (1978) compiled information concerning known and postulated interconversions of oxygen radical species; these are summarised in Fig. 1.8. The detailed reactions are presented in Table 1.2.

A Sources of free radicals

Oxygen free radicals can be generated from the ozone that escapes into the atmosphere (see Fig. 1.8). However, this is normally a negligible source. The major sources of body radicals are (i) radiation, (ii) food, and (iii) cellular metabolism.

i Radiation sources of free radicals: Radiation has been known for a long time to be hazardous. It leads to lipid peroxidation and these peroxides can destroy nucleic acids (Packer et al. 1967; Toubal and Tappel 1967) and biological membranes (Hunter Jr. et al. 1963; Mengel and Kann, Jr. 1966; Utsumi et al. 1966; Wills 1967). The radiation-induced lipoperoxidation is influenced by various factors including lipid composition, conformation and state of lipids in the biological structure, and a number of compounds particularly free radical scavengers. Irradiation has been found to stimulate hemolysis of RBC (Ting and Zirkle 1940; Minato 1955; Inouye et al. 1979).

A similar action was attributed to the superoxide radical (Kellog III and Fridovich 1977; Inouye et al. 1979). It has been shown by electron spin resonance (esr) that irradiation of alkaline aqueous solutions leads to production of O_2^- (Nilsson et al. 1969b; Draganic and Draganic 1971; Klug-Roth et al. 1973). Solutions of a number of compounds have also been shown to generate O_2^- on irradiation (Willson 1970,

Table 1.2. A partial list of known and postulated interconversion reactions of singlet oxygen ($^1O_2$) and related species (Singh 1978)[a].

$$O^-_{2aq} + H_2O_2 \rightarrow \dot{O}H + OH^-_{aq} + O_2$$

$$O^-_{2aq} + H_2O_2 + H^+_{aq} \rightarrow \dot{O}H + H_2O + O_2$$

$$Fe^{2+}_{aq} + H_2O_2 \rightarrow \dot{O}H + Fe^{3+}_{aq}\ OH^-_{aq}$$

$$Fe^{3+}_{aq} + O^-_{2aq} \rightarrow Fe^{2+}_{aq} + O_2$$

$$\dot{O}H + H_2O_2 \rightarrow HO^{\cdot}_2 + H_2O$$

$$HO^{\cdot}_2 \overset{H_2O}{\longleftrightarrow} O^-_{2aq} + H^+_{aq}$$

$(pK = 4.75 \pm 0.05)$

$$Fe^{2+}_{aq} + \dot{O}H \rightarrow Fe^{3+}_{aq} + OH^-_{aq}$$

$$H_2O_2 \overset{H_2O}{\longleftrightarrow} HO^-_{2aq} + H^+_{aq}$$

$$Fe^{3+}_{aq} + HO^-_{2aq} \rightarrow Fe^{2+}_{aq} + HO^{\cdot}_2$$

$$Fe^{3+}_{aq}\ (ADP) + O^-_{2aq} \rightarrow Fe^{2+}_{aq}\ (ADP) + O_2$$

$$Fe^{2+}_{aq}\ (ADP) + H_2O_2 \rightarrow Fe^{3+}_{aq}\ (ADP) + \dot{O}H + OH^-_{aq}$$

$$HO^{\cdot}_2 + \dot{O}H \longleftrightarrow H_2O_3$$

$$\dot{O}H + O^-_{2aq} \rightarrow {}^1O_2 + OH^-_{aq}$$

$$\dot{O}H + HO^{\cdot}_2 \rightarrow H_2O + O_2$$

Table 1.2 (Continued)

$$HO_2^{\cdot} + HO_2^{\cdot} \rightarrow H_2O_2 + O_2$$

$$O_2^- (H_2O)_n + O_2^- (H_2O)_n \rightarrow O_2^- (H_2O)_n + O_2$$

$$2O_{2aq}^- + 2H_{aq}^+ \rightarrow H_2O_2 + O_2$$

$$SOD\text{-}Cu^{2+} + O_{2aq}^- \rightarrow SOD\text{-}Cu^+ + O_2$$

$$SOD\text{-}Cu^+ + O_{2aq}^- + 2H_{aq}^+ \rightarrow SOD\ Cu^{2+} + H_2O_2$$

$$^{\cdot}OH + {}^{\cdot}OH \rightarrow H_2O_2$$

$$H_2O_2 \xrightarrow{h\nu} {}^{\cdot}OH + {}^{\cdot}OH$$

$$^{\cdot}OH + RH \rightarrow {}^{\cdot}R + H_2O$$

$$R^{\cdot} + Fe_{aq}^{3+} \longleftrightarrow R_{aq}^+ + Fe_{aq}^{2+}$$

$$R^{\cdot} + Fe_{aq}^{2+} \longleftrightarrow Fe_{aq}^{3+} + R_{aq}^-$$

$$R^{\cdot} + O_2^{\cdot} \rightarrow RO_2^{\cdot}$$

$$RO_2^{\cdot} + Fe_{aq}^{2+} \rightarrow RO_{2aq}^- + Fe_{aq}^{3+}$$

$$RCH_2OH + {}^{\cdot}OH \rightarrow R\dot{C}HOH + H_2O$$

$$R\dot{C}HOH + H_2O_2 \rightarrow RCHO + {}^{\cdot}OH + H_2O$$

$$R\dot{C}HOH + O_2 \rightarrow RC(\dot{O}_2)\ HOH$$

Table 1.2 (Continued)

$$RC(\dot{O}_2)\ HOH \rightarrow RCHO + HO_2^{\cdot}$$

$$RO_2^{\cdot} + RO_2^{\cdot} \rightarrow RO_2 - RO_2 \rightarrow {}^1O_2$$

$$RO_2^{\cdot} + RO_2^{\cdot} \rightarrow 2RO^{\cdot} + O_2$$

$$RO_2^{\cdot} + RH \rightarrow ROOH + R^{\cdot}$$

$$RO_2^{\cdot} + ROH \rightarrow ROOH + RO^{\cdot}$$

$$ROOH + O_{2sol} \xleftrightarrow{CH_3CN} RO^{\cdot} + OH^-_{sol} + O_2$$

$$RO_2^{\cdot} + Mn^{2+}_{aq} \rightarrow RO^-_{2aq} + Mn^{3+}_{aq}$$

$$3{}^1O_2({}^1\Delta g) \rightarrow 2O_3$$

$$2{}^1O_2({}^1\Sigma^+_g) + O_2 \rightarrow 2O_3$$

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

$$O_3 \xrightarrow{h\nu} {}^1O_2 + O$$

$$O_3 + H_2O \xrightarrow{h\nu} O_2 + H_2O_2$$

$$O_3 + OH^-_{aq} \rightarrow O^-_{2aq} + HO_2^{\cdot}$$

$$O_3 + HO_2^{\cdot} \rightarrow 2O_2 + \dot{O}H$$

$$O_3 + H_2O_2 \rightarrow \dot{O}H + HO_2^{\cdot} + O_2$$

$$O_2 \xrightarrow{h\nu} {}^3O_2^* \rightarrow O + O$$

Table 1.2 (Continued)

$$^3O_2^* + H_2O \rightarrow HO_2^{\cdot} + {}^{\cdot}OH$$

$$^3O_2^* + RH \rightarrow HO_2^{\cdot} + R^{\cdot}$$

$$O + O_2 \rightarrow O_3$$

$$O + H_2O \rightarrow {}^{\cdot}OH + {}^{\cdot}OH$$

$$O + RH \rightarrow {}^{\cdot}OH + R^{\cdot}$$

$$ArOH + {}^1O_2 \xrightarrow{CH_3OH} O_{2sol} + ArO^{\cdot} + H^+_{sol}$$

$${}^{\cdot}OH + Cl^-_{aq} \rightarrow OH^-_{aq} + {}^{\cdot}Cl$$

$${}^{\cdot}Cl + Cl^-_{aq} \rightarrow Cl_{2aq}^{\cdot -}$$

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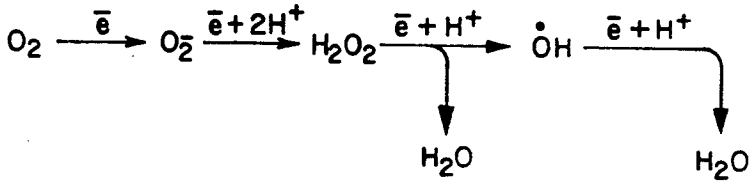
[a]All ions in polar solvents are shown as solvated unless the inference was clearly otherwise in the original references.

1971; Baxendale and Wardman 1971; Plonka et al. 1978). Even some single crystals like those of thymidine can form free radicals on irradiation (Spines and Henriksen 1970). Thus irradiation of the animal body may produce free radicals. Petkau (1978) and others have shown that injections of SOD can provide some protection against radiation effects. In addition Inouye et al. (1979) observed that vitamin E and SOD inhibited hemolysis due to irradiation of human red cells. Oxygen is known to enhance radiation lethality (Goscin and Fridovich 1973) and the lethality can be inhibited by SOD (Misra and Fridovich 1976).

ii Food sources of free radicals: A number of plant foods contain compounds which autoxidise to produce free radicals. Hydrolysable tannins are broken down to tannic acid and subsequently may produce pyrogallol and pyrocatechol. Pyrogallol has been shown to autoxidise in alkaline medium producing O_2^- (Marklund and Marklund 1974). Other plant components like ascorbic acid have been shown to catalyse autoxidation of other compounds to produce free radicals (see Borg et al. 1978). Plant subcellular organelles like chloroplasts have also been shown to generate O_2^- (Asada and Kiso 1973; Epel and Neuman 1973; Halliwell 1975). Recently (Svingen et al. 1979) it was shown that soybean lipoxygenase can produce alkyl (L.), alkoxy (LO.) and hydroperoxy (LOO.) radicals from linoleic acid. Thus active enzymes in poorly processed plant foods may be deleterious by enhancing denaturation of food nutrients and also producing free radicals on ingestion until the food is acidified in the stomach. In ruminants, this may result in destruction of some useful microflora.

iii Free radical-generation in cellular metabolism: The superoxide anion is a common intermediate of oxygen reduction in biology. This is because the dictates of quantum mechanics lead to a spin restriction that prevents the divalent reduction of oxygen and favours the univalent pathway (Taube 1965). The univalent pathway of



$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \} \text{Superoxide Dismutase}$$

$$H_2O_2 + H_2O_2 \longrightarrow 2H_2O + O_2 \} \text{Catalases}$$

$$H_2O_2 + RH_2 \longrightarrow 2H_2O + R \} \text{Peroxidases}$$

Fig. 1.9. Univalent pathway of oxygen reduction and the enzymatic defenses against the intermediates (Fridovich, 1978)

oxygen reduction and the defenses against the intermediates encountered (discussed in section 1.4 (D)) are presented in Figure 1.9. The superoxide anion is a minor, but not a trivial product of biological oxygen reduction; oxidative enzymes have been evolved that accomplish the divalent and even tetravalent reduction of oxygen without release of intermediates - most of the oxygen consumed by respiring cells is utilised by cytochrome oxidase which reduces 0_2 to H_20 without release of either 0_2^- or H_20_2 (Antonini et al. 1970). However, 0_2^- is produced in respiring cells by a number of reactions (see Fridovich 1978):

(1) autoxidation of hydroquinones, leukoflavins, catecholamines, thiols (e.g. GSH), tetrahydropterin, reduced ferredoxins, hemoglobin and myoglobin; since hemoglobin and myoglobin in their oxygenated forms liberate O_2^- as they are converted to methemoglobin and metmyoglobin it is suggested that they be referred to as ferri-superoxy and not ferro-oxy compounds. Production of methemoglobin must be substantial since there is a special methemoglobin-reductase which utilises NADH (Fridovich 1978); and a special NADH- and NADPH-diaphorase system (Beutler 1978) has been shown to exist in human red cells. Both systems reduce methemoglobin back to the ferrous form. The diaphorase system transfers an electron from NADPH or NADH to cytochrome b_5. The reduced cyt. b_5 then reduces metHb non-enzymatically; (2) oxidase enzymes including xanthine oxidase, aldehyde oxidases, dihydro-orotic dehydrogenase, flavin dehydrogenases, galactose oxidase, indoleamine dioxygenase, 2-nitropropane dioxygenase, to name a few, do generate O_2^- during their catalysis; (3) fragments of subcellular organelles like mitochondria and peroxisomes also produce O_2^-. This is expected as these organelles, particularly peroxisomes contain high levels of oxidase enzymes; (4) polymorphonuclear leukocytes (granulocytes) produce O_2^- during their respiratory burst that precedes phagocytosis (Cheson et al. 1977); and (5) lipid peroxidation seems to produce $^1O_2$ (King et al. 1975). The strong oxidising radical ($\dot{O}H$) may be produced through the interaction of O_2^- with H_2O_2, other organic peroxides (Peters and Foote 1976), or through a reaction similar to the Fenton's and Haber-Weiss reactions (Haber and Weiss 1934; Fridovich 1978) (Fig. 1.10). It is now generally agreed that the direct reaction of O_2^- with H_2O_2 is a slow process

(Ferradini and Seide 1969; Halliwell 1976). Other interconversions are possible as shown in Fig. 1.8.

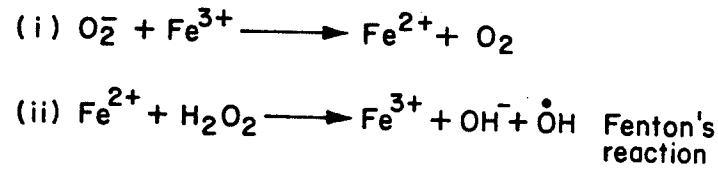


Fig. 1.10. Production of $\dot{O}H$ in the Fenton's reaction and the involvement of O_2^-.

B Methods for detecting free radicals

Free radicals have been detected by both direct and indirect methods. The direct methods consist of electron spin resonance (esr) and chemiluminescence, while the indirect methods employ oxidation, reduction and/or transformation of specific compounds to specific products.

i Direct methods for detecting free radicals

(a) Electron spin resonance (e.s.r.): Esr or electron paramagnetic resonance (epr) employs a principle similar to that of nuclear magnetic resonance (nmr) (Metzler 1977). Free radicals contain unpaired electrons; the latter have magnetic moments. Therefore they will absorb electromagnetic radiation of microwave frequencies. Microwave frequencies of the order of 10^{10} Hz are used in esr, while radio frequencies are employed in nmr. Thus the energies used in esr are

about 100 times those used in nmr.

The absorption spectra of the radical(s) generated may be observed directly or a spin label or trap (compound that can bind a free radical) may be used to stabilise and/or help characterise the radical. The radical is then identified based on the spectral characteristics of free radical standards. The conditions for absorption of energy in the esr spectrometer are given by the equation $h\nu = g\beta H_o$, where:

ν = resonance frequency at which absorption occurs in the spectrometer

h = Planck's constant

H_o = strength of the external magnetic field

β = a constant called the Bohr magneton, and

g = spectroscopic splitting factor.

The nature of the free radical is decided from the g value and hyperfine structure of the esr spectrum. The value of g is exactly 2.000 for a free electron, but may be somewhat different in radicals and substantially different in transition metals. Due to "spin-orbit coupling", g values may vary with environment. "Spin-orbit coupling" arises as a result of the p and d orbitals of atoms having directional character; therefore g sometimes has three discrete values for the three different directions (called g value anisotropy). In other instances, the g value parallel to the direction of H_o ($g_{||}$) differs from that in the perpendicular direction ($g_{\perp}$). Both values can be ascertained experimentally.

"Hyperfine structure" results from coupling of the magnetic moment of the unpaired electron with nuclear spins. It is given as the hyperfine splitting constant (A). Splitting may be caused by a magnetic

atom nucleus about which the electron is moving, or by some adjacent nucleus, or other unpaired electron. Sometimes the absence or presence of splitting may be used in making important chemical conclusions.

In esr spectroscopy, the first derivative of the absorption, rather than the actual absorption is usually plotted; thus the peaks of the bands of the spectrum mark the points of maximum slope on the actual absorption spectrum. To reduce noise due to molecular motion and instability of some radicals rapid freeze methods can be employed (Bray 1961). Computer simulations are commonly used to match observed band shapes under varying conditions, with those predicted for particular theories of motional broadening of lines (Devaux and McConnell 1972; Sackmann and Trauble 1972).

Spin trapping (Janzen 1971) was envisaged as a technique which could specifically identify and quantitate O_2^- and OH radicals (Harbour et al. 1974; Harbour and Bolton 1975; Fee and Valentine 1977). In order to do so, spin trapping must be able to distinguish the difference between the free radicals. The resultant nitroxide should be relatively stable and the rate constant for the reaction of the radical with the spin trap should be high enough to allow for the efficient detection of short-lived free radicals. Thus nitrone spin traps, particularly DMPO (5, 5-methyl-1-pyrroline-N-oxide) have been widely used as spin traps (Lai and Piette 1977, 1978; Saprin and Piette 1977; Harbour and Hair 1977; Harbour and Bolton 1978; Sealy et al. 1978). Recent reports (Finkelstein et al. 1979), however, have shown that although DMPO can trap both O_2^- and $\dot{O}H$, the DMPO adduct of superoxide anion (DMPO-OOH) is highly unstable and decomposes into the DMPO-hydroxyl radical adduct

(DMPO-OH); α-4-pyridy-1-oxide N-test-butyl nitrone (r-POBN) described by Janzen et al. (1978) also formed an unstable adduct with O_2^-, but it did not form hydroxynitroxide (4-POBN-OH) on decomposition. However, it was found that TMPO (2, 5, 5-trimethyl-1-pyrroline-N-oxide), structurally similar to DMPO, but lacking a β-hydrogen, formed a stable nitroxide upon reaction with superoxide. It was also found that spin trapping was an extremely inefficient method for detecting superoxide. During these investigations it was further shown that EDTA as a chelating agent lowered the signal due to DMPO-OOH, but DETAPAC (diethylenetriamine pentaacetic acid) as a chelator had no other effects. Buettner et al. (1978) found that DETAPAC-iron is inactive as a SOD in contrast to EDTA-iron. Thus choice of the spin trap should be examined considering the purity of the buffers, enzyme preparations chelating agents and the radical to be trapped.

(b) Chemiluminescence: Some free radicals like $^1O_2$ (Nakano et al. 1975) emit light as they fall to their ground states. This principle has been employed by a number of researchers (Handler et al. 1964; Brown and Johns 1968; Whillans and Johns 1969; Nakano et al. 1975), but its specificity has been challenged (Nilsson 1969).

ii Indirect methods for detecting free radicals

Free radicals individually or in combination(s) are known to reduce, oxidise and/or convert certain compounds to specific products; these transformations are inhibited by compounds that specifically either catalyse the removal of or react (quench) with the radicals directly. These properties have been employed in detecting the presence

of oxygen radicals particularly O_2^-, $\dot{O}H$ and $^1O_2$.

The reduction of ferricytochrome C to ferrocytochrome C (McCord and Fridovich 1968), nitro blue tetrazolium to a formazan (Misra 1974); and the oxidation of epinephrine to adrenochrome (Misra and Fridovich 1972b)and sulfite to tulphate (Fridovich and Handler 1961) have been employed in combination with SOD and other proteins or free radical scavengers to detect the presence of O_2^-. The reduction of hydroxylamine to nitrite is facilitated by a combination of $\dot{O}H$ and O_2^- (Elstner and Heupel 1976), therefore the presence of both radicals may be demonstrated. Unlike esr, these indirect methods can only detect one specific radical which must be specific for the reaction, and must be the only one capable of reacting with the indicator compound like cytochrome C. Hence such methods can not be used in tissue incubation mixtures. In addition recent evidence has challenged the specifity of certain transformations; the oxidation of 2, 5-diphenylfuran to 0-dibenzoylethylene, and/or 1, 3-diphenylisobenzofuran to 0-dibenzoylbenzene were thought to be specifically carried out by $^1O_2$ (King et al. 1975), however, other agents have been proven to have the capacity to carry out these oxidations (Chan 1971, Hawco et al. 1977; Takayana et al. 1977; Borg et al. 1978).

C Cellular effects of free radicals

The possibility that free radicals play an important role in normal metabolism, albeit at low concentrations, and in the development of a variety of forms of tissue damage is now being accepted with increasing momentum. Free radicals have been implicated in the following

(see Willson 1977a,b): (1) photosensitisation of the skin or retina during various pathological disorders, or following the ingestion of various artificial or naturally occurring compounds; (2) lethal effects of ionising radiation; (3) liver damage induced by carbon tetrachloride and the toxic action of chloroform and DDT; (4) carcinogenic, mutagenic and toxic action of various nitrogen-containing compounds including, nitro, nitroso, hydroxylamine and azo derivatives; (5) carcinogenic action of polycyclic aromatic hydrocarbons; (6) toxic effect of oxygen on anaerobic microorganisms and of hyperbaric oxygen, ozone and peroxides on mammalian tissue; (7) disorders resulting from metal imbalance such as iron overload or zinc deficiency; (8) alcohol poisoning and alloxan-induced diabetes; (9) herbicidal and toxic effect of paraquat and related viologens; (10) prostaglandin synthesis, inflammation and the action of aspirin; (11) the ability of granulocytes to destroy invasive bacteria; and (12) neural disorders and the action of phenothiazines.

Although evidence for the role of free radicals in some of the above effects is circumstantial and in others derived from studies on the protective effects of known free radical scavengers, they are supported by studies of the effects of free radicals on cells. Free radicals, particularly O_2^- and $\dot{O}H$ have been shown to inactivate viruses (Lavelle et al. 1973), induce lipid peroxidation (Kellogg III and Fridovich 1975), damage cell membranes (Goldberg and Stern 1976; Kellogg III and Fridovich 1977), kill cells and attack DNA (Michelson and Buckingham 1974), and cause oxidation of GSH, lipids and lysis of RBC (Fong et al. 1973). It is generally felt that $\dot{O}H$ formed from $^-O_2$

and H_2O_2 or other reactions, is the most potent oxidising radical and may account for the major portion of direct cellular damage (Beauchamp and Fridovich 1970). It is important to note, however, that free radicals may protect the body against viral (Lavelle et al. 1973) and bacterial (Cheson et al. 1977) infection. The production of free radicals during the respiratory burst preceding phagocytosis by leukocytes is thought necessary to kill the bacteria; and O_2^- may donate its electron to methemoglobin or metmyoglobin.

D Defenses against free radicals

It is apparent from the preceding sections that, even in the absence of foreign compounds, the biological cell contains many chemicals which, if able to react with each other, would produce potentially damaging free radicals. The iron-catalysed oxidation of thiol groups by oxygen with the possible formation of O_2^- and $\dot{O}H$, is a common example (Willson 1977a,b). However, such reactions appear to be controlled to a large extent by the molecular compart mentalisation of the cell (Willson 1977a, b). The catalytic activity of iron is normally carefully controlled by the incorporation of the metal into elaborate macromolecular structures. Willson (1977a, b) postulates that the apomoieties of enzymes, such as catalase, the various oxidases, and cytochrome P_{450} may play a greater role in preventing undesirable reactions than in actual catalysis; similarly, vital sites sensitive to iron-catalysed oxidation are carefully protected from interactions with iron, by being buried in some macromolecular structure or by being complexed to zinc ions. It is not surprising that zinc is known to be part of over 80 known enzymes, and binds with thiols, blocking the

binding of iron which makes the latter susceptible to oxidation. Zinc also inhibits lipid peroxidation and stabilises cellular membranes making them more resistant to free radical attack (Willson 1977b).

In addition to compartmentalisation, the body defends itself by a number of enzymes and compounds like GSH and thiol proteins synthesised in the body. Superoxide dismutase, peroxidases and catalase are the most important enzymes (see Fig. 1.9). Since free radicals mainly arise by univalent oxygen reduction, the O_2^- seems to be the "mother" of the other radicals. It is not surprising that the aerobic cell has developed a "special" enzyme, SOD, to remove the superoxide radical. Superoxide dismutase is a copper-zinc protein formerly known as the cupreins, whose nomenclature reflected the tissue of origin and the content of copper; for example the erythrocuprein of human RBC (see Fridovich 1974). Normal cells with organelles (unlike RBC) contain a manganese-containing SOD in the peroxisomes and mitochondria. The assays for SOD are primarily based on its ability to inhibit reactions carried out by O_2^- and Fridovich (1975) has reviewed this and other aspects of SOD that are beyond the scope of this thesis. The location of SOD is not strategic for combating O_2^- produced in plasma. It would seem therefore, that iron and/or other metal proteins in plasma play a dismutation role similar to that observed with the EDTA-iron complex (Buettner et al. 1978).

Catalase is an iron-protein which catalyses the reduction of H_2O_2 to water. Catalases of many eukaryotic cells are localised in subcellular organelles, in most cases in peroxisomes (Ruis 1979). Thus both SOD and catalase seem to have been developed to protect subcellular

organelles containing high levels of the oxidase enzymes whose reactions produce O_2^-.

Peroxidases reduce H_2O_2 utilising various types of thiol compounds. Glutathione peroxidase, a selenium-containing enzyme is thought to be the most important. The peroxidases are considered the major scavengers of H_2O_2 because of their location in the cytosol of the cell, and the plasma. Glutathione reductase is a flavin enzyme responsible for regenerating GSH from GSSG utilising NADPH as the hydrogen donor. Thus GSH and other reduced thiols help in neutralising the peroxides produced during oxidation by the free radicals. This prevents spread of the chain reactions. The methemoglobin and metmyoglobin reductase systems (Beutler 1978; Fridovich 1978), too help regenerate ferrous hemoglobin and therefore prevent its further denaturation and subsequent formation of heinz bodies.

A number of proteins and enzymes mentioned above contain metals or vitamins like riboflavin in their molecules. Therefore the levels of certain nutrients in the diet may be important in prevention of or predisposition to free radical toxicity. Some metals like copper are known to dismutate O_2^- (Rabani *et al*. 1973) and iron can do so when complexed with certain chelating agents and perhaps plasma proteins. The protective effects of zinc and the role played by selenium have already been cited. The importance of iron to health is common knowledge, but the lack of an upper limit to the amount of iron that can be added to, particularly, human foods may be dangerous in view of the fact that iron catalyses oxidation of thiol molecules to produce free radicals.

Many vitamins, particularly the group B vitamins, are coenzymes; riboflavin is part of the GSSG-red enzyme. However, vitamin E is presently the most important among vitamins in protecting against free radicals and the effects of hemolytic drugs (Rose and Gyorgy 1950, 1952; Brownlee et al. 1977; Dashman and Kamm 1979; Inouye et al. 1979), and its deficiency in chick and rat diets leads to spontaneous hemolysis (Rose and Gyorgy 1952; Scott et al. 1976; Scott 1978). Ascorbic acid (vitamin C) also reacts rapidly with free radicals (French et al. 1978; Packer et al. 1979; Winterbourn et al. 1979). These effects are due to the antioxidant properties of these vitamins (see Packer et al. 1979). It has also been suggested that the unique structure of vitamin E may enable it to penetrate to a precise site in the membrane (Lucy 1972; Slater 1978) and provide unique protein protection to "target" molecules essential for membrane integrity. In addition, vitamin E is considerably more lipophilic than vitamin C and hence is a more potent antioxidant, particularly with respect to lipid peroxidation. Recent evidence points to a possible role of vitamin E in the conversion of cyanocobalamin (vitamin B_{12}) to its coenzyme form (5-deoxyadenosylcobalamin), which is a cofactor of the enzyme methylmalonyl-COA mutase (responsible for converting methylmalonyl-COA succinyl COA). Packer et al. (1979) have shown that in addition to scavenging other radicals, ascorbic acid may be the specific regenerator of reduced vitamin E after the latter is converted to the quinone radical in its redox reactions (see Fig. 1.11). This finding may account in part for the fact that clinically overt vitamin E deficiency has not been demonstrated in man. Under certain conditions the vitamin C radical can be enzymatically

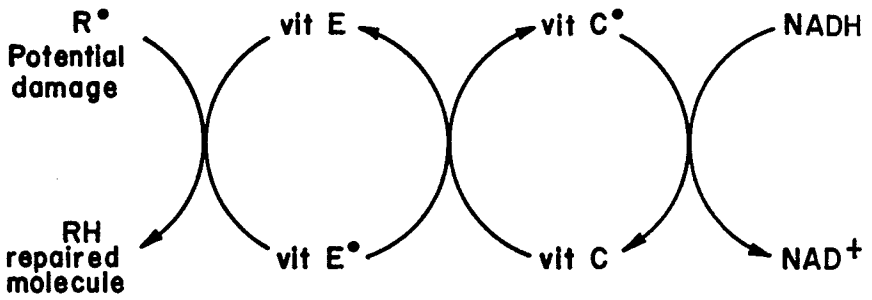


Fig. I·II Regeneration of active vit E by ascorbic acid (vit C) (Packer et al, 1979)

reduced back by an NADH-dependent system (Schneider and Staudinger 1965). Tryptophan, thiols and other compounds with reducing properties seem also to provide some protection against free radical toxicity. Therefore nutritional adequacy/inadequacy may play an important role in predisposition to free radical damage and the effects of hemolytic drugs in general.

In view of the literature cited experiments were initiated to:

(1) Develop a method for conveniently obtaining large quantities of vicine material from fababeans, since it was not available commercially.

(2) Study the effects of dietary vicine on chicks and laying hens.

(3) Try to establish the mode of action of vicine and convicine and their aglycones in causing egg weight depression in laying hens.

CHAPTER 2

QUANTITATIVE ISOLATION OF VICINE AND THE PREPARATION OF DIVICINE

2.1. Isolation of vicine

INTRODUCTION

A. Perspective

The methods reviewed in the literature for isolation of vicine from fababeans are either tedious and/or they use chemicals containing lead or mercury which may be hazardous both to man in large scale operations, but also to experimental animals if these compounds are not completely removed from the vicine sample. In addition, the costs of chemicals employed in some of the methods may be prohibitive to large scale isolations. Finally the purity of the products was not properly checked in some isolations and in others the properties of the preparations were far from those generally accepted and the yields low. Olaboro (1978) found that an ethanol/water (1:1) extract of FBPC could be further fractionated with acetone/water (3:1) into a precipitate and supernatant, both of which caused egg size depression (i.e. contained vicine) when added to diets of laying hens. The precipitate was more potent and contained more vicine per unit weight than the supernatant. It was therefore hypothesised that a simplified isolation method based on that of Olaboro (1978) could be developed.

Throughout the development of the method FBPC was employed. It was prepared by air classification (Craig 1974; Marquardt et al. 1975) at the University of Manitoba. The FBPC contained 93.0% dry matter and 67%

protein. The isolation method was divided into three stages, viz. (1) the extraction of vicine from the FBPC with solvent; (2) obtaining crude vicine from the extract; and (3) preparation of pure vicine from crude vicine. The development of the method was organised under these categories. However, it is easier to present stages 1 and 2 together and the third one separately. The initial problem was to choose between ethanol and acetone as precipitants of protein, amino acids and other undesirable compounds. The choice was influenced by the solubility of vicine in the solvent-water mixtures and the ease of evaporating the solvents. Therefore 0.5 gm of crude vicine (80% pure) prepared by the Olaboro method (Olaboro 1978), was dissolved in 50 ml of water and the pH adjusted to 7.1. Ethanol or acetone were then added and mixed until a white precipitate was evident. This was considered to be the upper limit of the ratio of solvent to water for purposes of vicine isolation. The samples were left to equilibrate for 15 minutes between additions of the solvent. This test assumed that the solubility of vicine was not affected by other compounds and that this data would apply to vicine in the fababean protein concentrate. However, this test was intended as a guide. These tests implied that 4:1 mixtures of either acetone or ethanol and water did not cause vicine precipitation from the solution; increasing the amount of acetone slightly beyond this ratio resulted in some precipitation after 15 minutes. Since acetone is more volatile than ethanol and therefore easier to evaporate it was employed throughout the initial extraction stage. In addition a 3:1 ratio of acetone to water was employed to avoid working at the extreme limit.

B. Development of method based on extraction with acetone-water mixtures

In developing this method it was assumed on the basis of the Higaz-Read assay (Higazi and Read 1974) that FBPC contained a maximum of 2% total vicine (vicine + convicine) by weight, and that the solubility of vicine was 1 g/100 ml of water. Therefore 1 kg of FBPC would contain 20 g of vicine which would require 2 litres of water for complete dissolution. The volume of acetone would depend on the amount needed to precipitate out the proteins and compounds other than vicine, and that necessary for the convenient handling (e.g. filtration) of the insolubilized FBPC. Keeping the volume of water constant (2 litres/ kg FBPC), it was found that 5 volumes, (based on weight of FBPC) of acetone were necessary for the extraction; thus 7-8 volumes of water/acetone mixture was required. Lower volumes (by reducing acetone) gave a thick slurry that was difficult to filter and the resultant filtrates were thick and almost impossible to filter through polyester cloth (particularly with 4 and 5 volumes of the mixture). Therefore 7 (2.5:1) or 8 (3:1) volumes of acetone/water mixture were thought to be adequate.

A clear supernatant did not form on top of the slurry, after one hour, when 7 volumes of extraction mixture were used, and therefore the supernatant could only be obtained by centrifugation or by squeezing. Centrifugation was not a viable alternative as the volume of sample was too large to be conveniently centrifuged. However, with 8 volumes a considerable quantity of clear supernatant was formed and was filtered through cheese cloth. The rest of the slurry was pressed in cotton bags with a mechanical press. Therefore 8 volumes were employed in the

initial trials and the evolution of the rest of the initial complete isolation procedure is subsequently described.

The first extraction stage involved suspending the FBPC in the extraction solvent, mixing for 5 minutes and then homogenising (Teckmar-SD 4SN) for 10 minutes. The mixture was left at room temperature for 30 minutes, after which the clear supernatant and the slurry were filtered through a double layer of cheese cloth and finally squeezed out. The second stage involved concentrating the filtrate in a rotary evaporator until the volume was low enough for the vicine to crystallise out. Centrifugation (14,600xg for 20 min) before concentration prevented the filtrate from becoming unmanageably thick before it was reduced to the desired volume (0.072) volumes, based on weight of original FBPC). The final concentrate was dark brown in colour, and when the right volume was attained white needle-shaped crystals were immediately formed even at room temperature. After a day of crystallisation at $4^{o}C$, the contents were centrifuged (15,500xg for 15 min) and the pellet and supernatant (residual supernatant) saved. Acetone was added to the residual supernatant until turbidity was observed and the sample was recentrifuged. The pellet obtained was pooled with the first pellet and washed twice with one volume of cold distilled deionised water. It was then washed with 2 volumes of ethanol and 5 volumes of acetone; with centrifugation after each washing. The volumes of solvents were based on the weight of the wet pellet. The product obtained up to this stage contained 88% vicine with the balance being mainly convicine, (amino acid method) and was referred to as crude vicine. This term was adapted to refer generally to the vicine material obtained at the end of the second stage.

The third stage for producing pure vicine involved recrystallisations (Olaboro 1978). Crude vicine was dissolved in 7 volumes of water (based on wet weight), and the pH adjusted to 9-10. The sample was then heated with constant mixing, and frequent checking of the pH to make sure it was in the desired range. This was followed by centrifugation (15,500x g for 10 minutes). Crystallisation was allowed to occur at $4^{o}C$. The crystals were harvested by centrifugation as above and then oven-dried ($60^{o}C$) or lyophilised. Two additional recrystallisations yielded what was considered to be pure vicine. The major problem with this method was that the yield of crude vicine and subsequently the pure vicine was low; crude vicine was only 0.08% of FBPC which was only 5% of total vicine if it is assumed that FBPC on average contained 1.5% total vicine; and centrifugation before the second stage of the isolation procedure was time-consuming. Finally, concentrating in a rotary evaporator was slow and meant that small quantities would be concentrated at a time.

The latter problem was solved by switching to a cyclone evaporator. Centrifugation before the second stage was eliminated by filtering the initial filtrates through fine polyester cloth or by using cotton bags instead of cheese cloth; this did not completely solve the problem as a considerable amount of impurities were present in the crude vicine and therefore larger washing volumes were needed to obtain clean crude vicine, and even then some dark material could not be completely removed. Therefore centrifugation was again employed except that in order to avoid large volumes, the filtrates were concentrated to one volume and the pH raised to 9-10 with 3N NaOH to increase the solubility of the crude vicine. The filtrates were then concentrated further to

0.2 volumes and then centrifuged (14,600xg for 20 min) at 30°C. The pH of the supernatant was then lowered to 6.55 with 2N HCl and was stored at 0°C to allow crystallisation. The yield of crude vicine using these modifications was 0.2% of FBPC, but it needed several recrystallisations to eliminate some dark coloured contaminants.

Therefore it was thought that acetone before the second stage could be employed to precipitate out some of this dark material. The acetone was added after raising the pH of the concentrate to 9-10, and concentrating to 0.1-0.2 volumes. It was found that vicine precipitated out of solution when more than one volume (based on the volume of concentrate) of acetone was added. Trying to exploit this method for isolation was unsuccessful because the quality of the resulting crude vicine was poor. Therefore a better alternative was to try and precipitate out the impurities and leave the vicine in solution. This stage needs experience because in some cases even 1 volume of acetone depending on the concentration and the purity of vicine could cause vicine precipitation. However, it was found that acetone could be added so long as no white precipitate was perceptible. The viscous contaminants in the presence of acetone were converted into a fluffy yellow floating material and were scooped off. The samples were then concentrated to about 0.06 volumes and after cooling the pH was lowered to 7.0 with 3N HCl. The yield and quality of crude vicine were slightly improved.

It was then difficult to improve the method because it was not clear where the vicine was being lost due to lack of a quick assay method. However, assuming that vicine was not destroyed during heating in the

cyclone evaporator (Olaboro 1978), and considering the volumes of residual supernatant and water washings, it was concluded that most of the vicine was left in the FBPC. Therefore it was decided to investigate the effect of (1) pH at different steps of the initial extraction stage, (2) effect of volume of solvent, and (3) the effect of double extraction on the yield of crude vicine. Since the amount of FBPC used had been raised, the procedure for washing at stage two was modified; the crystallite was centrifuged (14,600xg for 20 min) and the supernatant discarded. The vicine pellet was washed twice with 2 volumes of ethanol and 5 volumes of acetone. The sample was centrifuged as before after each wash, but for 30 minutes after the acetone wash because the pellet was not firm enough after 20 minutes of centrifugation. The pellet (crude vicine) was dried to constant weight at 60°C. The method employed in the subsequent investigations is schematically shown in Figure 2.1.

MATERIALS AND METHODS

2.1.1. Effect of pH of filtrate on the yield of crude vicine

In all the subsequent studies 5 kg of FBPC were extracted with 8 volumes of acetone/water (3:1) mixture unless specified otherwise. In this trial various amounts of base (3N NaOH) were added to the filtrates so that after acetone was evaporated, the concentrates had a pH of 7.02, 8.1 or 10.0. The samples were mixed and left at room temperature for 30 minutes before further processing.

2.1.2. Effect of adding base to the FBPC in water

A preliminary study was conducted to establish the titration

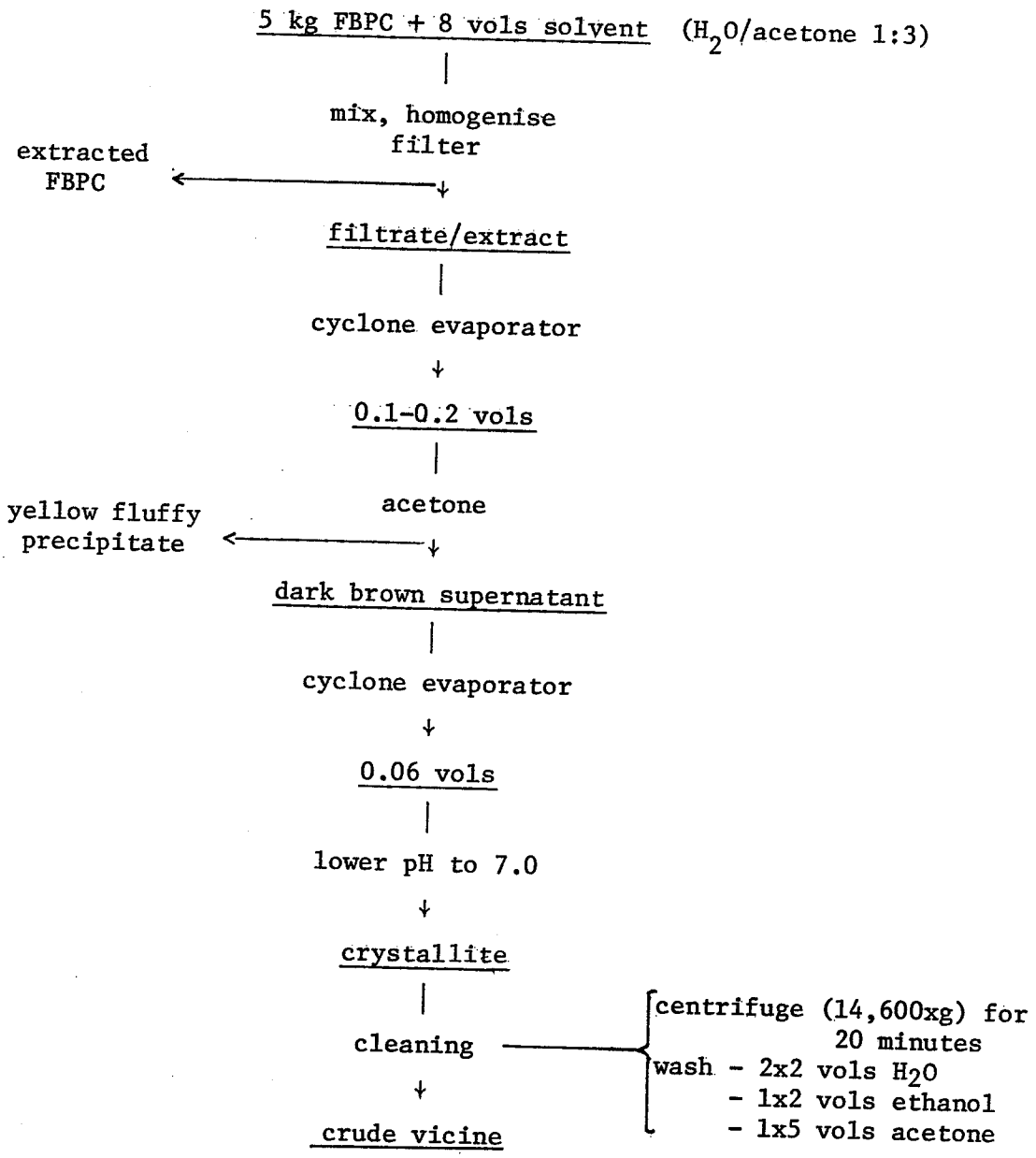


Fig. 2.1. Flow diagram of the initial method for isolation of crude vicine.

properties of FBPC. For this trial 5 g of FBPC were dissolved in 20 ml of distilled deionised water and allowed 15 minutes to equilibrate at room temperature. The pH of the solution was 6.26. The solution was then titrated with base first (0.058N NaOH) to pH 11.0, and then back titrated with acid (0.11N HCl) to pH 2.0 (see appendix Figure 2.1A). This data was employed to estimate the amount of base required to raise the pH of FBPC in water. Thus water was first added to the FBPC, the sample mixed and homogenised, and base was added during the homogenisation procedure. Acetone was then added and the sample mixed. The rest of the procedure was as described previously (section 2.1 A ii and Figure 2.1).

2.1.3. Effect of double extraction on the yield of crude vicine

The first extraction was the same as in Experiment 2.1.2. It involved the use of 500 ml of base since the highest yield of crude vicine was obtained with this amount of base (Table 2.2). The extracted FBPC was kept frozen until it was reextracted with half the volume of solvent employed in the first extraction without further addition of base. The sample was then processed as before except that the volume at each stage was proportionally lower.

2.1.4. Effect of volume of solvent on the yield of crude vicine

An experiment was conducted to establish if a single extraction using large volumes would provide the same yield as the double extraction method. Therefore 10.5 volumes of acetone/water (2.1:1) mixture (this includes the 500 ml of base) were employed. The volume of acetone was

reduced so that the amount of water could be raised to the total of that used in both extractions of Experiment 2.1.3. The rest of the isolation procedure was as already described, and the volumes at various stages were similar to those of the first extraction in Experiment 2.1.3.

2.1.5. Effect of pH of crystallisation on the yield of crude vicine

The isolation procedure was the same as that described for the first extraction in Experiment 2.1.3. However, the pH of the concentrated extract was lowered to either 7.2 or 9.0.

2.1.6. Effect of mixing time on the yield of crude vicine

The isolation procedure was the same as described for the first extraction in Experiment 2.1.3 except that the sample was mixed for 0, 1, 5 or 10 minutes.

2.1.7. Effect of pH, volume and ratio of acetone/water of the extraction solvent on the amount of vicine extracted

The objective was to establish the most suitable combination of total volume of solvent, ratio of water to acetone, and the amount of base needed to obtain good yield of vicine considering the work involved. To a homogeneous suspension of 2 g FBPC in 6 ml of water was added acetone, followed by base. Samples were then homogenised for 30 sec with a Silverson homogeniser set at full speed. The slurry was centrifuged (29,000xg for 10 minutes) and 50 µl of the supernatant were dried at 40°C under nitrogen. The dried residue was weighed, then dissolved in 10 ml of 0.1N HCl and absorbance determined at 274 nm. Solubility of vicine was calculated using ε = 16392.8 and a molecular weight of 322.

2.1.8. Modified method for isolation of crude vicine

From the preceeding experiments it was decided that 7.55 volumes of solvent (water:3N NaOH:acetone, 9.2:1:20) be tried. Therefore in the usual isolation employing 5 kg of FBPC, 11.5, 0.9 and 25 liters of water, 3N NaOH and acetone, respectively were used. Water was added first, base second and acetone last. The samples were respectively mixed for 5, 1 and 4 minutes. The slurry was placed in cotton bags and squeezed in a mechanical press. After several preliminary trials it was found unnecessary to further filter through polyester cloth. It was also found that at this pH and volume, the extract could be concentrated to 0.075-0.08 volumes (instead of 0.06) without need for the acetone clearing step. After cooling, the pH of the concentrate was lowered to 7.0-7.2 with 8N HCl (instead of 3N HCl) so as to reduce the volume changes. The higher pH (7.2) prevented some contaminants from coprecipitating with vicine and therefore was selected. The samples were then left to crystallise at $0^{o}C$ for 1-2 days.

Due to the elimination of the acetone clearing step in stage 2 of the isolation procedure there were more impurities in the crystallite, therefore the procedure for cleaning the crystallite was modified. After centrifugation (14,600xg for 20 min), the vicine pellet was washed twice each with 2.7 volumes of water (based on wet weight of the vicine pellet), and once with 5 volumes of acetone. The crude vicine was dried as before.

2.1.9. Preparation of pure vicine and convicine from crude vicine

(a) Ion exchange chromatography

The main objective here was to establish whether a concentrated extract of FBPC could be separated into vicine, convicine and other fractions. The eluted fractions would then be concentrated and crystallised. Preliminary work was performed with crude vicine to establish (a) the capacity of different resins for vicine/convicine and (b) their resolutions for vicine and convicine. Trials with the concentrated extracted were then carried out.

(i) Capacity of different resins for vicine/convicine

In all cases a column bed volume of 53.0 ml, packed in glass columns of equal length and diameter were employed. Cation exchange resins were equilibrated with 0.1N sodium citrate buffer (pH 3.25) while 0.1N Tris buffer (pH 9.0) was employed for anion exchange resins. Dilute solutions of crude vicine in their respective initial eluting buffers (0.25 g/100 ml) were added to the columns and the elution profiles were monitored at 280 nm (ISCO optical unit type 6). The sample was added until the UV absorbing material was eluted indicating that the column was saturated with vicine and/or convicine.

(ii) Resolution of vicine and convicine

To avoid potential hydrolysis at low pH, it was decided to use anion exchangers for further investigations. In all cases the column bed volume was as in the previous section, and the columns were equilibrated with 0.1N Tris buffer (pH 9.0). One milliliter of crude vicine in the equilibrating buffer (0.5 or 1.0 g/100 ml) was applied to

the column, followed by 130.0 ml of the same buffer, then 100 ml of Tris buffer (pH 7.0), sodium citrate buffer (pH 4.0) and 0.1N HCl. The eluents were monitored as before at room temperature.

(iii) Chromatography of a concentrated extract of FBPC

The extract was obtained as described in section 2.1.8. However, instead of lowering the pH to 7.2, it was raised to 9.0, and 0.5 ml were diluted to 50 ml with 0.1N Tris buffer (pH 9.0). One milliliter of the sample was applied to the column and eluted successively with 180.0 ml Tris buffer (pH 6.6) and 40 ml of 0.1N HCl. The eluents were monitored as before.

(b) Purification of vicine and convicine from crude vicine by recrystallisation

Due to the poor results obtained with ion exchange column chromatography, it was decided to try to prepare pure vicine from the crude vicine by recrystallisation. In all these trials, crude vicine was ground using a motor and pestle. Various combinations of volume of water, pH and temperature were tried out during the development of the method.

(i) Crystallisation of vicine and convicine as affected by pH

The aim was to selectively dissolve vicine, convicine and impurities by varying the pH. Therefore 1 g of crude vicine was suspended in 10 ml of water and the pH adjusted to 8.5, 9.0 or 9.5; the samples were equilibrated at room temperature ($27^{o}C$) for 15 minutes, and then the pH of the solutions were readjusted as before. This was followed by centrifugation (20,000xg for 10 min) to remove undissolved

vicine and other insoluble materials; the pH of the resulting supernatants was lowered to 7.2 and stored overnight at 0°C. The crystals were harvested by centrifugation as before and dried to a constant weight at 60°C.

In the second test, the aim was to dissolve all the crude vicine at room temperature by increasing the pH, and then to separate vicine and convicine by a stepwise drop in pH. Sufficient time for crystallisation was allowed between each of the two steps. Therefore 20 g of crude vicine were suspended in 200 ml of water and the pH raised to 11.0 with 6N KOH solution. The solutions were centrifuged (13,200xg for 20 min), the pH of the supernatant lowered to 9.5 with 3N HCl and left at room temperature (27°C) for 1 hour. The samples were then centrifuged as before and the supernatant saved. The pellet was washed successively, twice with four, once with four and twice with five volumes (based on dry weight of crude vicine) of water, ethanol and acetone, respectively, and then dried at 60°C. The water washes and the supernatant were pooled, the pH was lowered to 7.2 and the solution was stored at 0°C overnight. The resulting crystallite was processed as before.

(ii) Recrystallisation of vicine and convicine

Ground crude vicine was suspended in 9 volumes of water and the pH raised to about 11.0 with 12.5N NaOH (with stirring) until all particulate matter was solubilised. The solution was centrifuged (14,600xg for 30 min), the volume of the supernatant determined, and its pH lowered to 9.0 with 8N HCl. It was stored at 0°C for 3 hours or

overnight. The crystals were harvested by centrifuging at 14,600xg for 30 minutes. The pellet obtained was resuspended in 14.5 to 15.0 volumes (based on its wet weight) of distilled deionised water, the pH re-adjusted to 11.0 and the sample centrifuged as before. The pH of the resulting supernatant was lowered to 9.0 and left to crystallise as before. The crystals were harvested as before and washed successively with six, nine and 11.5 volumes (based on wet weight of pellet at the start of the second recrystallisation) of water, ethanol and acetone, respectively; and then dried in the oven as before. These were designated "pure" vicine. The supernatant obtained after removal of vicine was then processed so as to obtain a convicine rich fraction. The pH of this supernatant was lowered to 7.2 with 8N HCl and stored at $0^{o}C$ overnight. The crystals were harvested by centrifugation at 14,600xg for 30 minutes. The pellet was then washed twice with 11.5 volumes (based on wet weight of pellet after the first recrystallisation) of water, and centrifuged as before after each wash. The resulting pellet was resuspended in 5.7 volumes of water and the pH raised to 11.0 with 12.5N NaOH. After centrifugation (as before) the pH of the supernatant was lowered to 7.2 and then stored at $0^{o}C$ overnight. The crystals were harvested as before and successively washed with 5.7 and 11.5 volumes of ethanol and acetone, respectively; the volumes were again based on the wet weight of the pellet after the first recrystallisation. The samples were dried as before. These were considered to be "pure" convicine. An attempt was made to isolate vicine and convicine from dehulled fababeans in a similar manner.

2.1.10 Characterisation of vicine

(a) Dry matter of "pure" vicine

Three-tenths of a gram of "pure" vicine were dried in a vacuum oven at 90°C for 4 hours in preweighed pans. After cooling in the dessicator for 2 days the samples were reweighed.

(b) Nitrogen content

Nitrogen content was determined by the micro-Kjeldahl method.

(c) Melting point

The melting points of vicine and convicine were determined by placing a small amount of sample into a capillary tube with one end sealed. The capillary tubes and thermometer, side by side were immersed in concentrated sulfuric acid in a micro-Kjeldahl flask. Heat was applied gently until the sample decomposed.

(d) UV spectrum of vicine and convicine

One milliliter of a solution of vicine or convicine in 0.1N HCl (5 mg/ml) was added to 15.0 ml of 0.1N NaOH, 0.1M phosphate buffer (pH 6.2) or 0.1N HCl, just before scanning. Samples were again scanned twenty minutes later to check for hydrolysis.

(e) Effect of pH on the stability of vicine

A standard solution of vicine in water (50 mg/100 ml) was prepared, and 3 ml in duplicate were diluted to 50 ml with 0.1N HCl, water, 0.1M phosphate buffer or 0.1N NaOH. Three milliliters of water were similarly diluted for the blanks. The absorbance of the solutions at 274 and 280 nm were monitored at various intervals and the pH at the completion of the test.

(f) Effect of pH on the molar extinction coefficient (ε) of vicine

In the first trial, the solutions prepared in section (e) were used to calculate the ε value of vicine in 0.1N HCl, 0.1M phosphate buffer (pH 7.2) and 0.1N NaOH. In the second trial the changes in ε value were investigated in the 5 to 11 pH range using two appropriate buffers, tris and phosphate. Buffers of different pH were prepared from their stock solutions such that their concentration was the same (0.2M). Dibasic sodium phosphate was used to prepare phosphate buffers of pH 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0, while tris buffer was employed to prepare buffers of pH 7.0, 8.0, 9.0 and 10.0. Equal volumes (3 ml) of buffer and vicine in water (6 mg/100 ml) were mixed and the absorbance and pH determined.

(g) Optical rotation of vicine and convicine

Solutions of vicine or convicine in 0.2N NaOH were stored overnight at $0^{o}C$. Optical rotation was determined in a Carl Zeiss polarimeter and specific rotation calculated according to the formula:

$$(\alpha)_D^T = \frac{(\alpha) \text{ observed}}{1 \; c}$$

where T = temperature (^{o}C) = 27

D = Na line = 5,461 Å

C = concentration (g/100 ml)

1 = optical path length in decimeters

(h) Effect of pH on the solubility of vicine and convicine in water

(i) Vicine

Three-tenths of a gram of vicine were suspended in 3.0 ml of water (28°C) and thoroughly mixed. Precautions were taken so as to assure that the solution would be saturated with vicine at all pH levels. The pH of the solution was adjusted to the desired level (with 0.1N HCl or NaOH) with constant stirring and then stored sealed overnight at 28°C with shaking. The pH was then recorded before the sample was centrifuged (29,000xg for 10 min). Fifty microliters of the supernatant were diluted with 15.0 ml of 0.1N HCl and the absorbance was determined at 274 nm. The normalisation of the pH of all solutions permitted the use of one ε value in the solubility calculations. The ε value of 16393 in 0.1N HCl(Table 2.7) and a molecular weight of 322 were used in these calculations.

(ii) Convicine

Excess convicine was suspended in water such that the solution was saturated at all pH levels. The experiment was carried out under conditions similar to those of vicine. After 30 minutes of equilibration, 1.0 ml of suspension was centrifuged (29,000xg for 10 min) and 50µl of the supernatant were diluted as for vicine and absorbance determined at 273 nm. The supernatant and pellet obtained were added back to the original suspension and thoroughly mixed. The pH was adjusted (with stirring) with 0.1N NaOH, the sample left to equilibrate for 30 minutes and then processed as before. The above procedure was repeated for the different pH levels tested. The molecular weight of convicine was calculated as 322 from the formula by Bien et al. (1968).

The ε value in acid medium (pH 1.19) at 273 nm was estimated to be 17378 which is similar to that obtained by Bien et al. (1968). This value was employed in calculating the solubility (g/unit volume) of convicine.

(i) Effect of temperature, pH and type of salt on the solubility of vicine in water

The effects of temperature and pH were examined in the first of the two experiments described below. A saturated solution of vicine in water was incubated for 12 hr, with mixing, at the required temperature either "as is" or after adjusting the pH to about 9.0 with HCl. The samples were centrifuged (29,000xg for 10 min) in a temperature-regulated centrifuge. Saturated solutions of vicine in water (0.2 g/3 ml) were warmed to 60°C and maintained at this temperature for 12 hr with constant mixing. The sample was then centrifuged (29,000x g for 10 min) in a centrifuge set at 40°C. The supernatants were allowed to stand at 4, 29 or 60°C for 4 hours; they were then centrifuged as before and the supernatant pH checked. The pH recorded from the supernatant was used as the "as is" pH for both 4 and 29°C. For the 60°C sample, the "as is" pH was taken immediately after centrifuging, when the supernatant was still warm (the pH meter was adjusted to the same temperature). Fifty microliters of the supernatant were diluted to 20.0 ml with 0.1N HCl. Absorbance was determined as before and solubility calculated using ε = 16393 and a molecular weight of 322.

molecular weight of 322.

In the second experiment the effect of sodium chloride and urea on the solubility of vicine in water was examined at pH 7.0 and 10.0. Two-tenths of a gram of vicine were suspended in 10.0 ml of water and the pH was lowered to 7.0 with 3N HCl; and 0.4 g of vicine were suspended in 10.0 ml of water and the pH was raised to 10.0 with 3N NaOH. The samples were mixed for 15 minutes and NaCl or urea were added to 1.0 ml of the suspensions to a final concentration of 0, 0.1, 1.0 or 4.0 M. The solutions were incubated at 28°C with mixing for 24 hr and then centrifuged (29,000xg for 10 min). Fifty microliters of the supernatant were diluted in 15.0 ml of 0.1N HCl and the absorbance was determined at 274 nm. Solubility was calculated as in previous section. Salt solutions without vicine were processed for blanks.

(j) Solubility of vicine in different solvents and the effect of pH

In the first experiment, the solubility of vicine was determined in pure methanol, ethanol and acetone. An excess of vicine was suspended in the various solvents and mixed for 20 minutes at 28°C. One milliliter of the sample was centrifuged and 0.1 ml of the supernatant evaporated to dryness. The dried pellet was then dissolved in 10.0 ml of 0.1N HCl, the absorbance was determined at 274 nm and the solubility was calculated as before.

In the second experiment, the effect of the above solvents on the solubility of vicine in water at different pH levels was investigated. One-tenth of a gram of vicine was suspended in 0.3 ml of 3N NaOH and mixed for 15 minutes at 28°C. After recording the pH of the solution,

3.0 ml of acetone were added, the sample was mixed and then incubated with mixing for 30 minutes. The sample was centrifuged (29,000xg for 10 min), and 50 µl of the supernatant were dried at 40°C. The dried pellet was dissolved in 20.0 ml of 0.1N HCl and absorbance determined at 274 nm. This procedure was repeated using 0.3 ml of water or 3N HCl instead of 3N NaOH, and for each of the above, 3.0 ml of methanol instead of acetone were tried. Ethanol instead of acetone was tried with 3N NaOH or water, but not with acid.

The third experiment further explored the solubility of vicine in water/methanol mixtures and the effect of pH. One-tenth of a gram of vicine was suspended in 1.0 ml of water and the pH adjusted to 9.0, 9.5, 10.0 or 10.5. Two milliliters of methanol were then added, the solution mixed and then centrifuged at 29,000xg for 10 minutes. Since methanol did not interfere with absorbance at 274 nm, 50 µl of the supernatant were diluted in 20.0 ml of 0.1N HCl and then absorbance was determined directly. The rest of the supernatant was added back to the pellet and 2.0 ml of methanol further added, followed by mixing. The sample was then processed as before. Finally the methanol was evaporated from the rest of the supernatants, volumes increased with water and the pH determined. Solubility (g/100 ml) was calculated as before. In all cases a saturated solution was maintained by having an excess of undissolved vicine in the preparation.

(k) Titration of vicine and crude vicine

The titration curve of vicine and the effect of vicine concentration (3 or 5 mg/ml) on the titration properties was examined.

Solutions of vicine in water were titrated first with base (0.058N NaOH) to pH 11.0 and then back titrated to pH 2.0 with 0.11N HCl. Fifty milliliters of water were titrated for a blank. A solution of crude vicine in water (4 mg/ml) was similarly titrated.

(1) Isoelectric focusing of vicine and convicine

In all cases focusing was performed with polyacrylamide gels in cylinders that were connected to a high voltage apparatus (Buchler Instruments). In the first trial a standard solution of vicine in water (1.0 mg/ml) was diluted to produce solutions of 0.33 and 0.17 mg/ml of vicine. An aliquot of the sample (2.3 ml) was mixed successively with 1.5 ml, 0.5 ml and 20 µl of freshly prepared concentrated mixture B (Appendix Table 2.1A), 40% sucrose and ammonium persulfite (100 mg/ml), respectively. This mixture was applied to top of the tube, followed, gently by a water layer. One hour was allowed for polymerisation to occur before applying the voltage (see Table 2.1A). The running buffers were 5% phosphoric acid (top, + ve) and 5% ethylene diamine (bottom - ve). The focusing occurred overnight at 150 V and 1.0 mamp. To increase focusing, the samples were run for two nights; after the first night the voltage was raised to 300 for 30 minutes and then reduced back to 150 V for the second overnight run. The high voltage helps keep samples at their isoelectric point (IEP) without overheating or loss of the pH gradient. A blank without vicine, and one with cyt C were run for the pH gradient and to indicate focusing, respectively.

In the second trial vicine (4.9 mg/ml), crude vicine (5.1 mg/ml)

and convicine (5.1 mg/ml) were run as described in the first trial except that 0.1 of the sample was applied either on top of the gel or it was added prior to gel formation as in the first trial. After focusing, the gels in both trials, were automatically (Gilson Instruments) sliced in 5 mm fractions, mixed in 5.0 ml of water and the pH determined. Fifty microliters of the solution were diluted in 15.0 ml of 0.1N HCl and absorbance determined at 274 nm.

(m) Thin layer chromatography (TLC) of vicine and convicine

Initial trials with the TLC method of Jamalian et al. (1976) were unsuccessful because spots were not visible under UV light or even after staining with fluorescein and silver nitrate. Therefore the TLC method of Olsen and Andersen (1978) was employed. Ten microliters of crude vicine, vicine and/or vicine in water (2 mg/ml) were spotted on cellulose plates (20x20 in avicell microcrystalline cellulose, Fisher Co.). When vicine and convicine were spotted together, 10 µl of each were used. The plates were developed in methanol:ammonia:water (14:1:5) mixture for 2 hr, and then spotted with 0.1% flourescein in ethanol and then bromine gas. The spots were viewed under UV light.

(n) Column chromatography of vicine, convicine and fractions containing various proportions of both

About 0.02 g of sample was dissolved in 100 ml of 0.2N sodium citrate buffer (pH 2.2, 3.25 or 3.49) as indicated in the results section. One-quarter of a milliliter of the sample was eluted on an amino acid analyser column (see amino acid analyser method under section 1.2) (Beckman Instruments) successively with 0.2N Na citrate (pH 4.12)

and then 0.2N NaOH. However, in the case of FBPC, 2 g of the sample were thoroughly mixed in 20.0 ml of water, while another sample was suspended in 15.0 ml of water. The pH of the second sample was raised to 12.7 (with 8N NaOH) and the volume made up to 20.0 ml with water. To 2.0 ml of each sample was added 1.0 ml each of 0.67N H_2SO_4 and 10% sodium tungstate ($Na_2WO_4.2H_2O$). The samples were centrifuged (3020xg for 20 min) and 0.25 ml of the supernatant applied to column as before except that the initial buffer had a pH of 3.25. The absorbance of the eluents was monitored at 280 nm (ISCO, type 6 optical unit). The flow rate and chart speed were 70 ml/hr and 30 cm/hr, respectively. The reference cell contained 0.2N Na citrate (pH 3.49). For the blank chromatogram 0.25 ml of Na citrate buffer were applied to the column and then eluted as the samples.

(o) Ninhydrin test (Rosen 1957)

Vicine and/or convicine were dissolved in water (73 µg/ml) and 1.0 ml of the solution employed in the analysis.

(p) Reducing test with phenol reagent

Two-tenths of a milliliter of vicine and/or convicine or β-marcoptoethanol in 0.1N HCl (0.21 mg/ml) were added to 1.0 ml of phenol reagent diluted 1:1 with water. In addition vicine was heated (100°C) in the phenol reagent for 30 minutes.

2.2. Preparation of divicine from vicine and its characterisation

2.2.1. Preparation of divicine

Divicine was prepared from vicine according to the method of

Flohe et al. (1971) and the pellet of divicine sulphate was obtained by centrifuging at 755xg for 10 min. The wet pellet was employed in the characterisation.

2.2.2. Some properties of divicine

All tests in this section were performed at room temperature ($26^{o}C$).

(a) UV absorption spectra of divicine

A saturated solution of divicine or vicine in 0.1N HCl was either diluted with 0.1N HCl or 0.1N NaOH solution and then scanned between 200-450 nm.

(b) Effect of pH on the stability of divicine at 280 nm

A saturated solution of vicine or divicine was prepared in a degassed (Mettler Electronics Corp. Sonicator) solution of 0.01N HCl. One-half of a milliliter of the saturated solution was added to 10.0 ml of degassed solutions of 0.01N HCl, 0.01M phosphate buffer (pH 5, 6, 7 or 9) or 0.01N NaOH. Absorbance changes over time were monitored at 280 nm.

(c) Effect of gas medium and pH on the stability of divicine at 280 nm

A saturated solution of divicine in 0.01N HCl was prepared as before, and then further bubbled with nitrogen to ensure that most of the air was eliminated. Twenty milliliters of 0.01N HCl, 0.01M phosphate buffer (pH 7.0) and 0.01N NaOH were degassed and then introduced into sealed bottles by a needle and syringe; nitrogen was then

bubbled through the solution. Another set of non-degassed solvents was prepared in beakers and equilibrated with air. Using a needle and syringe, 1.0 ml of divicine solution was transferred to the various solvents and the changes in absorbance monitored as before.

(d) Effect of type of solvent and pH on the stability of divicine at 240 or 250 nm

There are reports that the absorbance at 280 nm of divicine solutions decreases with time, while initially it concomitantly, increases at 240 nm before it starts to decrease (Bendich and Clements 1953). Therefore the changes in absorbance with time were investigated at 240 and 250 nm. One-half of a milliliter of a saturated solution of divicine in 0.1N HCl was diluted in 20.0 ml of 0.1N HCl, 0.128M phosphate buffer (pH 8.0) or 0.1N NaOH. The concentrations of the solvents were changed to those that were employed in Chapter 5. Absorbance was determined immediately (20 seconds) and then monitored for 3 minutes.

RESULTS AND DISCUSSION

Isolation of crude vicine

When the pH of the extract was increased from 7.0 to 10.0 the yield of crude vicine was increased by 21% (Table 2.1). It would therefore seem that some vicine precipitated out and was lost during the second stage. The maximum yield obtained at pH 10.0, however, was only 20% of the theoretical yield. Therefore it was decided to raise the pH of the slurry of FBPC. A preliminary study was carried out to establish the amount of base needed to achieve a certain pH (appendix figure 2.1A). It was found however, that in large extractions slightly

Table 2.1. Effect of pH of extract on the yield of crude vicine (section 2.1.1)

Base added (ml)	pH of supernatant without acetone	Yield of crude vicine (g dry wt)	Yield of crude vicine (% of FBPC)
30.0	7.02	13.8	0.28
40.0	8.10	15.0	0.30
51.0	10.0	17.1	0.34

Table 2.2. Effect of adding base at the beginning of the extraction on the yield of crude vicine (section 2.1.2)

Base added (3N NaOH) (ml)	pH of supernatant after acetone removal	Yield of crude vicine (g dry wt)	Yield of crude vicine (% of FBPC)
35	5.26	11.5	0.23
40	5.28	17.0	0.34
375	7.48	24.3	0.49
400	7.82	24.6	0.49
500	8.38	26.4	0.53
860	9.48	21.4	0.43

more base was required than that predicted from the curve, and the pH after evaporating the acetone was slightly lower than it was before adding the acetone.

Table 2.2 shows that a greater increase in yield was achieved when the pH was raised at the beginning of the extraction. The maximum effect of this treatment was achieved between pH 8 and 9.5. The yield of crude vicine under optimal pH conditions was still low, being only 33% of the theoretical yield. It would seem from these results that vicine did not solubilise fast or that its solubility properties were modified by compounds in the FBPC. There is also a possibility that when acetone is added in a single batch, it precipitates out the protein together with vicine due to a local concentration effect. The precipitated vicine could have been trapped in the proteins and therefore solubilise at a slower rate. The decrease in yield of crude vicine at the high pH might also be due to hydrolysis of vicine.

FBPC was extracted twice in an attempt to determine if the low yield of crude vicine was attributable to its low solubility (section 2.1.3). This would not only establish if vicine was left in the FBPC following the first extraction but also if vicine was either destroyed during the isolation or did not completely crystallise out of solution. The pH of the second extract after acetone had been evaporated was lower (7.86) than that of the first extract (8.14). This was expected since base was not added in the second extraction. In addition, the proportion of prismatic crystals (convicine) was much higher in the

crystallite of the second extraction. The total yield of crude vicine in the second extraction was 0.28% of the original FBPC. This gave a total yield of 0.80% together with that from the first extraction which was more than 50% of that theoretical yield. These results suggest that vicine was not completely solubilised from FBPC in the first extraction. The yield of the second extraction was about 50% of the first which could be due to differences in volume of extraction solvent used.

Double extraction was tedious because the entire isolation process was repeated. Therefore it was decided that one extraction should be tried, in which the volume of water was equal to the total of that employed in the double extraction (section 2.1.4). The pH of the extract after removal of acetone was 8.46, which was comparable to that of the first extraction in 2.1.3. The crystallite formed a thick paste after 2 days at $0^{o}C$. Both needle- (vicine) and prismatic- (convicine) shaped crystals were observed. The average yield of crude vicine was 0.75% of the original FBPC, which is close that obtained by the double extraction method.

The optimum pH for obtaining the crystallite was subsequently investigated (section 2.1.5). The yield of crude vicine at pH 9.0 was very low (10 g, which is 0.2% of the original FBPC) even after 2 days of crystallisation. The yield at pH 7.2 was in the range obtained in the first trial of Experiment 2.1.3. Therefore it was decided to employ pH 7.2 and to allow the sample to stand for one day at $0^{o}C$ so as to promote a maximum degree of crystallisation.

Employing the first isolation of section 2.1.3, it was found that

mixing for 1 minute improved the yield of crude vicine by about 50% compared to no mixing; there was however, only a slight improvement in yield after 10 minutes. Although preliminary small scale trials indicated that homogenising did not dramatically improve the yield, it might be advantageous in large scale isolations. However, only mixing in contrast to homogenisation was employed in the subsequent isolations. In these routine procedures FBPC was mixed with water and base for 5 minutes, acetone was added and the mixing continued for another 5 minutes.

From the information obtained, the procedure described in section 2.1.4 and depicted in Figure 2.2 (method 1) was employed in isolating crude vicine that was used in the feeding trials of Chapters 3 and 4.

The above procedure however was reevaluated by investigating various combinations of the factors so far discussed so as to further establish factors affecting the solubilisation of vicine from FBPC (section 2.1.7 and Table 2.3). It is evident from Table 2.3 that more than 70% acetone in the extraction solvent causes a marked decrease in the amount of vicine solubilised from the FBPC. In addition an acetone/water ratio of 2 or less would be better if the lower level of acetone does not cause a loss in quality of the crude vicine. Increases in pH lead to increased solubilisation of vicine up to pH 9.0 so long as the acetone/water ratio is generally below 3. However, beyond pH 9.0, and acetone/water ratios exceeding 3, the effect of pH seems to be negative; the reasons for this effect are not obvious. The high pH may lead to hydrolysis of vicine, or interionic and/or

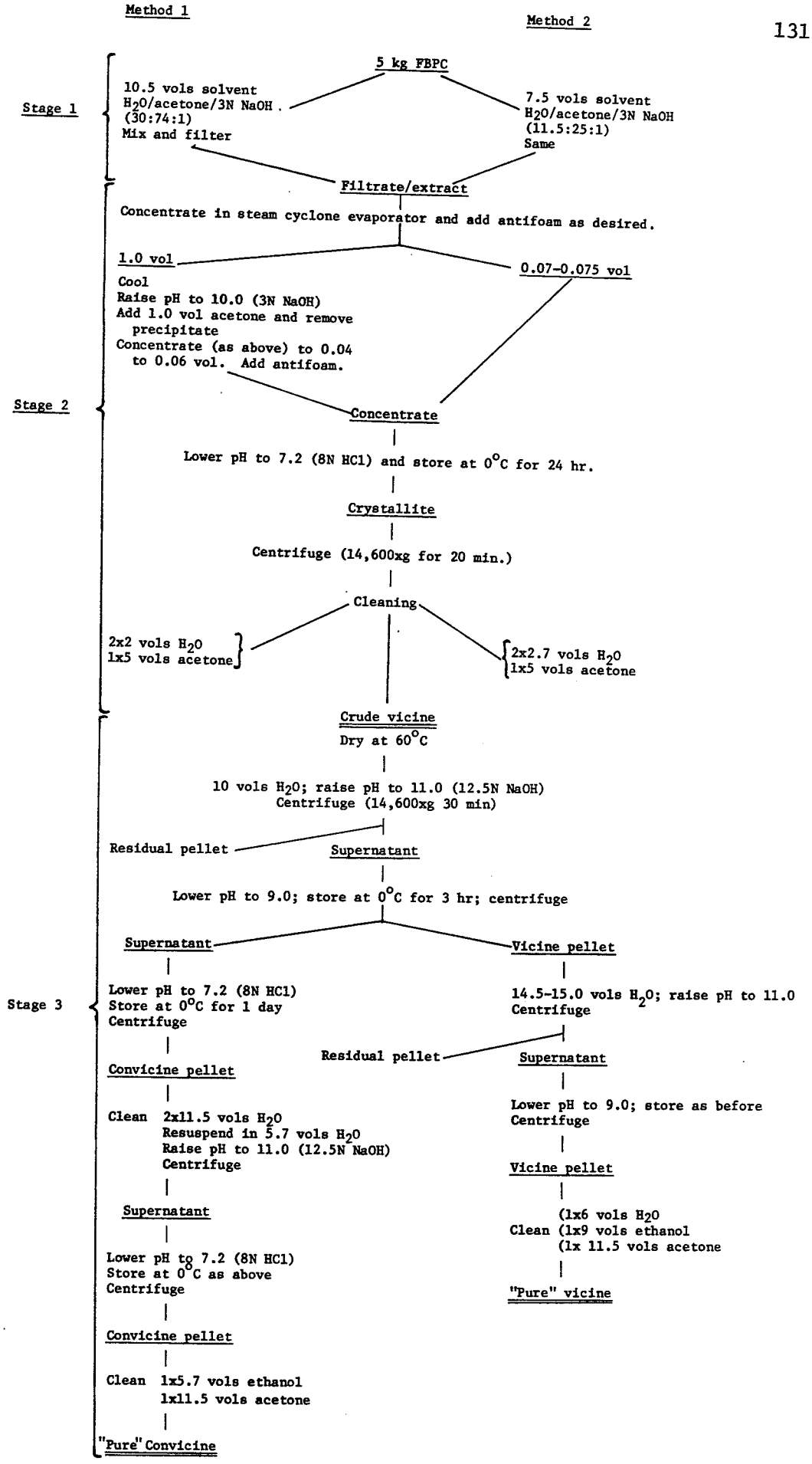


Fig. 2.2. Flow diagram of the method for isolation of vicine and convicine from FBPC. Samples were centrifuged between washes; stage 3 is described in detail in section 2.1.9 (b) ii.

Table 2.3. Effect of volume, ratio of acetone/H_2O and pH of extraction solvent on the extractability of vicine from FBPC

Volume of solvent/g FBPC	Ratio acetone/water	Base added (ml)	pH of supernatant		Solubility[a] (g/100 ml)	Total vicine extracted (mg/g FBPC)
			Before acetone added	After acetone evaporated		
6	2.0	0	6.20	5.74	0.23	13.8
6	2.0	0.2	7.84	7.82	0.24	14.4
6	2.0	0.4	9.36	8.92	0.25	15.0
6	2.5	0.6	10.16	9.58	0.21	12.6
7	2.5	0.2	7.78	7.83	0.18	12.6
7	2.5	0.8	11.11	9.83	0.20	14.0
7.5	2.0	0	6.16	5.80	0.22	16.5
7.5	2.0	0.2	7.62	7.71	0.22	16.5
7.5	2.0	0.4	9.24	8.97	0.23	17.3
8	1.7	0.2	8.20	7.71	0.22	17.6
8	1.9	0.2	8.00	7.78	0.21	16.8
8	2.5	0.2	8.07	7.81	0.18	14.4
9	2.0	0	6.38	5.91	0.19	17.1
9	2.0	0.2	7.69	7.79	0.19	17.1
9	2.0	0.4	9.42	9.00	0.20	18.0
10.5	2.5	0.2	8.17	7.82	0.16	16.8
10.5	4.25	0.6	9.61	9.55	0.08	8.4
10.5	4.25	0.8	10.48	9.81	0.07	7.35

[a]Solubility in the acetone/water mixture.

hydrophorbic interactions between acetone and water and perhaps vicine itself, all of which may reduce the solubility of the latter.

The above information suggested that it would be possible to develop another method employing 7.55 volumes of extraction solvent (section 2.1.8 and figure 2.2, method 2) instead of the 10.5 volumes in section 2.1.4. The average yield with this method was 0.6% of the FBPC used. The total volume of water and base was 12.4 liters, therefore the yield was 2.4 g/liter as opposed to 2.7 g/liter in method 1. Thus confirming that extremely high pH reduces the yield of crude vicine even at low ratios of acetone/water. However, considering the total volumes (acetone + H_2O + base) handled the yield in method 2 would be 3.97 g/unit volume compared to 3.81 g in method 1. The proportion of convicine relative to vicine obtained in method 2 is lower than that in method 1. It would therefore appear that a higher proportion of vicine than convicine was extracted from the FBPC. The low extractability of convicine relative to vicine may be attributed to its lower solubility.

Therefore it may be concluded that crude vicine can be extracted from FBPC utilising acetone/water mixtures in ratios below 3. Increasing the pH during the extraction increases the amount of recoverable vicine and to a lesser degree convicine. However, pH levels above 9.0 may lead to decreased yield of crude vicine. Finally, the present methods recovered at most, about 50% of the vicine plus convicine in FBPC.

These methods should therefore be improved if higher yields are required. However, yields obtained by these methods are higher than

those obtained by previously published methods, considering the relative time and volumes of solvents employed. The high yields of crude vicine are essential for animal feeding trials.

Attempts to use either of the two methods to extract vicine from dehulled ground fababeans were unsuccessful because the extract was difficult to filter, the yield was extremely low and the crude vicine was very dirty and contained a highly sticky material. Although this area needs investigation if the method is to be applied in areas without the expensive air classification equipment, the low concentration of vicine material, particularly in dry beans, may require large amounts and volumes of fababean powder and extraction solvent. This would make the operation very expensive and tedious.

Preparation of pure vicine and convicine from crude vicine

Data from ion exchange chromatography were exploratory and since they were not encouraging, they are briefly presented here. In the study to determine the capacity of different resins for vicine (section 2.1.9 (a) (i), the following resins were tested: Dowex, 50Wx8, H^+(20-50 mesh); 50x1-100; 50x4-50; Dowex 21K; 1x1-100 (Dowex-1); MSA-1 (mesh 20-50); and DEAE (cellex-D). Among these resins 1x1-100 (Dowex-1) had the highest capacity for crude vicine (20.8 mg/ml of resin). Therefore to process crude vicine from one isolation (30 g) would require a bed volume of 624 liters. Such large columns are very expensive, the isolation would require a considerable amount of time and would generate a large volume of solution which would have to be evaporated.

Further tests with the crude vicine nevertheless demonstrated that

either anion or cation exchanges (DEAE or Dowex 1x1-100) were capable of resolving vicine and convicine into two distinct peaks provided the columns were not overloaded and the pH and ionic strength of buffer were within a certain range (section 2.1.9 (a) (ii)).

Studies with concentrated extract from FBPC were also tried on both resins (section 2.1.9 (a) (iii)). There were problems with both columns. The extract contained dark material which tended to bind irreversibly to the resins which eventually lead to loss of resolution and a drastic reduction in flow rate. These results indicated that a column would be used only once or twice, or a special clearing column would be necessary to remove the dark material. This would further raise the costs of the purification procedure. Trials to bind the dark material with PVP were abandoned because its capacity to bind these substances was low and occurred only if the extract was passed through a column packed with PVP, but not when a batch method was employed.

In addition, the amount of vicine that was irreversibly bound to PVP was not established. Based on these trials it was decided that ion exchange chromatography would be a long and expensive method and would only yield small quantities of vicine and convicine. Therefore re-crystallisation was thought of as the best alternative (section 2.1.9 (b)) for the resolution of crude vicine into pure vicine and convicine.

It is evident from Table 2.4, that even at pH 9.4, about 48% of the crude vicine was not solubilised when the concentration was 1 g/10 ml. Within 2 hours at room temperature ($27^{o}C$) crystals had formed when the pH of the supernatant solutions were lowered to 8.5, but not in the other two groups. However, when left overnight at $0^{o}C$, copious

Table 2.4. Effect of pH on the amount of crude vicine solubilised, and the yield of recrystallised vicine[a]

Observations	pH		
	8.5	9.0	9.5
Initial pH	6.8	7.1	6.8
Final pH of suspension mixture after pH adjustment	8.47	8.96	9.42
Dry residual pellet, wt (g)	0.70	0.61	0.48
Calculated amount of vicine solubilised	0.30	0.39	0.52
pH of supernatant; initial	8.03	8.77	9.64
final after adjustment	7.25	7.17	7.12

[a]See first trial of section 2.1.9 (b) (i) for the methods.

Table 2.5. Recrystallisation of vicine as affected by a stepwise drop in pH[a]

pH of crystallisation	Yield of vicine	
	Wt (g)	% of initial crude vicine
9.51	4.52	22.6
7.20	8.56	42.8
Water wash crystals	0.56	0.03
Total recovery	13.64	65.0

[a]See second trial of section 2.19 (b) (i) for the procedure.

quantities of crystals formed from the pH 8.5 supernatant, and some crystals had formed in the other two supernatants. The crystals obtained from the supernatants were very clean. They were also cleaner than the pellet, and yet the latter still had most of the vicine, particularly for samples titrated to pH 8.5 and 9.0.

Although these methods would be unsatisfactory for routine crystallisation of vicine from crude vicine, they suggested that a method could be developed if the solubility of vicine could be initially increased (i.e. increase in temperature or pH of the solution) so as to produce a highly concentrated solution of vicine. Once in solution vicine could be induced to crystallise by lowering both pH and/or temperature.

In section 2.1.9 (b) the volumes of water were kept low so that evaporation of excess liquid would not be necessary before crystallisation. The recovery with this method was only 65% most of which was obtained at pH 7.2 (Table 2.5). Crystals obtained at pH 9.5 appeared to be "pure" vicine; microscopic examination indicated the presence of only needle-shaped crystals. In contrast there appeared to be a much higher proportion of convicine, as indicated by the abundance of prismatic crystals, in the pH 7.2 fraction. These results suggested that it should be possible to obtain pure vicine and convicine by successive crystallisations at selected pH levels.

Therefore a method involving two successive crystallisations was evolved (section 2.1.9 (b) (ii)). The first crystallisation at pH 9.0 yielded a pellet rich in vicine and a supernatant rich in convicine. The pellet and supernatant obtained above were processed as described

in section 2.1.9 (b) (ii) and Figure 2.2. This method gave 70% recovery of crude vicine of which 5% was convicine. This method was adapted for routine preparation of "pure" vicine and convicine (see Figure 2.2, Stage 3).

Characterisation of the "pure" vicine and convicine

The orginal aim was to develop a method for isolating vicine and to establish some of the properties of vicine. However, when it was discovered that convicine could also be readily purified some of its properties were also studied. The "pure" vicine and convicine samples were found to be 99.5% dry (section 2.1.10 (a)) therefore the water content of the sample did not contribute significantly to the other properties studied. The nitrogen content (section 2.1.10 (b)) of vicine and convicine were 16.6 and 12.7%, respectively. The values for vicine are slightly lower than the literature values (17.39-18.67%) (Bendich and Clements 1953). This could be due to insufficient degradation time or the sample not being pure. It is well known that complete decomposition of aromatic compounds requires high temperatures. The nitrogen value for convicine was close to the theoretical value of 13% and those obtained by others (Bien et al. 1968). The melting points of vicine and convicine were 242-244 and 276-278^{o}C, respectively. Although the values for vicine were within literature values (Bendich and Clements 1953), those of convicine were about 10 degrees lower (Bien et al. 1968) which might indicate lack of absolute purity with regards to convicine. The samples did not actually melt, but directly decomposed; they first crumbled, turned brown and finally black. The melting point was that temperature when the sample began to crumble. The slight variations in the decomposition temperatures,

particularly for vicine could be due to differences in rate of heating since an ordinary bunsen burner was used.

The UV spectra of vicine and convicine are shown in Figure 2.3. The changes in vicine spectra with changes in pH are similar to those reported by Bendich and Clements (1953). The changes in convicine spectra were similar to those of vicine but less pronounced. These spectral patterns were not changed after 20 minutes at room temperature ($27^{o}C$). The changes in spectral patterns at different pH levels may reflect changes in the distribution of electrons within the ring since the ionic states of a number of its groups change with pH. The difference between vicine and convicine may be due to their difference at carbon 5.

Similar to data reported by Bendich and Clements (1953), vicine was found stable in dilute mineral acid and base, and alkaline buffers even after 4 days (Table 2.6). The consistent decreases in absorbance at 280 nm in 0.1N HCl are difficult to explain in view of a lack of change in absorbance at 274 nm. It could be an indication of limited hydrolysis followed by the formation of a new compound that has a slightly different absorption maximum. The absorbance values in the other solvents fluctuated slightly and randomly with no pattern, which might reflect changes in the accuracy of the machine rather than changes in the samples.

It is evident from Table 2.7 that the ε value of vicine at λ max decreased with increases in pH of the solution and that these changes appeared to be independent of the type of solvent. This trend has already been reported (Bendich and Clements 1953; Olaboro 1978).

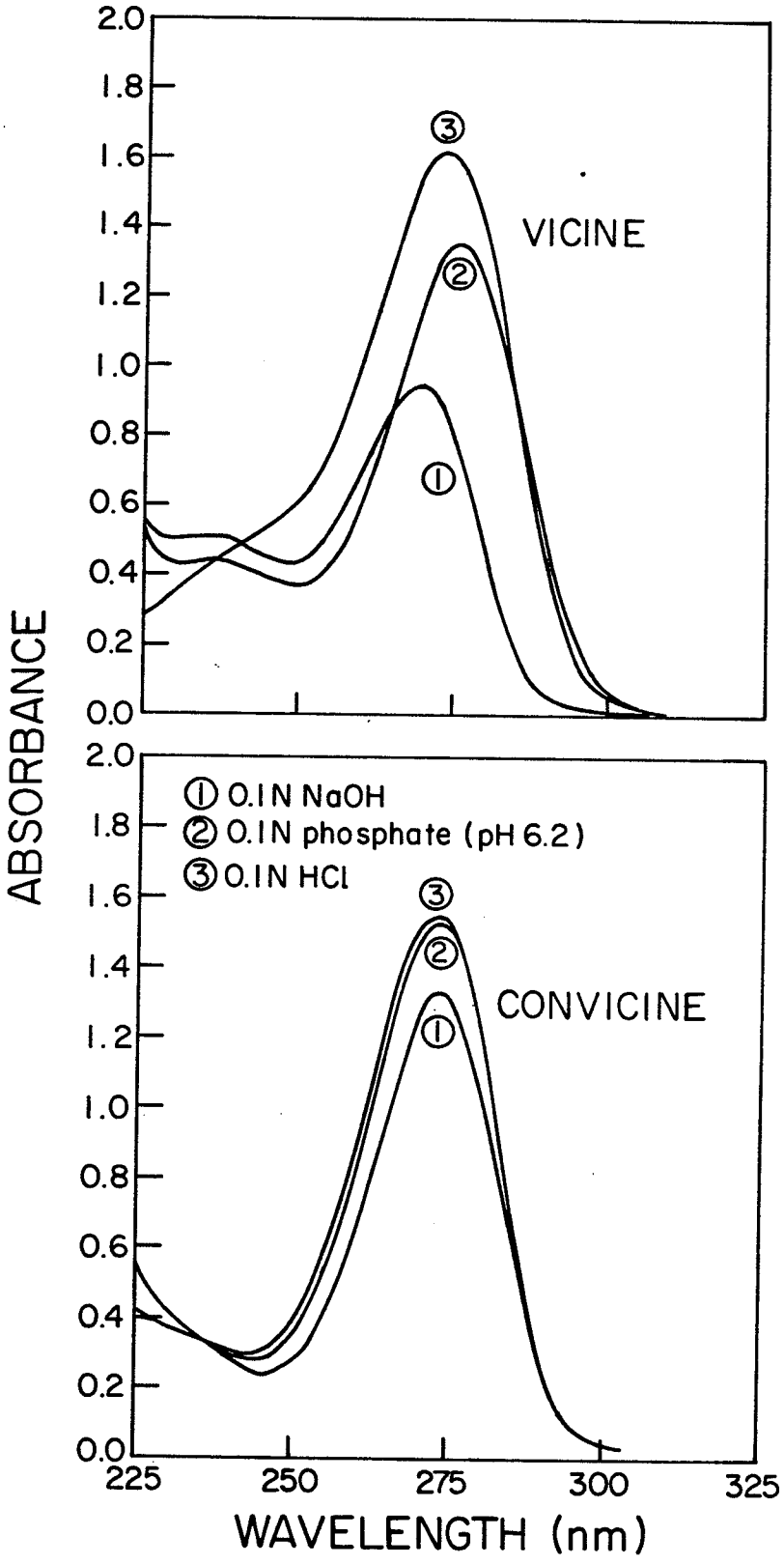


Fig. 2.3 UV spectra of vicine and convicine at various pH. See section 2.1.10 (d) for the procedure.

Table 2.6. Effect of pH on the stability of vicine solutions[a]. See section 2.1.10 (e) for the procedure

Time (hr)	Solvent and pH							
	0.1N HCl (1.20)[b]		55.5M H_2O (6.87)		0.1M phosphate (7.19)		0.1N NaOH (12.24)	
0.5[c]	1.7	1.45	1.24	1.14	1.46	1.30	0.78	0.44
3.0	101.4	100.8	101.3	100.6	99.5	101.5	100.4	99.9
6.0	101.6	98.8	100.7	100.3	100.3	101.7	99.9	97.2
30.0	102.2	95.8	101.9	99.1	101.1	99.0	99.6	92.8
72.5	101.0	99.0	100.2	101.5	97.3	100.1	101.6	102.4
97.0	100.5	96.6	99.3	100.5	98.7	99.6	101.5	97.7

[a]The first and second column give absorbance at 274 and 280 nm, respectively for each pH level.

[b]pH values are shown in brackets.

[c]Actual absorbance values are given at 0.5 hr and the absorbance units at other times are expressed as a percentage of the 0.5 hr values.

Table 2.7. Effect of pH on the molar extinction coefficients (ε) of vicine at 274 nm. See section 2.1.10 (f) for the procedure.

Solvent	pH[a]	ε value[b]
0.1N HCl	1.19	16392.8
0.1M phosphate	7.20	13717.3
0.1N NaOH	12.10	8494.5
0.2M phosphate	5.25	15306.5
" "	6.21	15427.4
" "	7.14	15377.5
" "	8.04	14132.2
" "	9.01	12875.8
" "	10.09	10248.4
" "	11.10	9567.7
Tris buffer	6.99	14591.9
" "	7.97	14398.4
" "	9.10	13446.7
" "	10.00	11070.9

[a] pH determined after absorbance.

[b] ε value calculated according to Maron and Prutton (1958):

$\varepsilon = \frac{A}{C1}$ where, A is the wavelength of light (nm), C is the concentration of vicine solution (moles/litre), and 1 is the light path length/cuvette width (cm). A molecular weight of 322 for vicine plus one molecule of water, was employed.

The ε values obtained in 0.1N HCl, 0.1M phosphate buffer and 0.1N NaOH are close to those reported in the literature. However, those in 0.1N NaOH deviated most from the literature values and this may be because the λ max of vicine in 0.1N NaOH is 269 nm (Bendich and Clements 1953; results in this thesis) whereas the values reported here were determined at 274 nm. The shifts in ε value with changes in pH may be explained on the same grounds as in section 2.1.19 (d) - electron shifts in the pyrimidine ring and perhaps changes in the sugar molecule too.

The optical rotations (α) of vicine and convicine were -19.6 and -16.6, respectively. Bendich and Clements (1953) reported $(\alpha)_D^{26^o} = -11.7$ in 0.2N NaOH and C = 3.9; while Winterstein (1919) reported $(\alpha)_D^{15^o} = -8.77$ in 2N H_2SO_4 with C = 10, and $(\alpha)_D^{15^o} = -12.2$ in 0.5N NaOH with C = 6.6. There were no values for (α) for convicine in the literature available, however, it is evident that the (α) values for vicine obtained in this thesis are higher than the literature values. The glucose molecule must contribute considerably to the (α) value of both vicine and convicine. Since this molecule changes its (α) value in different solvents due to changes between α and β forms at its anomeric carbon (mutarotation) it is possible that during the isolation, the sugar molecule is altered a bit leading to this discrepancy or that some isomerization to other types of carbohydrates occurred in the basic solutions. In addition, reversible changes in the pyrimidine structure from lactim to lactam forms may contribute to the variation. However, direct comparison of this data is not possible because Bendich and Clements (1953) did not fully describe the method they employed, while the methods employed by Winterstein (1919) were somewhat different from

those employed in this thesis.

The effects of pH, temperature and salt on the solubility of vicine in water are summarised in Figure 2.4 and Tables 2.8 and 2.9. The solubility of vicine in water (Figure 2.4) was 0.55 g/100 ml between pH 4 and 9, and rose to 3 and 6.6 g/100 ml at pH 10.2 and 10.86, respectively. In addition, lowering the pH to 1.68 raised its solubility to 1.9 g/100 ml. This data may partially explain the occurrence of convicine and vicine in the different fractions (section 2.1.9 (b) (i) and Table 2.4) during the development of a method to purify vicine. However, the generally stated solubility of vicine in water (1 g/100 ml) (Lin and Ling 1962a) is higher than that obtained in the current study and is probably an overestimation. Lin and Ling (1962a) did not give the method they employed, but it would seem that they did not correct the absorbance and ε values for the pH of sample being tested. Also they did not control the pH of the solvent.

The solubility of convicine was determined over a narrower pH range. Its solubility was fairly constant between pH 6.8 and 7.7 (0.14 g/100 ml) and increased to 0.17, 0.40 and 0.57 g/100 ml at pH 8.3, 8.76 and 10.5, respectively. Thus at low pH levels vicine is more soluble than convicine. There is, however, a pH level when their solubilities are equal, and yet at a higher pH convicine is more soluble than vicine. The solubility of convicine should be established across a wider pH range to facilitate further improvement of the method for separating them and to facilitate other studies with convicine. It is evident from Table 2.8 that at any pH the solubility of vicine increased with increasing temperature and vice versa.

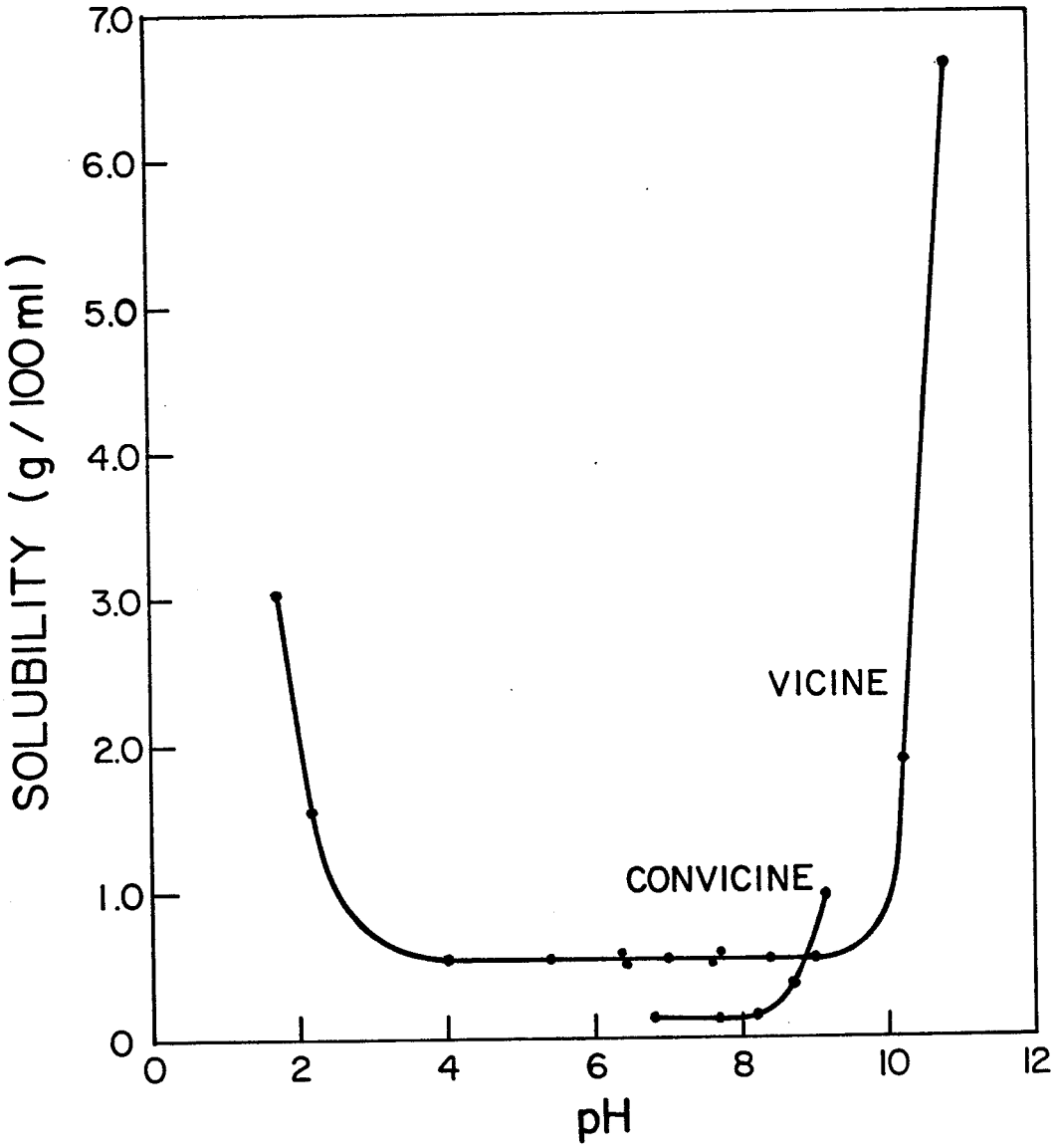


Fig. 2.4 Effect of pH on the solubility of vicine and convicine in Water. See section 2.1.10(h) for the procedure.

Table 2.8. Effect of temperature and pH on the solubility of vicine in water. See the first experiment in section 2.1.10 (i) for the procedure

Temp (^{o}C)	pH of supernatant	Solubility (g/100 ml)
60	7.54	1.29
	8.86	1.68
29	7.48	0.57
	9.18	0.96
4	7.68	0.26
	9.92	0.78

Table 2.9. Effect of salt on the solubility of vicine in water. See experiment 2 of section 2.1.10 (i) for the procedure

	Solubility (g/100 ml)	
	pH	
Type and concentration of salt	7.0	10.0
0	0.56	1.05
NaCl 0.1M	0.55	1.14
1.0M	0.58	1.12
4.0M	0.59	1.06
Urea 0.1M	0.54	1.05
1.0M	0.66	1.14
4.0M	0.98	1.42

These results would suggest that it is possible to increase or decrease the solubility of vicine by altering one or both of these factors. Table 2.9 would indicate that at pH 7.0 and 10.0 NaCl or urea at concentrations of 0.1 to 4.0M had no effect on the solubility of vicine in water. In fact urea tended to increase the solubility; its effect being greater at low compared to high pH. The effect of urea could be due to increases in pH and not a salt effect per se or due to hydrogen bonding. The pH of the solutions after adding the salts was not checked which could clarify the situation. This possibility however, seems unlikely as urea is a neutral salt.

Table 2.10 shows that vicine was only slightly soluble in ethanol or acetone, but relatively highly soluble in methanol. Similar results have been reported (Bendich and Clements 1953). Raising the pH with NaOH increased the solubility in both methanol and ethanol, but not in acetone. Lowering the pH with either water or HCl lead to a dramatic decrease in solubility of vicine in methanol but not in ethanol or acetone. It is possible that hydrogen bonding between water and methanol may reduce the solubility of vicine in either solvent. Further investigation into the solubility of vicine in methanol/H_2O mixtures (Table 2.11) revealed that water reduced the solubility of vicine in methanol even at high pH levels. Although the solubility of vicine at pH 10.0 in a 1:2 water/methanol mixture approached that in pure methanol, its solubility in a 1:4 mixture at the same pH was about 50% of that of methanol. It would seem therefore that a method employing 1:2 H_2O/methanol solvent could be promising for extraction of vicine from FBPC.

Table 2.10. Solubility of vicine in different solvents at various pH[a]

Solvent	Solubility (g/100 ml)			
	Pure solvent	+ NaOH	+ HCl	+ H_2O
Methanol	1.53	2.22	0.17	0.11
Ethanol	0.05	0.27	-	0.05
Acetone	0.02	0.03	0.02	0.02

[a]For the procedure, see section 2.1.10 (i), first and second experiments. The pH of vicine solution in NaOH, HCl and H_2O was 11.08, 1.19 and 8.60, respectively.

Table 2.11. Solubility of vicine in methanol/H_2O mixtures at various pH

Ratio water/methanol and pH[a]	Solubility (g/100 ml solvent)
1/2	
9.08	0.25
9.45	0.35
10.01	0.78
10.45	1.40
1/4	
8.98	0.13
9.38	0.18
9.92	0.43
10.18	0.77

[a]pH of the samples with 1/2 ratio was taken after the pH was adjusted but before methanol addition and that for 1/4 was determined after methanol had been evaporated from the sample.

In an attempt to establish the isolectric pH (p^I) of vicine, solutions were titrated (Figures 2.5a and 2.5b) and focused with fractions containing various amounts of convicine (Figures 2.6 and 2.7). The p^I as determined by taking the midpoint of the steep portion of the titration curve was calculated to be 6.4 (Figure 2.5b). However, the curves in Figure 2.5b are not smooth in their latter parts and this is more pronounced with the more concentrated vicine solution. Therefore there could be p^{ka} values that are obsecured, making the p^I value obtained rather unreliable. The results of IEF are presented in Figures 2.6 and 2.7. The p^I obtained with different concentrations of vicine following IEF (Figure 2.6) were quite close and gave a mean p^I of 6.86 which is close to that obtained from the titration curves. However, the absorbance at 280 nm was spread throughout the gel. In the second trial the samples applied to the top of the gel did not migrate and most of the absorbance was concentrated at the top of the gel. Data from those samples that were applied as in the first trial (Figure 2.7) show that convicine had a lower p^I (4.7) than vicine. This is consistent with the column chromatography data of crude vicine and mixtures of vicine and convicine reported by Olaboro (1978). The vicine sample had a p^I of 6.6 which is slightly lower than that obtained in the first trial. In addition it showed another broad peak - a similar peak is evident with curves 3 and 4 of Figure 2.6. These results might indicate incomplete focusing or the formation of more than one compound, or contamination with another compound such as vicine in the convicine sample and vice versa. The proportionally lower degree of spreading during IEF (Figure 2.6) that occurred in gels

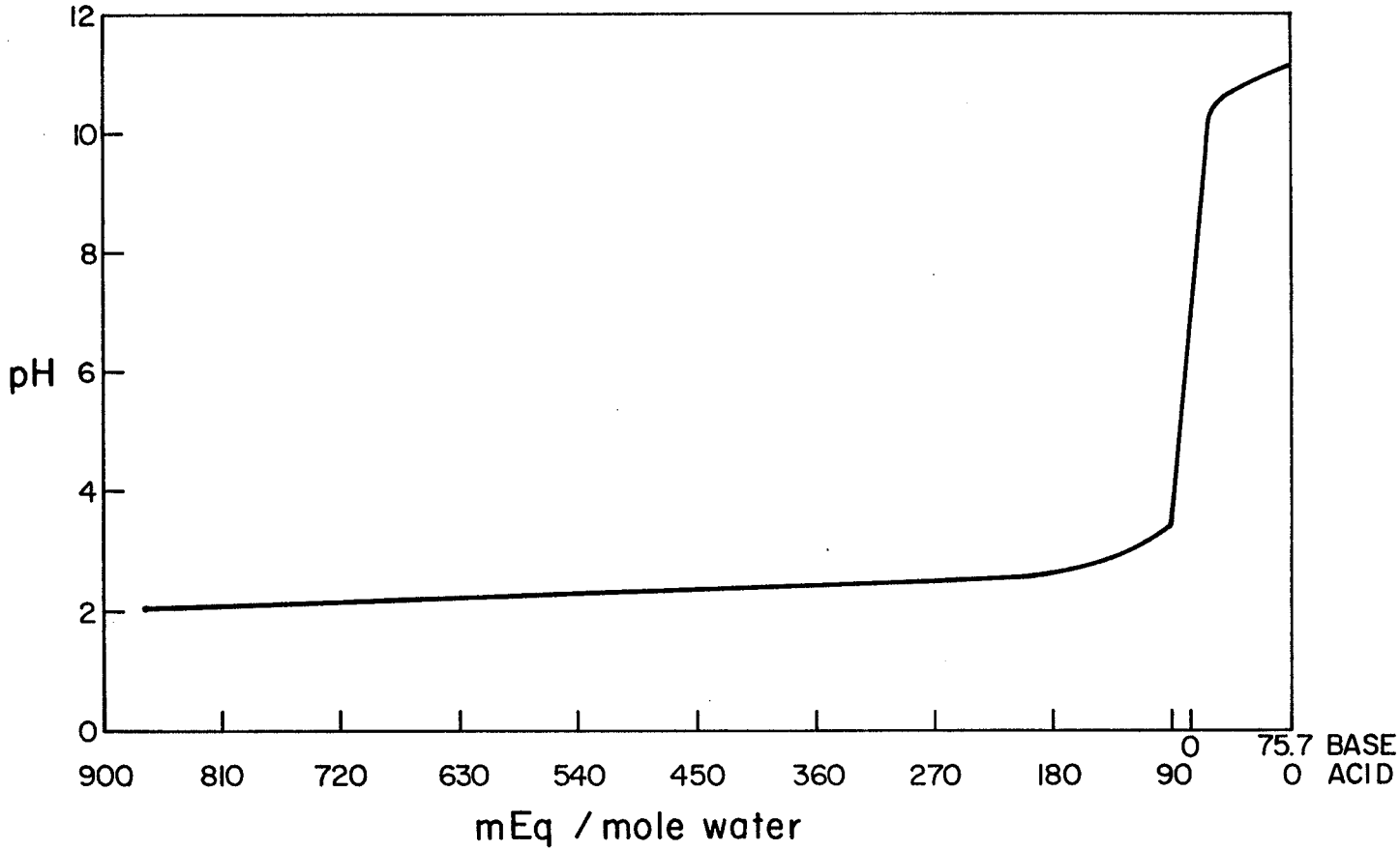


Fig. 2.5a Titration curve of water. See section 2.1.10(k) for the procedure.

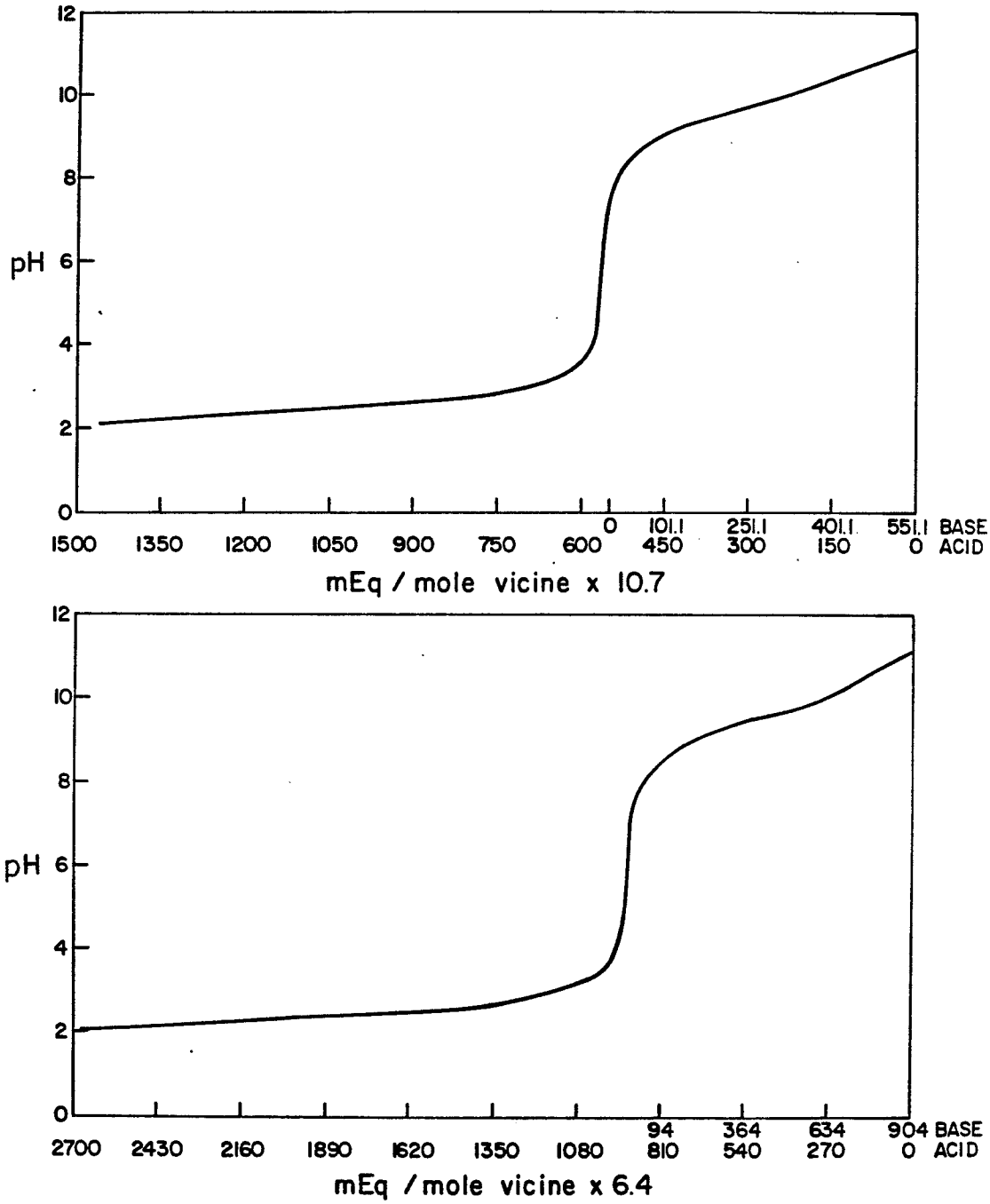


Fig.2.5b Titration curves of 9.3 mM (top) and 15.5 mM (bottom) solutions of vicine. See section 2.1.10 (k) for the procedure.

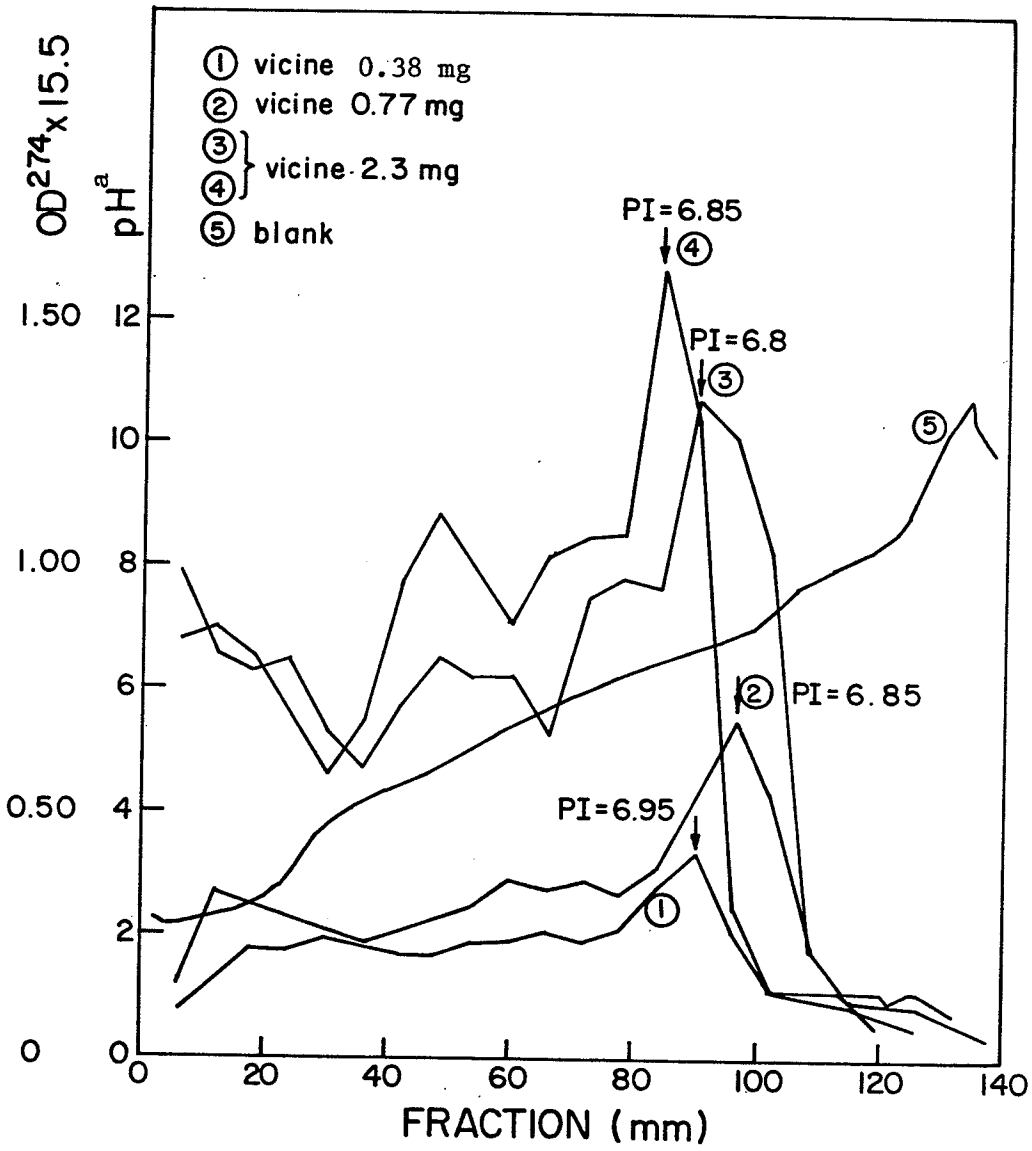


Fig. 2.6 Effect of vicine concentration on its isoelectric pH. See section 2.1.10 (l) for the procedure.

[a]pH of blank (gradient, curve 5).

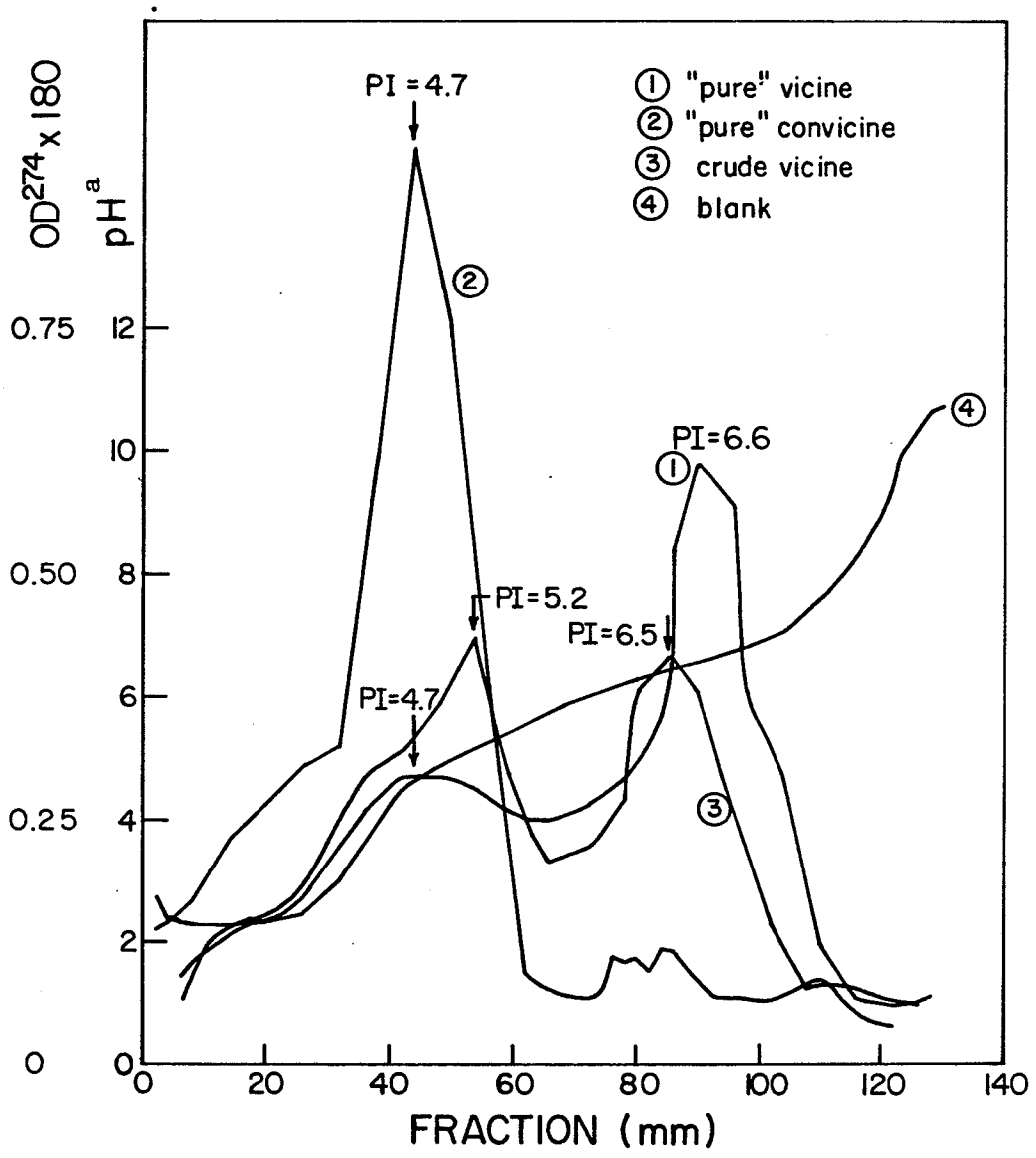


Fig. 2.7 Isoelectric pH of vicine, convicine, and crude vicine. See section 2.1.10 (l) for the procedure.

[a]pH of blank (gradient, curve 4).

that contained a low concentration as compared to those that contained a high concentration of vicine would suggest that convicine interaction is an important factor that contributes to the spreading of the vicine peak. Crude vicine showed two p^I at 5.2 and 6.5 which were higher and lower than values obtained for convicine and vicine, respectively. These results further support the conclusion that there are not only interionic interactions among vicine molecules and probably convicine molecules, but also between vicine and convicine molecules.

Additional analysis were carried out so as to more clearly establish the purity of the samples (sections 2.1.10 m, n, o, p). Results with TLC (Figure 2.8) show that crude vicine contained both vicine and convicine as confirmed by spots from a mixture of "pure" vicine and convicine. It also indicated that "pure" vicine and convicine did not contain any detectable contaminants. However, Rf values of the "pure" vicine and convicine were not very reproducible and different from those obtained with mixtures of vicine and convicine. The latter result is similar to data obtained in IEF with crude vicine and may be similarly explained, whereas in the former differences may be due to slight variations in technique.

The amino acid method (Figure 2.9) which is much more sensitive than the TLC method, demonstrated that the "pure" vicine was 97% pure and the rest was convicine, while "pure" convicine was only 91% pure and the rest was vicine. It is interesting that the FBPC that was extracted at the high pH (FBPC (2)) showed a smaller peak before the convicine peak, while the latter decreased indicating some conversion of convicine to either another form or compound. The ninhydrin

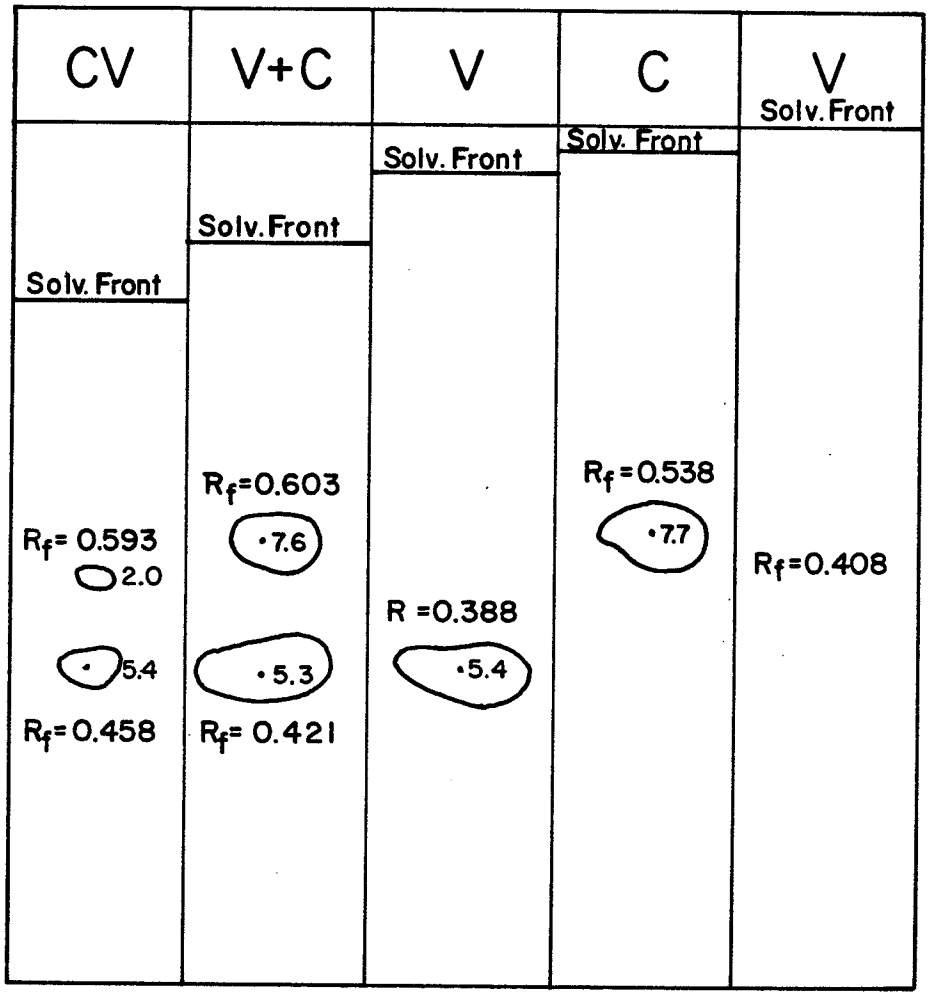


Fig. 2.8 Thin Layer chromatograms of crude vicine, vicine, convicine and a mixture of the latter two (1:1). See section 2.1.10(m) for the procedure. CV= Crude Vicine, V = Vicine, and C = Convicine.

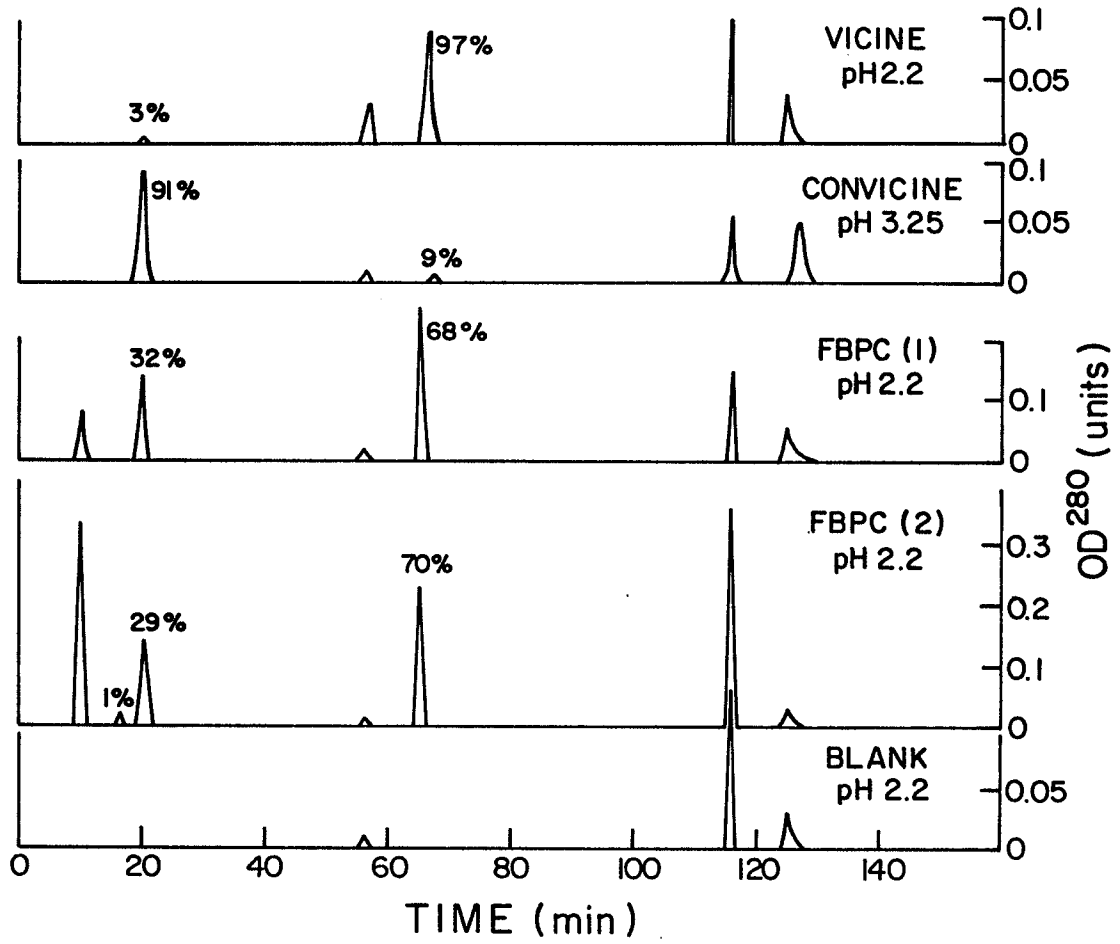


Fig. 2.9 Column chromatograms of FBPC, Vicine and Convicine. See section 2.1.10(n) for the procedure. The pH shown is that in which the sample was dissolved if not specified in the materials and methods section. The percentages were calculated from the peak areas.

(section 2.1.10 (o)) and reducing (section 2.1.10 (p)) tests were negative indicating that both compounds isolated were not contaminated with amino acids, nor were they hydrolysed to their aglycones. However, a bluish green colour was obtained when vicine was heated in the reducing test indicating partial hydrolysis of the sample.

It is evident therefore, that although many of the characteristics determined confirmed that the compounds isolated were vicine and convicine, they were not absolutely pure, but were contaminated with each other. Considering the amounts used in the analyses, the effect of the contamination by the compounds themselves would be minimal. One of the larger discrepancies with literature values (i.e. specific rotation) would suggest that some isomerisation of the molecule (probably the glucose portion) occurred during the isolation procedure. This possibility should be examined and if such a reaction occurs precaution should be taken to eliminate it. This could probably be done by ensuring that the sample is not exposed to both high pH and temperature for long periods of time. This possible change in glucose molecule does not invalidate the data from the feeding trials of Chapters 3 and 4 because vicine and convicine were not hydrolysed and as discussed in the literature review the toxic effects of these compounds are due to their aglycones. Also the total fraction of vicine and convicine that would be affected would be relatively small. Work is underway to further purify these compounds and reexamine some of their characteristics.

Some properties of divicine

The UV absorption spectra of divicine and vicine are shown in

Figure 2.10. Vicine and divicine in HCl showed peak absorption at 274 and 280 nm, respectively. These spectra are similar to those reported (Bendich and Clements 1953; Olaboro 1978). While the spectrum of vicine remained stable in both acid and base, that of divicine changed in acid and extremely fast in base (Figure 2.10, bottom). Such data has already been reported (Bendich and Clements 1953), and further confirm that the compound isolated from the FBPC was essentially vicine.

A detailed study of the effect of pH on the stability of divicine confirmed that in solutions equilibrated with air, the absorbance at 280 nm decreased with time, the rate of which increased with increases in pH (Figure 2.11). Thus within 5 min, more than 50% of the reduced divicine was oxidized; confirming observations by Lin (1963) that the reaction between GSH and divicine was clearly observed only if divicine was used within 5 minutes of its preparation. It was also found that the gas medium influenced the rate of oxidation of reduced divicine at all pH levels (Figure 2.12). Oxidation was virtually inhibited in an atmosphere of nitrogen as opposed to that of air. Bendich and Clements (1953) have made similar observations.

Bendich and Clements (1953) observed that a fall in absorbance at 280 nm was concomitantly associated with an increase in absorbance at 240 nm of a solution of vicine in phosphate buffer (pH 6.0). The absorbance at 240 nm was attributed to oxidized divicine. The absorbance at 240 nm started decreasing after 10 minutes, indicating that oxidized divicine was an intermediate product which was subsequently

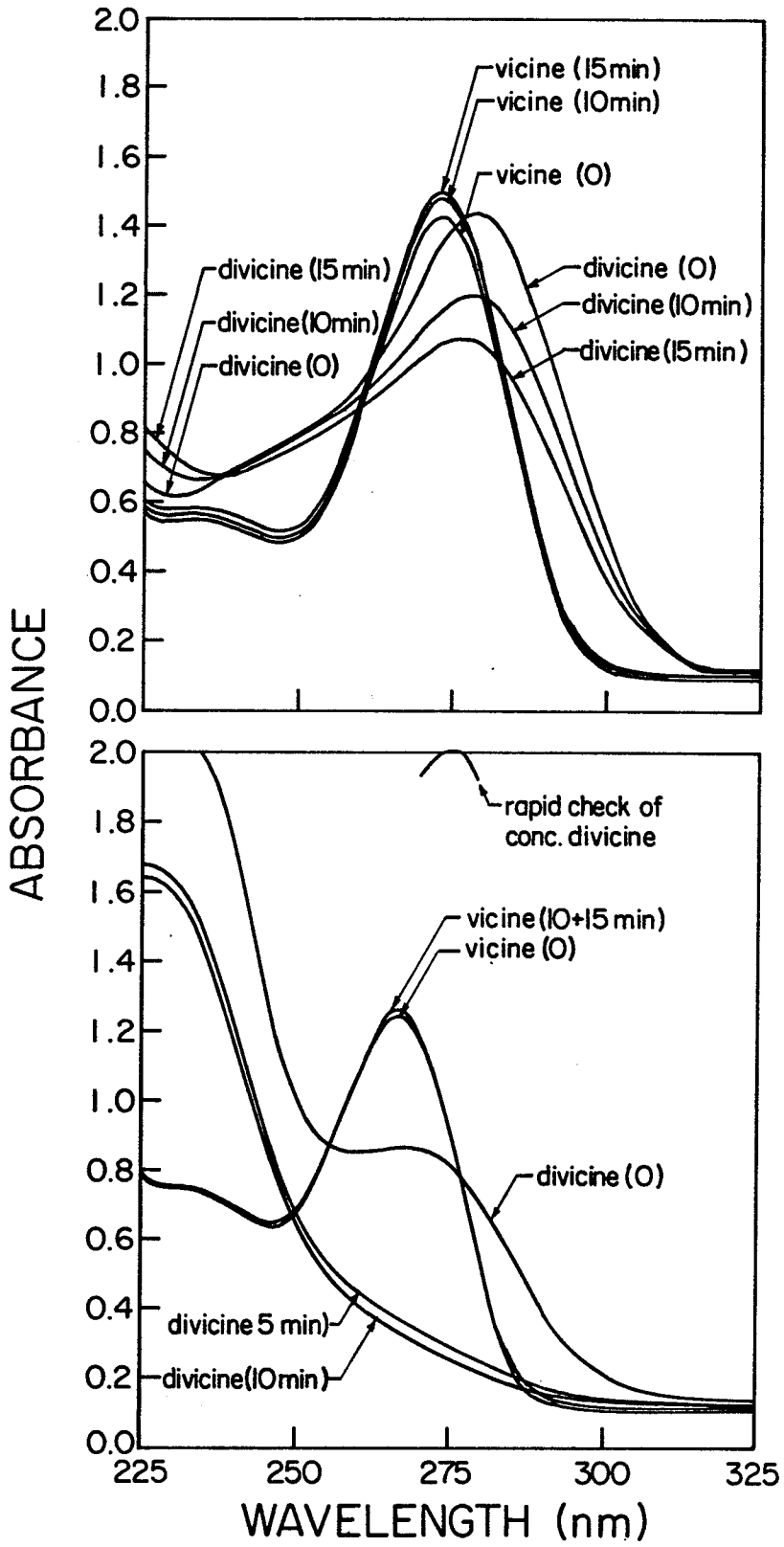


Fig. 2.10 UV absorption spectra of vicine and divicine in 0.1 N HCl (top) and 0.1 N NaOH (bottom) solutions. See section 2.2.2 (a) for the procedure.

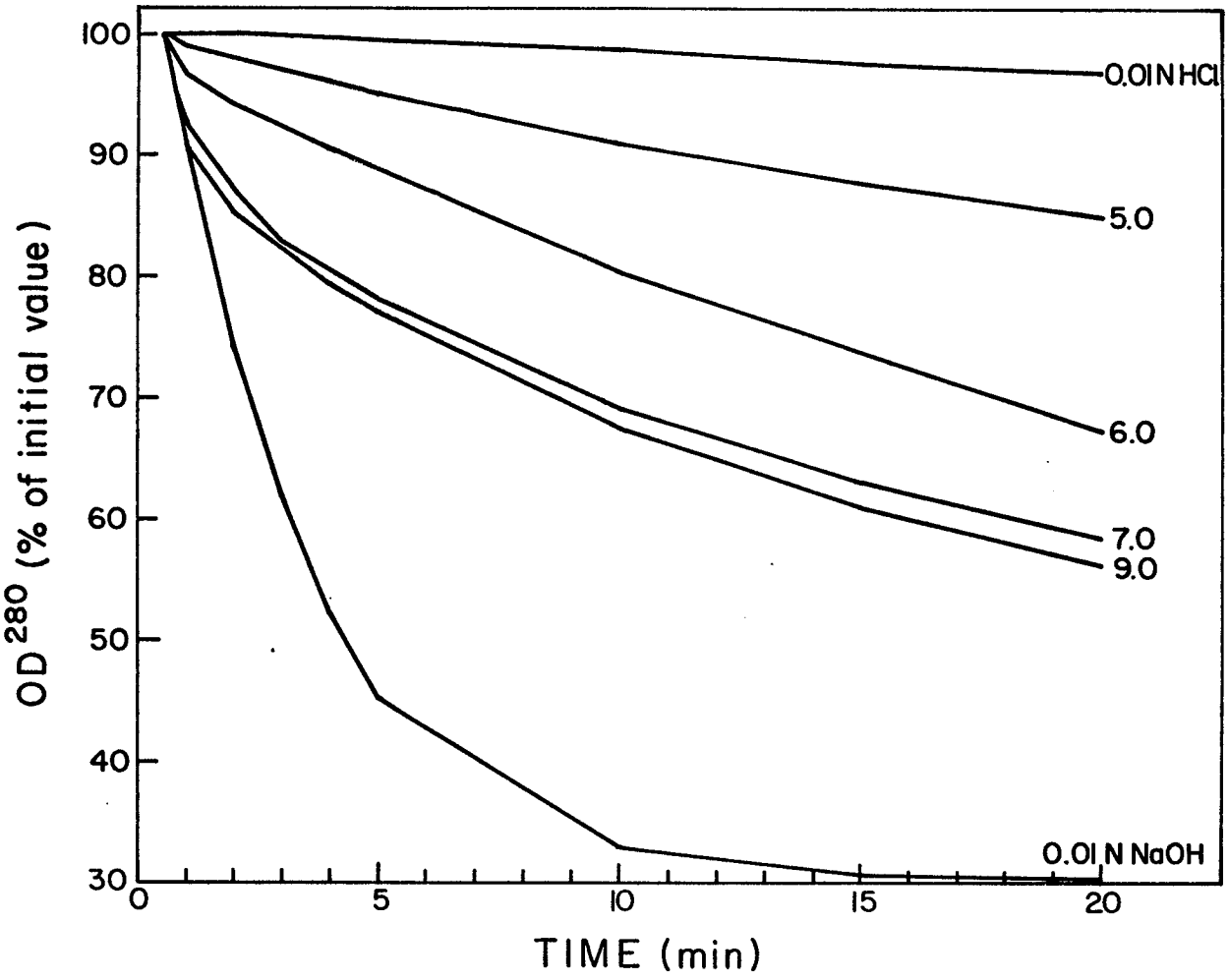


Fig. 2. 11 Effect of pH on the stability of divicine. See section 2.2.2 (b) for the procedure.

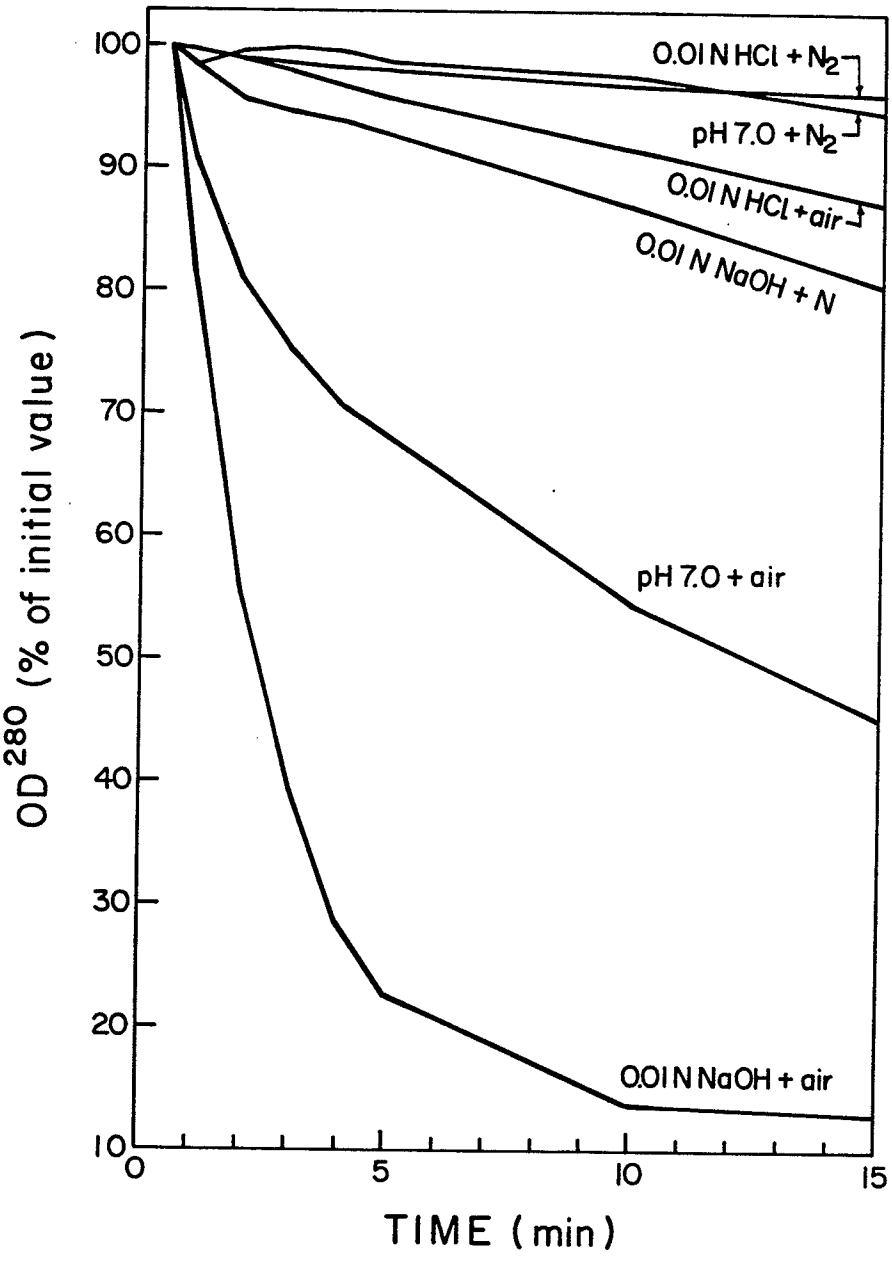


Fig. 2.12 Effect of gas medium and pH on the stability of divicine. See section 2.2.2 (c) for the procedure.

transformed into some product that did not absorb at 240 nm. Therefore it was decided to investigate the changes in absorbance at 240 nm of vicine in acid, base and phosphate buffer (Figure 2.13). There was an initial increase in absorbance with all the solvents. However, while the absorbance in phosphate buffer continued rising over the experimental period, that of HCl did not change much. In contrast, that in NaOH started decreasing almost immediately, indicating that the H^+ ions in buffer and acid may help reduce the rate of autoxidation or further destruction of the oxidised divicine.

This chapter presents a new and simplified method for the preparation of large quantities of crude vicine (approximately 88% vicine and 9% convicine). This should facilitate increased number of studies with animals. The method also for the first time outlines the simultaneous preparation of pure vicine and convicine by selective crystallisation. The purity of each was over 97 and 91%, respectively; the balance of the impurities were attributed to the presence of convicine or vicine. Recrystallisation would further purify the compounds. The crystalline structure, nitrogen content, melting point, UV spectrum, stability properties and effect of pH, the molar extinction coefficient and the solubility in different solvents indicated that the compounds isolated were vicine and convicine. However, data for the specific rotation indicated that some minor molecular alterations particularly to the sugar portion might have occurred.

The TLC method was found less sensitive and therefore less reliable than the amino acid method, in separating vicine and convicine.

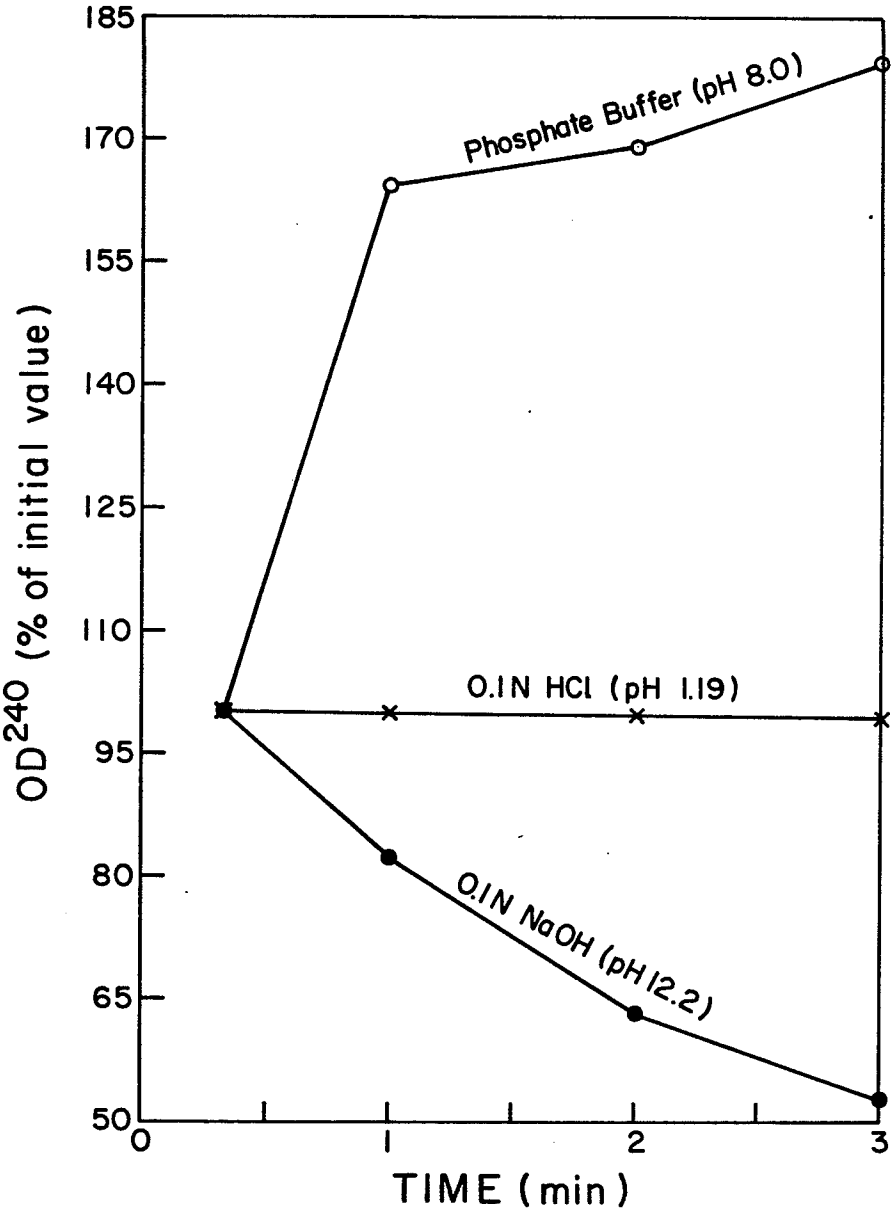


Fig. 2.13 Effect of type of solvent and pH on the stability of divicine at 240 nm. See section 2.2.2 (d) for the procedure.

The compound prepared from vicine was confirmed to have been divicine by its spectral and stability properties, and was further confirmed in Chapter 5.

CHAPTER 3

EFFECT OF DIETARY VICINE ON THE PRODUCTIVE PERFORMANCE OF GROWING AND LAYING CHICKENS.

Studies were conducted to determine the effect of various levels of dietary vicine on selected parameters in the chicken at different physiological stages. Apart from the economic factor, it was hoped that a suitable stage could be selected for further studies on the effects of vicine.

MATERIALS AND METHODS

Experiment 3.1. Effect of dietary vicine on the productive performance of male chicks.

General management and feeding

Thirty-five 7-day old white leghorn male chicks weighing 63-65 g were randomised among five dietary treatments (1-5). The birds were fed a broiler starter diet (Table 3.1) ad libitum, containing 0, 0.25, 0.5, 1.0 or 2.0% (group 1-5, respectively) crude vicine for two weeks; crude vicine was estimated to contain 89% vicine-reactive material. Therefore in all diets the actual levels of vicine material were slightly lower than the indicated "vicine" levels. In addition, the term vicine as used in the feeding experiments implies vicine plus some convicine (see Chapter 2). The birds were kept in temperature-controlled cages and water provided free choice.

Tissue sampling

At the end of two weeks, the birds were starved for at least 12 hours, then bled by heart puncture and killed by cervical dislocation.

Table 3.1. Broiler starter diet (Expt. 3.1)

Ingredient	Kg/1000 kg diet
Wheat (13)	626
Soybean meal (47.5)[a]	252
Fish meal (72)[a]	40
Soybean oil	40
Calcium phosphate	14
Vitamin premix[b]	10
Mineral premix[c]	5
Proximate analysis	
Dry matter (%)	87.9
Protein (N x 6.25) (%)	21.8[d]
Ether extract (%)	4.6
Fiber (% acid detergent)	4.4
Calcium (%)	1.0
Total phosphorus (%)	0.82
ME (calculated kcal/kg)	3000
C/P ratio[e]	138

[a]% protein.

[b]Vitamin premix provided per kg diet: 8,287.3 I.U., Vit. A; 875.1 I.U., Vit. D_3; 5.5 I.U., Vit. E; 11.0 µg, Vit. B_{12}; 2.4 µg, menadione; 1.0 g penicillin-streptomycin; 249 mg, santoquin; 503 mg, methionine-DL; 1.25 g, Vit. B_{58} complex[f]; 99.4 mg, animal tallow; 503 mg, amprol; and 6.2 g wheat middlings.

[c]Mineral premix provided per kg diet: 166.3 mg, manganese oxide; 14.4 mg, zinc oxide; 31.0 mg, $FeSO_4.7H_2O$; 25.5 mg, $CuSO_4.5H_2O$; and 4.8 g iodised salt.

[d]The amino acid content of the crude vicine (3%) was too low to affect the protein values at the levels of vicine in the diets.

[e]Calorie/protein ratio.

[f]Vitamin B_{58} complex contained riboflavin, pantothenic acid, niacin and choline (1:2:3:50).

Their livers were immediately excised, gall bladders removed, dried between paper towels and weighed. Blood was kept in heparin-coated tubes at $4^{o}C$ until it was centrifuged (7710xg for 10 minutes) to obtain plasma.

Tissue analysis

Four drops of fresh blood samples were placed into test tubes and spontaneous hemolysis immediately determined according to Draper and Csallany (1969), except that samples were incubated for 48 hours. The absorbance of the plasma at 350 nm was used as an estimate of turbidity. This wavelength was selected after scanning hemolysed-clear, hemolysed-turbid, nonhemolysed-clear and nonhemolysed-turbid blood samples between 200-850 nm. Preliminary data had indicated a high positive correlation between plasma turbidity and plasma lipid content. Data from experiments in Chapters 3 and 4 were analysed by a one-way analysis of variance, and the means were compared using Student Newman-Keuls test according to Snedecor and Cochran (1967). The χ^2 was computed according to the method devised by Snedecor and Irwin (1933) (Snedecor and Cochran 1967).

Experiment 3.2. Effect of dietary vicine on the productive performance and some liver, plasma and ova characteristics of mature laying hens.

The objectives of the study were to further investigate the effects that vicine had on egg and yolk weights and to compare these results with those obtained by Olaboro (1978).

General management and feeding

Twenty mature shaver layers initially 59 weeks of age were individually caged and had access to water free choice. In order to establish normal egg production rates and egg and yolk weights, all birds were fed a breeder diet (control diet, Table 3.4) free choice, for 10 days (control period). During the latter part of the control period, birds

were allotted to one of four treatment groups (1-4) such that the average yolk weights of the groups were quite similar. The control period was followed by a test period in which birds in groups 1 and 2 were fed the control diet, while those in groups 3 and 4 were fed the breeder diet containing 0.5 and 1.0% crude vicine, respectively for 14 days. All groups were fed ad libitum, except group 2 birds which were pair-fed with group 3.

Tissue sampling

Eggs were collected daily and their weights and those of the yolks measured immediately. Egg and yolk data obtained in the last 9 days of the test period were employed in the statistics. Blood samples were obtained both before and after the test period; on both occasions the birds were prestarved for 24 hours. A solution of 2% EDTA in normal saline (pH 7.0) was used as the anticoagulant.

Ten milliliters of blood were drawn into 10 ml syringes, previously rinsed with the anticoagulant, and then transferred into test tubes containing 1.0 ml of the anticoagulant. The blood was dept at $4^{o}C$ until centrifuged (3020 x g for 10 min at $-5^{o}C$) during the same day. The plasma obtained was stored frozen ($-20^{o}C$) until thawed for various assays.

At the end of the test period, the birds were exsanguinated by severance of the juglar vein. Livers were immediately excised, separated from the gall bladder, wiped between paper towels, frozen in liquid nitrogen in preweighed polyethylene bags, and then weighed. Abdominal fat pads were similarly wiped and weighed. Ova measuring more than 0.75 mm (inclusive) at the narrow mid-region were counted.

Tissue analysis

Lyophilised liver samples were analysed for lipids by a modification of the method of Reshef et al. (1976); forty milliliters of chloroform-methanol (2:1) mixture were added to 1 gm of finely ground liver tissue in a conical flask with a glass stopper, thoroughly swirled, stoppered and left to stand overnight at room temperature. The mixture was filtered through 5 g of hyflosupercel layered between two fibre glass filters in a buchner funnel. Twenty milliliter aliquots of the filtrate were evaporated to dryness under a stream of nitrogen and left to cool and dry to constant weight in a dessicator for 2 days before weighing. Plasma total lipid content was determined according to Heald and Badman (1963) except that 4.0 ml instead of 0.5 ml were used.

Experiment 3.3. Effect of dietary vicine on the productive performance, egg fertility, and some liver and plasma characteristics of young layers.

General management and feeding

The experimental protocol in this study was generally similar to that of Experiment 3.2. Sixty shaver breeder hens 26 weeks of age were initially selected for uniform ratio of yolk to egg weights. During the control period all birds were fed the same breeders' diet (control) (Table 3.4). Before the test period birds were allotted to one of three treatment groups (1-3) as described in Experiment 3.2. During the test period, birds in group 1 were continued on the breeders' diet ad libitum, group 3 was given, free choice, breeders diet containing 1% vicine, and group 2 was given the control diet, but pair-fed with group 3. On day 10 of the test period all birds were each inseminated with 0.05 ml of

semen pooled from 12 males. The semen was deposited 1.5 cm into the oviduct and eggs were collected on two consecutive days, 3 days after insemination. The eggs were incubated in a Robbin's incubator, at the University hatchery, on the second day of collection.

Tissue sampling

The termination of the experiment was as described in Experiment 3.2.

Tissue analysis

Liver and plasma lipids, plasma turbidity and spontaneous hemolysis were determined as in Experiment 3.2. In addition, a subjective visual hemolysis score was conducted and compared with the conventional method. In the visual hemolysis score samples were rated 1 to 5 depending on their redness. Plasma lipid peroxides were determined by a modification of the Asakawa-Matsushita (1979) method. Briefly, 1.0 ml of plasma and 0.1 ml of butylated hydroxytoluene (BHT) were placed in Sorvell test tubes, followed by 0.1 ml ferrous sulphate, 2.0 ml thiobarbituric acid (TBA) and 1.0 ml of trichloroacetic acid (TCA), solutions. The test tubes were stoppered with glass beads and heated in boiling water for 15 minutes. Following cooling in water, 1.0 ml glacial acetic acid and 2.0 ml chloroform were added and the mixture vortexed. The mixture was centrifuged at 48,200 x g for 15 min., the volume of the supernatant noted, and its absorbance measured at 532 nm. The lipid peroxide level was estimated as malondialdehyde (MDA) using its molar extinction coefficient (ε) of 1.53×10^5. A non-incubated preparation was used as the blank.

The same method was employed to estimate lipid peroxide levels in liver homogenates. The homogenates were prepared as described by Trostler et al. (1979), except that 4 volumes, instead of 2, of ice cold normal saline solution were used. The tissues were homogenised for 30 seconds and 1.0 ml of homogenate was used in the assay.

Plasma vitamin E levels were determined by the method of Quaife et al. (1949), except that all volumes were raised by appropriate factors to facilitate the usage of standard pipittes. Liver GSH levels were estimated by the method of Beutler et al. (1963) using 1.5 ml of homogenates prepared as described by Noguchi et al. (1973). Preliminary trials indicated that reliable data could only be obtained if the homogenates were analysed for GSH within 15 minutes of their preparation. Liver and plasma protein levels were estimated by the Lowry method (Lowry et al. 1951); 0.025 ml of plasma was diluted to 20 ml with distilled-deionised water, while for the liver 0.02-0.05 gm of tissue was solubilized in 1.0 ml of 1N NaOH solution by heating at 70°C. One tenth of a milliliter of the solution was then diluted to 20 ml with distilled water. One milliliter of diluted sample was employed in both assays.

RESULTS AND DISCUSSION

Data from Experiment 3.1 are summarised in Tables 3.2 and 3.3. Body weight and feed intake (Table 3.2) of the chicks appeared to be reduced as the level of dietary vicine increased. However, dietary vicine at all levels increased ($P<0.05$) RBC hemolysis in vitro. Chicks fed 0.25% dietary vicine gained weight at the same rate as the controls,

Table 3.2. Weight gain and feed efficiency of broiler chicks fed a broiler starter diet containing various amounts of vicine[a] (Expt. 3.1)

Group	Crude vicine content (%)	Average body weight		Normalised final[b] body weight (percent) of initial weight)	Mean feed intake (g/bird/day)	Feed/gain ratio
		Start	End			
1	0	63.6±1.0	175.9±3.3[c]	100.0	18.6	1.82
2	0.25	63.1±1.5	175.3±4.7[c]	100.3	18.4	1.80
3	0.50	62.7±1.8	137.7±5.1[a]	79.4	16.5	2.42
4	1.00	63.1±1.9	174.5±5.2[c]	99.9	18.2	1.80
5	2.00	63.6±1.8	167.7±6.0[b]	95.3	17.5	1.85

[a]Chicks were not individually caged or banded, therefore no statistics were run except for the initial body and final body weights (P = .05). Regressing the treatment on body weight or feed/gain ratio was avoided due to group 3 which by error did not have access to water all the time.

[b]The value final weight x 100/initial weight of the control group was designated 100% and the other values are expressed relatively.

resulting in a slightly better feed efficiency compared to the control group. The birds fed 0.5% dietary vicine had no access to water (waterers not properly adjusted) which resulted in abnormally low weight gains and hence high feed/gain ratio. However, it would seem from birds fed 2% dietary vicine that high levels of vicine could depress weight gain enough to raise the feed/gain ratio above that of the controls. These results are similar to those obtained with chicks (Arscott and Harper 1963) and rats (Lee 1950) fed divicine and rats fed vicine (Lin and Ling 1962c). Thus vicine and/or convicine may account for part of the heat resistant growth depressing effect of fababeans. No differences ($P>0.05$) in liver weight or plasma turbidity were observed among the experimental groups (Table 3.3) but spontaneous hemolysis was higher ($P<0.05$) in the vicine-fed chicks. However, more research concerning the biochemical effects of vicine on growing chicks should be performed as the chick would be a convenient and economical animal model.

Results of Experiment 3.2 are summarised in Tables 3.5 to 3.7. Feed consumption (Table 3.5) in all groups, and particularly those fed the vicine diet ($P<0.05$) was lower during the test than control period. However, all groups registered slight gains in body weight. Egg weight (Table 3.5) was depressed in vicine fed birds during the test period, but egg production rate at both levels of vicine was the same ($P>0.05$) as the control ad libitum group, but lower ($P<0.05$) than that of the control restricted group. The large standard errors might have accounted for the lack of differences in egg production rates, particularly between group 4 (1% vicine) and the others. However, the differences in egg production rates were reflected in a lower ($P<0.05$) egg mass in

Table 3.3. Liver weight, spontaneous hemolysis and plasma turbidity of broiler chicks fed a broiler starter diet containing various levels of vicine[a] (Expt. 3.1)

Group[b]	Liver weight (% of body weight)	Spontaneous hemolysis (%)	Plasma turbidity (OD units at 350 nm)
1	3.1	6.4±0.9[a]	0.93±0.09
2	3.0	9.9±1.1[b]	0.85±0.10
3	3.0	8.5±1.0[b]	0.95±0.08
4	3.0	9.4±1.3[b]	0.81±0.09
5	3.0	9.2±0.5[b]	0.84±0.09

[a]Means ± SE with different superscripts are significantly different ($P<0.05$).

[b]Same as in Table 3.2.

Table 3.4. Chicken breeders' diet (Expt. 3.2)

Ingredient	Kg/1000 kg diet
Barley	693
Soybean meal (44)[a]	184
Tallow	17.5
Dehydrated alfalfa	10.0
Calcium carbonate	42.5
Dicalcium phosphate	17.5
Oyster shell	20.0
Vitamin premix[b]	10.0
Mineral premix[c]	5.0
Grit	5.0
Proximate analysis	
Dry matter (%)	89.8
Protein (N x 6.25) (%)	17.0[d]
Ether extract (%)	5.0
Fiber (acid detergent) (%)	6.9

[a]Percent protein.

[b]Vitamin premix provided per kg diet: 33,000 I.U., vitamin A; 3,520 I.U., vitamin D_3; 22 I.U., vitamin E; 2.0 g vitamin B_{58} complex[e]; 2.0 g, DL-methionine; 1.0 g, santoquin; 400 mg animal tallow; 33 g, wheat middlings; and 44 µg, vitamin B_{12}.

[c]Mineral premix provided per kg diet: 1.32 g, manganese oxide; 0.44 g, zinc oxide; and 38.6 g, iodized salt.

[d]The amino acid content (%) of the vicine was too low to affect the protein composition of the diet. These levels were below those tested by Olaboro (1978) and found to be harmless.

[e]Vitamin B_{58} complex contained riboflavin, pantothenic acid, niacin and choline (1:2:3:50).

Table 3.5. Feed consumption, feed efficiency, egg weight and production rate of Shaver laying hens fed a control breeder diet and vicine breeder diets[a] (Expt. 3.2)

Group[b]	Treatment	Feed consumption		Body wt.[c]	Egg weight		Egg production rate[d]	
		Control period (g/bird/day)	Test period (% of control period)		Control period (g)	Test period (% of control period)	Control period (No./bird/day)	Test period (% of control period)
1	Control ad libitum	117.9±4.1	98.7±4.1^{b}	102.9±2.1	60.9±1.1^{a}	100.4±1.0^{c}	0.83±0.05	101.8±8.0^{ab}
2	Control restricted	120.0±5.0	94.6±5.5^{ab}	100.5±0.9	65.3±1.5^{b}	100.7±0.7^{c}	0.86±0.00	106.7±4.1^{b}
3	0.5% vicine	120.5±5.3	90.5±3.5^{a}	101.0±1.0	62.0±1.1^{a}	96.1±0.6^{b}	0.83±0.03	104.0±6.6^{ab}
4	1.0% vicine	117.6±4.5	88.8±4.7^{a}	102.3±1.3	64.3±0.4^{b}	94.5±1.0^{a}	0.86±0.00	93.3±4.1^{a}

[a]Means ± SE with different superscripts are significantly different, $P<0.05$.

[b]Five birds per group.

[c]Test period as percent of control period

[d]Egg mass data is shown in next Table (3.6).

group 4 compared to the other groups (Table 3.6). Yolk weights (Table 3.6) were reduced during the test period in birds fed 0.5 or 1.0% dietary vicine; the degree of depression was greater ($P<0.05$) in birds fed 0.5% dietary vicine than any other group. However, the yolk mass was more depressed ($P<0.05$) in the 1.0% vicine group compared to the others. Therefore the yolk weight seems to be determined, in part by the rate of production, and hence egg or yolk mass, rather than either weight or production rate, might be a better index of the productivity of a laying hen. The data obtained are similar to those reported by Olaboro (1978).

The differences in number of developing ova (Table 3.6) were small but significant ($P<0.05$). It is difficult to attribute these differences to vicine since no data was obtained during the control period. A larger number of birds may be necessary to further support these results. The differences in abdominal fat pad (Table 3.7) did not seem to be related to dietary vicine, and their significance and authenticity are just as questionable as those of the ova. Plasma lipids (Table 3.7) were elevated during the test period in all but the control ad libitum group; however, the differences between 0.5% vicine and the control ad libitum group were not significant ($P>0.05$), perhaps due to the high variability within groups. Liver weight and lipid (both % and total) levels (Table 3.7) were similar ($P>0.05$) among all groups, although the vicine fed birds tended to show lower liver lipid levels. This latter observation was reported also by Olaboro (1978). In addition there seems to be an inverse relationship between plasma and liver lipid levels.

The results of Experiment 3.2 would suggest that vicine depresses

Table 3.6. Egg mass, yolk weight and mass, and number of developing ova of Shaver laying hens fed a control breeder diet and vicine breeder diets[a] (Expt. 3.2)

Group[b]	Egg mass[c,d]	Yolk weight		Yolk mass[c,d]	No. of developing ova[e]
		Control period (g)	Test period (% of control period)		
1	102.2±8.3[b]	18.0±0.4	100.2±0.9[c]	102.2±8.6[b]	5.6±0.2[b]
2	107.4±4.5[b]	18.1±0.6	100.8±2.1[c]	107.8±6.2[b]	6.4±0.4[c]
3	99.8±6.0[b]	18.1±0.4	95.1±0.8[a]	98.9±6.1[ab]	5.0±0.3[a]
4	88.1±3.3[a]	18.3±0.6	97.4±1.6[b]	90.9±4.5[a]	5.0±0.4[a]

[a]Means ± SE with different superscripts are significantly different, $P<0.05$.

[b]Same as Table 3.5.

[c]Production rate x weight.

[d]Test period as percent of control period.

[e]Ova having a narrow mid portion, diameter of greater than 0.75 mm.

Table 3.7. Effect of feeding Shaver laying hens, a control breeder diet and vicine breeder diets, on abdominal fat pad, plasma lipids, and liver weight and lipid levels[a] (Expt. 3.2)

Group[b]	Abdominal[c] fat pad (% body wt.)	Plasma lipids Treatment period (mg %)	Plasma lipids Treatment period (% of control period)	Liver weight[c] (% body wt.)	Liver lipid[d] (% DM basis)
1	2.9±0.1[a]	8.4±1.8[a]	75.0±20.7[a]	1.4±0.1	32.0±6.9
2	3.0±0.5[a]	11.1±2.3[ab]	175.3±39.8[b]	1.3±0.0	31.0±2.1
3	3.6±0.3[b]	13.8±4.1[b]	100.1±18.2[a]	1.5±0.1	27.6±1.7
4	2.7±0.4[a]	13.9±1.5[b]	194.4±21.5[b]	1.4±0.1	26.7±2.6

[a]Means ± SE with different superscripts are significantly different, $P<0.05$.

[b]Same as Table 3.5.

[c]Weighed only at end of test period.

[d]Total liver lipid showed same trend and was not also different among treatments ($P>0.05$).

egg yolk weights and their mass, and the number of developing ova. In addition vicine elevates plasma lipids. Inconclusive data were obtained with regards to liver weight and lipid levels, and abdominal fat pad weights. These effects can not be attributed to differences in feed intake since they did not occur in birds pair-fed a control diet.

The data from Experiment 3.3 are summarised in Tables 3.8 to 3.13. Changes in feed consumption, body weight, egg and yolk weight, production rate and mass (Tables 3.8 and 3.9) were generally similar to those observed in Experiment 3.2. However, in Experiment 3.3 body weights during the test period were more depressed ($P<0.05$) in the restricted and 1.0% vicine groups than in the control ad libitum group. In addition the egg production rate was clearly lower ($P<0.05$) in the 1.0% vicine group compared to the two control groups, resulting in an even greater difference in egg mass. The weight of the egg is mainly determined by the yolk size since other components of the egg are laid down in proportion to the yolk. Therefore vicine seems to cause production of small yolks and hence small eggs. This is supported by the fact that the depression in egg weight or mass is greater than in yolk weight or mass, indicating that other egg components in addition to the yolk are reduced when birds are fed vicine. The yolk is closely invested by the granulosa cells during the initial growth phase and therefore the granulosa cells may be involved in the transport of the large quantities of precursor material (protein, fat, etc.) required to form the yolk, and may be actively involved in yolk synthesis (Wyburn et al. 1965). Vicine may act by either reducing the amount of precursor material available to the granulosa cells, damage the granulosa cells and hence their activity

Table 3.8. Feed consumption, feed efficiency, egg weight, mass and production rate of Shaver breeder hens fed control breeder and vicine breeder diets[a] (Expt. 3.3)

Group[b]	Treatment	Feed consumption		Body wt.[c]	Egg wt.[e] (g)	Egg production rate		Total egg[c,d] mass
		Control period (g/bird/day)	Test period (% of control period)			Control period (No./bird/day)	Test period (% of control period)	
1	Control ad libitum	121.4±1.9	98.0±2.5[c]	91.2±0.8[b]	55.8±0.7[b]	0.96±0.02	102.1±2.9[b]	103.8±3.0[c]
2	Control restricted	119.9±2.2	83.7±1.7[b]	89.3±1.1[a]	55.5±0.8[b]	0.92±0.02	98.1±3.4[b]	98.1±3.0[b]
3	1.0% vicine	121.1±2.1	76.9±2.5[a]	89.7±0.7[a]	48.1±0.7[a]	0.95±0.02	89.7±4.5[a]	80.1±4.0[a]

[a]Means ± SE with different superscripts are significantly different, $P<0.05$.

[b]Twenty birds per group.

[c]Test period as percent of control period.

[d]Egg weight x production rate.

[e]Treatment data presented since there were no differences ($P>0.01$) during the control period.

Table 3.9. Yolk characteristics of Shaver breeder hens fed a control breeder diet and vicine breeder diet[a] (Expt. 3.3)

Group[b]	Yolk wt.[c] (g)	Total yolk[d,e] mass	Yolk index[f]	Frequency of blood[g] spotting
1	13.8±0.2[c]	108.6±4.1[b]	0.48±0.01[b]	0.086±0.07[a]
2	13.5±0.2[b]	102.8±3.8[b]	0.48±0.01[b]	0.068±0.04[a]
3	11.8±0.2[a]	81.9±4.1[a]	0.42±0.01[a]	0.35±0.06[b]

[a]Means ± SE with different superscripts are significantly different, $P<0.05$.

[b]Same as Table 3.8.

[c]Data for test period is presented since there were no differences ($P>0.01$) during the control period.

[d]Test period data as percent of control period data.

[e]Weight x production rate.

[f]Measured only during test period after noticing gradual changes in the yolk height; Yolk index = $\frac{\text{yolk height}}{\text{diameter}}$ (Moran 1936).

[g]Data for test period minus that for treatment period; because some had no incidences in one period it would be difficult to calculate % changes.

or destroy the ovum.

The yolk index was lower ($P<0.05$), while the frequency of blood spotting was higher ($P<0.05$) in vicine than control groups (Table 3.9). The yolk index is a reflection of yolk membrane strength, which seems to fall with deterioration in egg quality (Moran 1936). Since the membrane is composed mainly of protein and lipids, to a lesser degree (Moran and Hale 1936; Bellairs et al. 1963) it is possible that vicine causes damage or denaturation of these proteins and/or alter the structure of the lipid component. The fertility and hatchability of the eggs from vicine birds were lower ($P<0.025$) than those from the control groups (Table 3.10). However, the differences in hatchability of fertile eggs were lower among the experimental groups. Thus indicating that vicine destroys embryos either at the very early stages or prevents fertilisation by destroying the spermatozoa or the ovum. Some of the unhatched eggs were so macerated that it was difficult to distinguish between the fertile and infertile eggs. Tables 3.10 and 3.11 seem to show that low feed intake (group 2) may improve fertility, but could slightly reduce hatchability. However, this effect is negligible compared to that of vicine, and its validity is questionable without statistical analysis.

Plasma lipids (Table 3.12) during the treatment period were higher ($P<0.05$) in vicine fed than control fed groups; conversely the ratio of plasma vit. E/lipid levels was reversed ($P<0.05$). The ratio of plasma vit. E/lipid levels was used in this study as it is a better index of vit. E adequacy than either of their absolute levels (Horwitt et al. 1972). The low ratio of plasma vit. E/lipid in vicine fed birds was also associated with a high ($P<0.05$) hemolysis score. However, there were no

Table 3.10. Fertility and hatchability of eggs from hens fed a control breeder and vicine breeder diets free choice and a control breeder diet at restricted intake[a] (Expt. 3.3)

Treatments[b]	No. of eggs		Percent fertility (%)	No. of chicks hatched (No.)	Percent hatchability of	
	Set (No.)	Infertile (No.)			Total eggs (%)	Fertile eggs (%)
1	41	3	93	37	90	94
2	37	1	97	31	84	86
3	30	16	47	10	33	71

[a]Eggs from each group were hatched together, therefore only a χ^2 test was run and indicated that hatchability (% of total eggs) and fertility differed among the treatments ($P<0.025$).

[b]Same as Table 3.8.

Table 3.11. Mortality grouped by stages of embryonic development (Expt. 3.3)

Treatments[a]	Fertile eggs unhatched	Stages of development			
		0-7 days	8-14 days	15-20 days	Day 21-pipped[b]
1	1	-	-	-	1
2	5	1	-	1	3
3	4	2	-	2	-

[a]Same as Table 3.8.

[b]Pipped eggs are those where the chick was able to crack the shell but did not have enough strength to break out.

Table 3.12. Plasma characteristics of Shaver breeder hens fed control breeder and vicine breeder diets[a,b] (Exp. 3.3)

Group[c]	Plasma lipids (mg %)	Percent ratio vit.E/lipids	Hemolysis[d] score	Lipid peroxides[e] (n moles MDA/ml)	Protein (g %)
1	4.9±0.6[a]	6.5±1.0[b]	1.6±0.3[a]	1.7±0.1[a]	4.5±0.2
2	5.6±0.5[a]	6.0±0.7[b]	1.7±0.3[a]	1.5±0.1[a]	4.5±0.2
3	15.1±1.8[b]	3.1±0.4[a]	2.5±0.3[b]	8.0±2.5[b]	4.6±0.1

[a]Means ± SE with different superscripts are significantly different, $P<0.05$.

[b]Data at the end of the test period.

[c]Same as Table 3.8.

[d]Visual score, based on the degree of redness.

[e]MDA = Malondialdehyde.

differences ($P>0.05$) in plasma protein among the experimental groups (Table 3.12).

Vicine-fed birds had heavier livers, and higher GSH and lipid peroxide levels ($P<0.05$) than the control groups (Table 3.13). These GSH levels may indicate that vicine fed birds had normal or elevated levels of GSSG-reductase, but perhaps normal or lower levels of GSH-px (or their activities) than the control groups. Liver lipid levels in this experiment, unlike Experiment 3.2 were not different ($P>0.05$) among the experimental groups; this might be due to the fact that the lipids were determined on fresh samples whose dry matter might have varied or due to age differences between birds in the two experiments. Liver protein levels were slightly depressed in the control restricted and more in the vicine-fed birds. This was associated with heavier livers which might indicate an adaptation to a low production of some essential compound(s).

Therefore it may be concluded from data in this chapter that dietary vicine lowers the productive performance of both chicks and laying hens. In the chick it increases RBC fragility and retards the growth rate, while in the laying hen it causes depression in egg and yolk mass, by reducing mainly their weights, and partly the production rate; and reduces fertility and hatchability of eggs. In addition vicine increased the fragility of the yolk membrane and the number of blood spots on the yolk. It also leads to some undesirable biochemical changes in the blood and liver of the laying hen such as elevated levels of plasma lipid and lipid peroxides depressed ratio of plasma vitamin E/lipid levels and an increased degree of erythrocyte hemolysis.

Table 3.13. Liver characteristics of Shaver breeder hens fed control breeder and vicine breeder diets[a,b] (Expt. 3.3)

Group[c]	Liver wt. (% body wt.)	Liver lipids (%)	Liver GSH (μ moles/g)	Lipid peroxides[d] (n moles MDA/g)	Protein (mg/g)
1	1.57±0.05[a]	5.4±0.5	0.20±0.01[a]	17.25±0.40[a]	272.7±6.7
2	1.51±0.04[a]	6.4±0.9	0.19±0.01[a]	19.34±0.92[b]	241.6±8.8
3	1.71±0.05[b]	6.0±0.6	0.27±0.01[b]	22.30±0.85[c]	234.1±5.3

[a]Means ± SE with different superscripts are significantly different, $P<0.05$.

[b]Data at the end of the test period.

[c]Same as Table 3.8.

[d]MDA = Malondialdehyde.

CHAPTER 4

THE EFFECT OF DIETARY VICINE AND VITAMIN E SUPPLEMENTATION ON THE PRODUCTIVE PERFORMANCE OF BREEDER CHICKENS

Based on the data of Experiments 3.2 and 3.3, it was decided to investigate whether vitamin E, a known antioxidant and free radical scavenger, could protect against all/or some of the effects due to dietary vicine.

MATERIALS AND METHODS

General bird management and feeding

Since it had been consistently shown that dietary vicine leads to low egg weights in both mature and young layers, the control period was eliminated in this study. Thirty shaver breeder hens initially 26 weeks of age were randomised among three dietary treatments (1-3). Group 1 birds received a normal breeder's diet (see Table 3.4), those of group 2 received breeder's diet containing 1.22% crude vicine, and the third group received the same diet as group 2, but supplemented with 10 times more vitamin E; this level was raised to 40 times after 6 days when it was observed that the egg weight in this group was on the decline. Birds were individually caged and fed ad libitum, but two birds of the same treatment group shared a feeder, and hence five, instead of ten, observations for feed consumption were measured. The birds were inseminated on day 8, and eggs collected and incubated as before (Experiment 3.3).

Tissue sampling

Eggs were collected and weighed daily to give an indication as to when it was appropriate to inseminate the birds. The eggs collected

during the first four days were considered control; during these days egg weight increased in all groups, therefore the effect of vicine had not been translated into egg weights.

The termination of the experiment and procedures for obtaining liver and blood samples, were as already described. However, it was found that centrifugation at 1,000xg for 10 min. was adequate to obtain plasma and was therefore employed in this study. The RBC precipitates were washed three times with their plasma volume of normal saline solution, and stored frozen until hemolysed for assay. Subjective visual scores were made for plasma turbidity and hemolysis, and hematocrit values were determined by drawing blood from the wing vein directly into heparin-coated capillary tubes.

Tissue analysis

Liver and plasma lipids, liver GSH, plasma lipid peroxides and vitamin E levels were determined as described in Experiment 3.3. Liver catalase activity was determined according to Cohen et al. (1970); after some preliminary trials the following procedure was adapted: 1 g of liver tissue was homogenised, for 30 secs. in 10 volumes of normal saline-phosphate buffer (pH 7.4), centrifuged (700xg for 10 min.) and 9.0 ml of the supernatant drawn into a test tube. Following the addition of ethanol (0.09 ml), the samples were incubated for 30 min. in ice water, and then 10% triton-x was added to a final concentration of 1%. Two to three minutes later, an aliquot of the mixture was diluted 200 fold with cold saline-phosphate buffer (pH 7.4), and 0.5 ml of the resultant solution was utilised in the catalase assay.

Liver glutathione peroxidase (GSH-px) activity was determined as described by Noguchi et al. (1973), except that it was found necessary to dilute the homogenates 40 times rather than 10-fold, and the absorbance values obtained with 2 mM GSH were so low that 10 mM GSH was selected instead. In both catalase and GSH-px assays, water instead of homogenate were used as blanks. For the superoxide dismutase (SOD) assay, liver tissue was prepared according to VanHien et al. (1975), and SOD activity determined by the NBT reaction of Beauchamp and Fridovich (1971). Preliminary tests indicated that assaying the tissues without protein precipitation with ethanol-chloroform, gave negative inhibition with increasing concentration of the tissue in the assay medium. It therefore seemed like some proteins or compounds coprecipitating with proteins inhibit SOD activity or produce more O_2^- to override the SOD effect. Based on these trials, 1 g of liver tissue was homogenised for 30 secs. in 5 volumes of ice cold normal saline and then centrifuged at 20,000xg for 10 minutes. The excess protein was denatured by adding 0.3 volumes (based on supernatant volume above) of cold ethanol-chloroform (5:1) mixture to the supernatant. The vortexed sample was incubated in ice cold water for 15 min. and then centrifuged as before. The resulting supernatant was diluted 50-fold, and 0.1 ml was added to 2.9 ml of the NBT cocktail (Beauchamp and Fridovich 1971). In some assays, the changes in absorbance of the standard NBT mixture, were so low that the NBT concentration was raised 10-fold. One unit of SOD activity was equivalent to a 50% inhibition of the NBT reaction. Due to variations in the standard, relative activities were calculated within each assay and these were normalised in each group by considering the

highest value as unity. Potassium phosphate buffer (pH 7.4), instead of liver supernatant was processed for the blank reaction.

Hemolysates of RBC were prepared by adding 25 ml of distilled, deionised water to the RBC precipitates, allowed to stand for 10 min. at room temperature and then centrifuged at 20,000xg for 10 minutes. The supernatant was then frozen until assayed for hemoglobin, GSH and SOD activity. Hemoglobin was determined by using 50 µl of the hemolysate in the cyanmethemoglobin method (Sigma Chemical Co. 1978). Reduced glutathione was determined according to Beutler et al. (1963) except that 1.0 ml of the hemolysate was used without further dilution. For the SOD assay, the hemolysates were diluted twice with potassium phosphate buffer, and 0.1 ml of the solution added to 2.9 ml of the NBT cocktail (Beauchamp and Fridovich 1971).

RESULTS AND DISCUSSION

Since it was demonstrated in Chapter 3 that feed intake was not the major cause of the poor performance of birds fed vicine diets the pair fed group was eliminated from studies in this chapter. One experiment was performed and will be referred to as Experiment 4.1.

Data from Experiment 4.1 are summarised in Tables 4.1 to 4.6. Feed consumption during the test period was lower ($P<0.05$) in the vicine fed birds than those fed a control diet (Table 4.1). The body weights, however, did not decrease proportionally, leading to a lower ($P<0.05$) ratio of feed/kg body weight in the vicine fed birds compared to the control group. However, general feed efficiency was lower in the vicine-fed birds if egg mass is considered. Although egg weights were depressed ($P<0.05$) in both vicine fed groups, those of birds in the vitamin E supplemented vicine group were

Table 4.1. Feed consumption and egg weight of Shaver breeder hens fed a breeder diet containing 1.22% vicine with or without vitamin E supplementation[a] (Expt. 4.1)

Group[b]	Diet	Feed consumption		Egg weight	
		(g/bird/day)	(g/kg final body wt.)	Control period (g)	Test period (as % of control period)
1	Breeder's diet (Control)	107.0±3.0[b]	30.1±0.7[b]	54.2	103.9±1.6[c]
2	Breeder's diet + 1.22% vicine	84.6±5.4[a]	24.7±1.6[a]	53.0	94.1±1.5[a]
3	Breeder's diet + 1.22% vicine + extra vitamin E[c]	87.1±5.5[a]	25.1±1.14[a]	54.5	96.4±0.6[b]

[a]Means ± SE with different superscripts are significantly different $P<0.05$.

[b]Ten birds in each group.

[c]880 I.U./kg diet.

less severely affected ($P<0.05$). In addition vitamin E supplementation improved the fertility and hatchability of vicine fed birds (Table 4.2). Similar to results of Experiment 3.3, the differences among experimental groups were greater for fertility and percent hatchability of all eggs than for percent hatchability of fertile eggs. Table 4.3 further confirms that vicine affected the early stages of embryonic development. This is further supported by reports that peak embryonic mortality in eggs from hens fed vitamin E deficient diets occurs on the 4th day of incubation (Adamstone 1931).

Data on liver characteristics (Table 4.4) show that feeding vicine resulted in elevated ($P<0.05$) liver weights, lipid and GSH levels. Vitamin E supplementation reduces the first two but not GSH, to control levels ($P>0.05$). The effects of dietary vicine (group 2) on the above three parameters are similar to those observed in Experiment 3.3. However, unlike in Experiment 3.3, liver protein levels were slightly elevated ($P>0.05$) in birds fed vicine (group 2); but the levels were quite variable in the control and vitamin E supplemented vicine groups.

Liver GSH-px and SOD activities were lower ($P<0.05$) in birds fed vicine without vitamin E supplementation than the other two groups (Table 4.4). In addition catalase activity was lower ($P<0.05$) in both vicine groups compared to the control. This data indicates that the elevated levels of GSH in vicine fed birds are due to a low activity and/or level of GSH-peroxidase and that vicine does not affect the activity or synthesis of GSSG-reductase or vicine affects the latter less compared to GSH-peroxidase. It is however intriguing that vicine leads to low activities of the enzymes known to scavange intermediates and

Table 4.2. Fertility and hatchability of eggs from hens fed a control breeder diet and vicine breeder diets with or without vitamin E supplementation[a] (Expt. 4.1)

Group[b]	No. of eggs		Percent[c] fertility	No. chicks hatched	Percent hatchability of	
	Set	Infertile			Total eggs	Fertile eggs
1	25	0	100.0	23	92.0	92.0
2	22	13	40.9	5	22.7	55.5
3	22	4	81.8	15	68.2	83.4

[a]Same as Table 3.10; differences in hatchability (% of total eggs) were significant ($P<0.01$) while those of fertility were significant at $P<0.005$.

[b]Treatments are same as Table 4.1.

[c]Includes the unhatched fertile eggs and pips, shown in Table 4.3.

Table 4.3. Mortality grouped by stages of embryonic development (Expt. 4.1)

Group[a]	Fertile eggs unhatched	Stages of development			
		0-7 days	8-14 days	15-20 days	Day 21-pipped[b]
1	1	-	-	-	1
2	4	2	-	1	1
3	3	3	-	-	-

[a]Treatments are similar to Table 4.1.

[b]As defined in Table 3.11.

Table 4.4. Some liver characteristics of Shaver breeder hens fed a control breeder diet and vicine breeder diets with or without vitamin E supplementation[a] (Expt. 4.1)

Group[b]	Liver wt. (% of body wt.)	Liver lipid (%)	Liver protein (mg/g)	Liver GSH (μ moles/g)	GSH-px activity[c] (units/g liver)	GSH-px activity[c] (units/mg protein)	Catalase activity[d] (k units)	SOD activity (% inhibition per unit volume of supernatant)	SOD activity (units/mg protein)
1	0.80±0.04[a]	5.99±0.23[a]	199.8±12.9	0.50±0.02[a]	16.9±1.0	87.7±7.5[b]	0.46±0.04[b]	92.1±3.5[b]	92.6±3.3[b]
2	0.91±0.03[b]	7.13±0.51[b]	209.1±8.6	0.59±0.04[b]	15.0±1.7	70.3±5.6[a]	0.37±0.03[a]	84.7±2.7[a]	80.6±3.0[a]
3	0.84±0.02[a]	6.30±0.34[a]	213.0±11.6	0.62±0.03[b]	17.0±1.6	82.3±7.5[b]	0.32±0.03[a]	91.2±4.6[b]	86.0±5.3[ab]

[a]Means ± SE with different superscripts are significantly different P<0.05.

[b]Treatments are same as in Table 4.1.

[c]A unit of enzyme activity was equivalent to the disappearance of 1% of GSH per minute (Noguchi et al. 1973).

[d]Calculated according to Cohen et al. (1970), assuming 1st order kinetics for decomposition of H_2O_2; where k is the 1st order reaction rate constant.

[e]A unit of enzyme activity was equivalent to an inhibition of the NBT reaction by 50%, compared to the control.

products of oxygen reduction and other potential prooxidants.

Plasma lipid levels (Table 4.5) were higher ($P<0.05$) in both vicine groups than the control, although vitamin E supplementation of vicine diets tended to reduce these levels. Plasma vitamin E levels (Table 4.5) were elevated ($P<0.05$) in the vitamin E supplemented vicine group, leading to a higher ($P<0.05$) ratio of plasma vitamin E/lipid compared to the other groups. In spite of the higher plasma vitamin E levels in vicine fed birds (group 2) compared to the control, the ratio of plasma vitamin E/lipid was slightly lower ($P>0.05$) in the former suggesting that vitamin E levels were more adequate in the control group. This is reflected in a lower level of lipid peroxides (Table 4.5) and hemolysis, and a higher hematocrit (Table 4.6) ($P<0.05$) in the control group compared to both vicine groups. There were however, no differences in the parameters between the vicine groups ($P>0.05$). Thus vitamin E at these levels was not effective in protecting the erythrocytes against vicine. Addition of glucose to the incubation medium reduced in vitro hemolysis in all groups, but the effect was greater ($P<0.05$) in the vicine groups than the control. In some cases glucose increased hemolysis in the control but not in the vicine groups. Thus a lack of glucose or its reducing metabolites may in part account for the hemolysis induced by vicine. The above could result from inhibition of enzymes or destruction of membranes on which the enzymes that metabolise or transport glucose are bound.

Hemoglobin levels in the hemolysate (Table 4.6) were lower ($P<0.05$) in both vicine groups than the control, even after standardising the values to the same theoretical hematocrit. Conversely, the levels of

Table 4.5. Plasma lipid, lipid peroxide and vitamin E levels of Shaver breeder hens fed a control breeder diet and vicine breeder diets with or without vitamin E supplementation[a] (Expt. 4.1)

Group[b]	Plasma vit. E (mg %)	Plasma lipids (mg %)	Percent ratio vit. E/lipids	Plasma lipid peroxides[c] (n moles MDA/ml)
1	0.36±0.05[a]	7.67±0.52[a]	4.8±0.6[a]	0.88±0.11[a]
2	0.57±0.07[a]	13.34±1.56[b]	4.6±0.9[a]	1.63±0.19[b]
3	1.29±0.23[b]	12.89±2.99[b]	9.2±0.3[b]	1.58±0.15[b]

[a]Means ± SE with different superscripts are significantly different $P<0.05$.

[b]Treatments are similar to Table 4.1.

[c]MDA = malondialdehyde.

Table 4.6. Haematocrit, spontaneous hemolysis and levels of hemoglobin, GSH and SOD activity in RBC hemolysates of Shaver breeder hens fed a control breeder diet, and vicine diets with or without vitamin E supplementation[a] (Expt. 4.1)

Group[b]	Hematocrit (%)	Hemolysis		Hemoglobin[c] (Hb) (mg/ml hemolysate)	GSH[c] (μ moles/ml hemolysate)	SOD activity[c]	
		-glucose (%)	+ glucose (% of -glucose			(% inhibition/ unit volume)	(units/g Hb)[d]
1	31.3±0.8[b]	22.0±5.0[a]	89.3±21.5[b]	25.3±0.7[b]	0.51±0.02[a]	69.9±3.3[a]	1103.7±47.0[a]
2	22.3±1.0[a]	63.8±5.9[b]	32.2±4.9[a]	22.4±1.0[a]	0.74±0.05[c]	87.8±7.7[b]	1602.4±165.0[b]
3	22.7±1.1[a]	54.0±7.7[b]	33.1±6.0[a]	22.8±1.0[a]	0.65±0.02[b]	92.9±8.1[b]	1669.0±183.4[b]

[a]Means ± SE with different superscripts are significantly different, $P<0.05$.

[b]Treatments are same as in Table 4.1.

[c]Standardised at 30% haematocrit for all groups. In reality the total amount available to the animal would be lower in birds of low haematocrit values.

[d]A unit of enzyme activity was an inhibition equivalent to 50% compared to the control.

GSH and SOD activity were higher ($P<0.05$) in the vicine groups than the control group with or without standardising the values to the same hematocrit. This would seem to indicate that vicine in RBC accelerates destruction of Hb, but increases the activities of GSSG-reductase and SOD while inhibiting that of GSH-peroxidase. Since there is no protein synthesis in the RBC, vicine can not act by inhibiting synthesis, hence destruction of the enzymes would lead to increased molar activities of the surviving enzyme molecules.

It may be concluded from data in this chapter that vicine or its metabolites do not affect liver lipid levels but markedly elevate plasma lipid levels. These metabolites appear to be prooxidants that cause lipid peroxidation and cause destruction of some proteins both in liver and RBC, and the RBC themselves. Vicine or its metabolites also reduce fertility but the mechanism is not yet known. Vitamin E in the presence of dietary vicine provides protection against reduced fertility, but does not seem to substantially affect the other biochemical parameters. The increased SOD activity in RBC of vicine fed birds along with the other effects strongly indicates an increased generation of superoxide and other radicals.

CHAPTER 5

BIOCHEMICAL REACTIONS OF DIVICINE AND THE POSSIBILITY THAT FREE RADICALS MAY BE INVOLVED IN ITS BIOCHEMICAL EFFECTS

Flohe et al. (1971) were the first to propose that the action of divicine on RBC involved free radical reactions. The rapid rate of autoxidation in oxygen and alkaline pH, which is inhibited in a medium of carbon dioxide or nitrogen gas and/or low pH, is similar to that of other compounds known to yield O_2^- and H_2O_2 on autoxidation and are hemolytic. Therefore experiments were conducted to test the reactions of divicine with some compounds and enzymes that are known to be involved in free radical metabolism, and to test whether O_2^- is produced during the autoxidation of divicine.

This chapter is presented in a format somewhat different from Chapters 3 and 4 and is divided into two major sections (5.1 and 5.2). Section 5.1 is concerned with biochemical reactions of divicine with different biological compounds, and section 5.2 tries to establish that O_2^- is generated during the autoxidation of divicine; the latter section is presented under three subsections (5.2.1-5.2.3) representing different ways of establishing the presence of superoxide anion.

MATERIALS AND METHODS

5.1. Biochemical reactions of divicine

The divicine used in this and other sections of this chapter was prepared by acid hydrolysis (see section 2.2). A saturated solution was made in 0.1N HCl (SAT) and this stock solution was diluted (DIL) as desired for the assays. Divicine was prepared fresh daily and sometimes for each assay on the same day.

The objectives of this study were (a) to determine the effects of certain compounds on the autoxidation of divicine, and (b) the nature of the reactions between divicine and GSH, GSSG, oxyHb and methemoglobin. Based on the spectral characteristics observed in Chapter 2, it was decided that autoxidation would be followed as an increase in absorbance at 240 or 250 nm. This absorption was attributed to oxidized divicine since a fall in absorbance at 280 nm (the proposed λ max for reduced divicine) was concomitantly associated with an increase at 240 nm. The reactions between divicine and GSH and GSSG were monitored also at 305 nm since Lin (1963) observed that a product with a peak at this wavelength was produced when he incubated divicine with reduced glutathione. Changes in oxyHb and MetHb were monitored at their respective λ max (577 and 630 nm, respectively) (Winterbourn et al. 1979).

In sections 5.1 and 5.2.1, the compounds to be tested were dissolved in 0.128M K_2HPO_4 - EDTA (100 µM) buffer (pH 8.0) (in concentrations specified in the tables and figures); 2.9 ml of the solution was placed in a cuvette and the absorbance determined. This was followed by the addition of 0.1 ml of divicine in 0.1N HCl, or just 0.1 ml of 0.1N HCl for the blank. The sample was mixed and the absorbance was monitored at certain time intervals. A control that did not contain the compound being tested was always run first and its absorbance was used as a reference value. All values are from duplicate determinations.

5.2. Testing for production of free radicals

The assumption in this section was that O_2^-, $^{\cdot}OH$ and a divicine radical were the most likely products of divicine autoxidation. The structural resemblance to dialuric acid and ascorbic acid (Mager et al. 1969) suggested that O_2^- and H_2O_2 were the most probable initial products (Fee and Teitelbaum 1972). Therefore, it was decided to test for the production of O_2^- employing the reduction of cytochrome C (Jain and Hochstein 1979) and nitroblue tetrazolium (NBT) (Misra 1974), and also for any other free radical using electron spin resonance spectroscopy. All values in this section are from duplicate determinations.

5.2.1. Testing for presence of O_2^- by cytochrome C reduction

The experimental procedures for testing for the presence of O_2^- were similar to those described for section 5.1 and Figure 5.1. The modifications made are specified in each table and/or figure. The effects of GSH, Hb, metHb and bovine serum albumin (BSA) on the reaction between divicine and cytochrome C were investigated at different wavelengths to try and gain an insight as to the direction of the reaction components. Finally, the effects of divicine on the activity of SOD and catalase were also investigated.

5.2.2. Testing for presence of O_2^- by NBT reduction

The method of Misra (1974) was adapted and modified where necessary. Details of the procedures are given in each table or figure. Buffered enzyme solutions were denatured by heating in a boiling water bath for 20 minutes.

5.2.3. Testing for presence of free radicals with e.s.r. spectroscopy

A dual syringe, automatic plunger was employed. Divicine (SAT or DIL 2x) was prepared in 0.01N HCl and stored in one syringe and the second syringe contained buffers of pH ranging from 6-10. Due to the arrangement of the plunger, equal volumes of sample and buffer were employed and hence the sample concentration in the mixture was half that of the original sample. The pH of the reaction mixture was slightly lower than that of the buffer. In some cases a free radical trap (DMPO) was included in the buffer at 10 mM or 20 mM concentrations. The tests were performed at room temperature ($25^{o}C$).

RESULTS AND DISCUSSION

Data from section 5.1 are summarised in Figures 5.1 to 5.7 and Tables 5.1 to 5.4. Catalase seems to initially reduce the rate of divicine autoxidation, but its effect is overcome with time (Figures 5.1 and 5.2). A similar trend was observed with SOD (Figure 5.4). Since the study with catalase indicated that changes in absorbance during the autoxidation of divicine were similar at 240 and 250 nm (Figures 5.1 and 5.2), data obtained at either wavelength is representative of both. Addition of H_2O_2 to the incubation medium did not affect the initial rate of autoxidation but inhibited the latter part of it (Figure 5.3 and Table 5.1). The effect of H_2O_2 was substantially corrected by catalase (Table 5.1). Borg *et al*. (1978) have suggested that there are two types of reducing compounds that generate a superoxide anion on their autoxidation; the strong reducing compounds and the weak to moderate reducing compounds. In case of the strong reducing

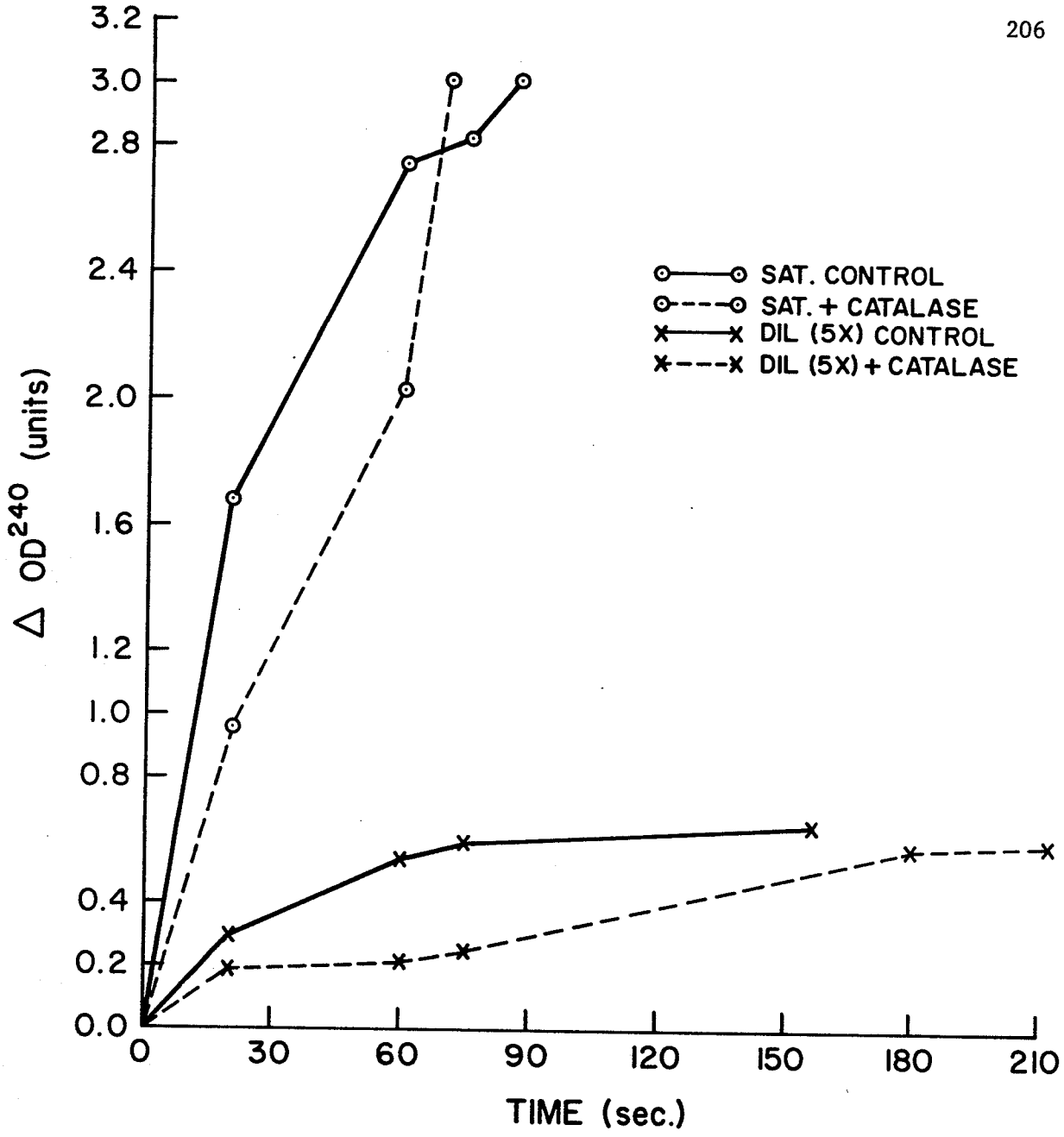


Fig. 5.I. Effect of catalase on the autoxidation of divicine at 240 nm. Catalase (0.88 mg/ml) was dissolved in 0.128M K_2HPO_4 (pH 8.0) buffer that contained 100 uM EDTA. The reaction was started by adding 0.1 ml of divicine in 0.1N HCl, to 2.9 ml of the buffer-catalase solution. Buffer without catalase was the control, and 0.1 ml of 0.1N HCl without divicine was used as a blank. Absorbance of the buffer alone and buffer with catalase were determined before addition of divicine. Absorbance changes were then recorded until maximum values were reached. All buffers and solutions without divicine had been previously equilibrated with atmospheric air.

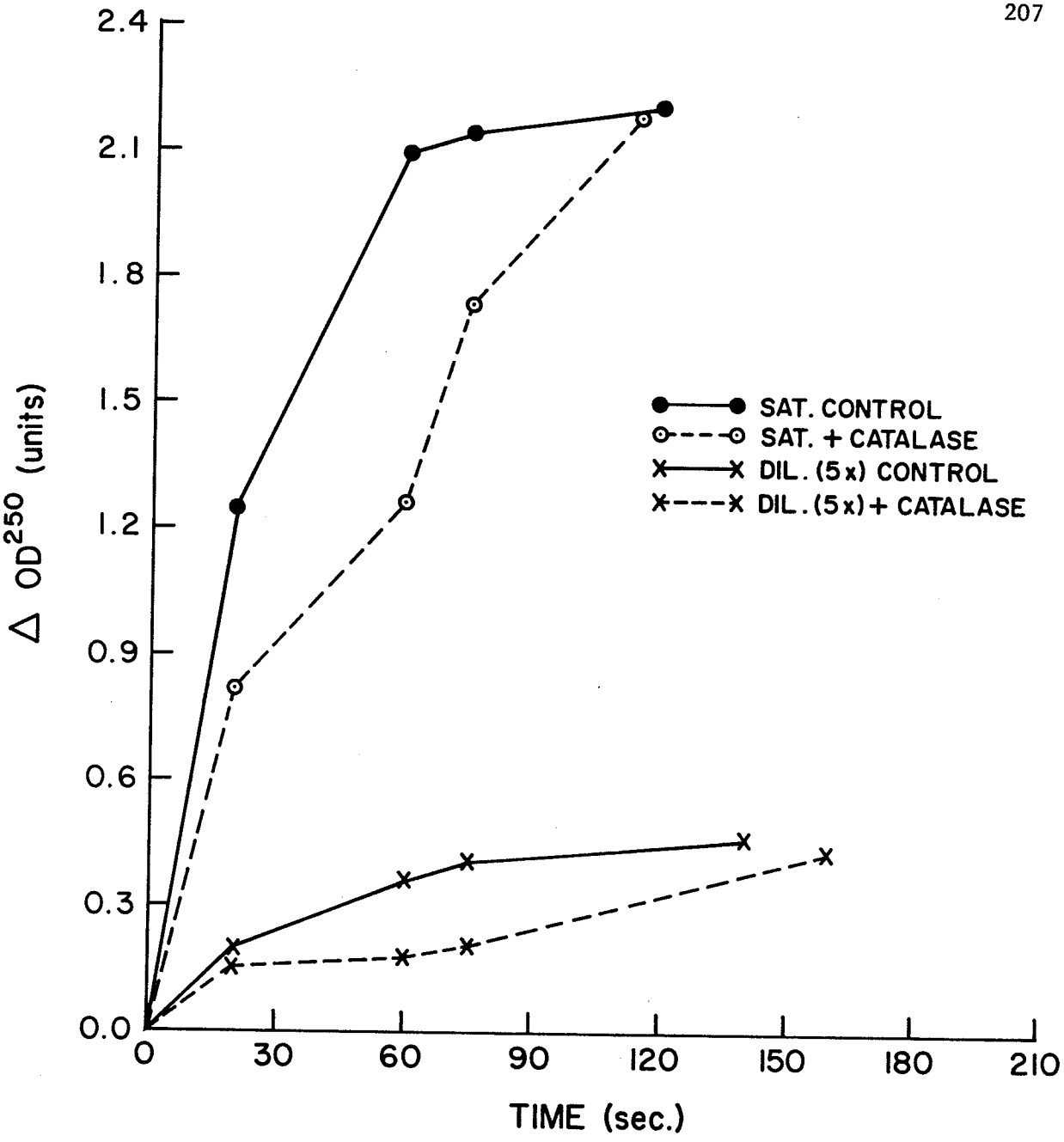


Fig. 5.2. Effect of catalase on the autoxidation of divicine at 250 nm. The experimental procedure was the same as in Figure 5.1.

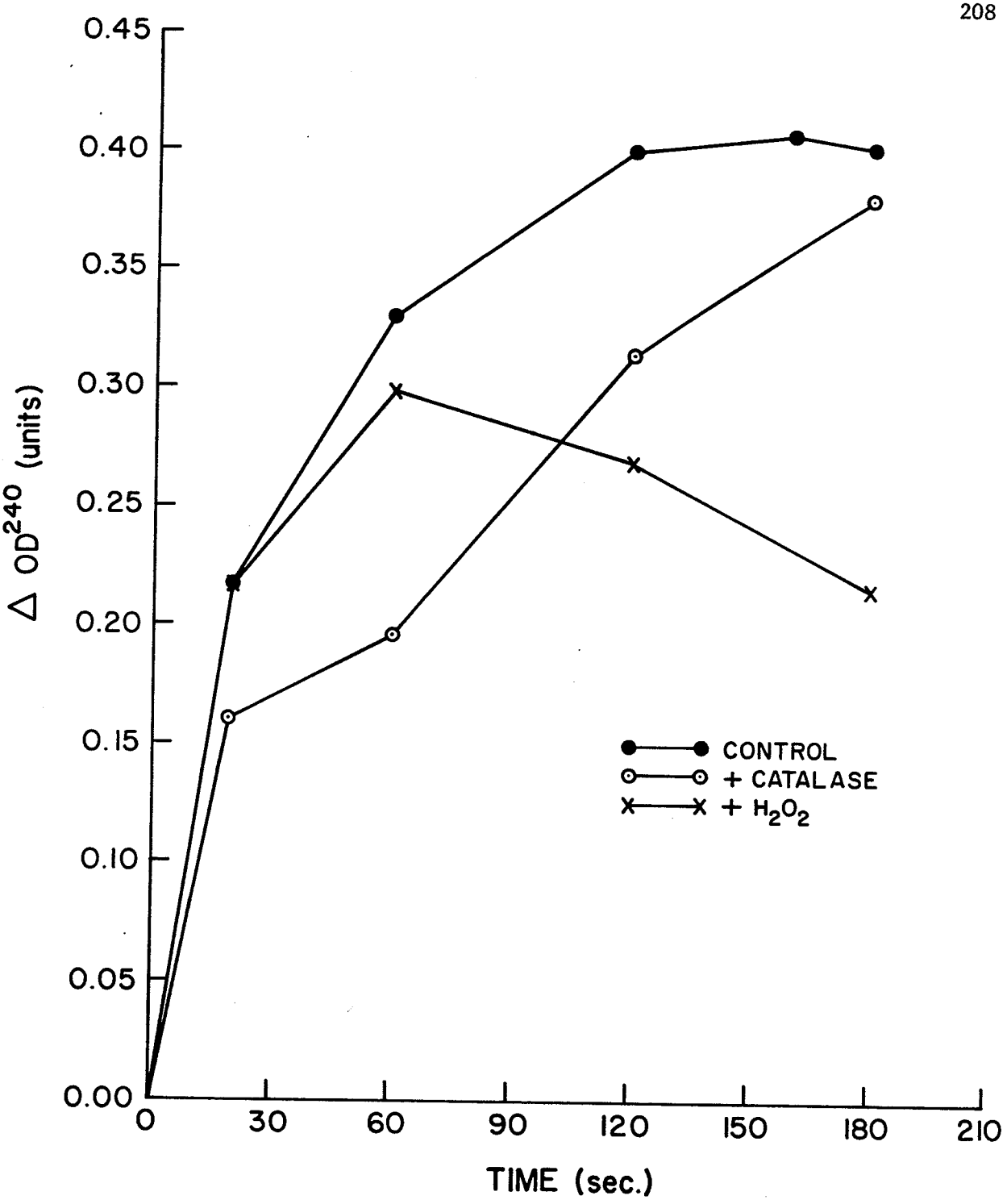


Fig. 5.3. Effect of exogenous H_2O_2 and catalase on the autoxidation of divicine at 240 nm. The procedure was generally the same as that described for Figure 5.1 However, 0.05 ml of divicine was added so as to give a concentration equivalent to DIL (5x) in Figure 5.1, this allowed the addition of an appropriate amount of H_2O_2 in 0.05 ml, therefore maintaining the volumes constant. When used catalase (0.88 mg/ml) was dissolved in buffer and 2.9 ml of that solution used. The combination of H_2O_2 and catalase produced considerable quantities of oxygen bubbles for the first 2 min., therefore sequential absorbances could not be determined, instead the maximum attained values are reported in Table 5.1.

Table 5.1. Effect of exogenous H_2O_2 and catalase on the autoxidation of divicine at 240 nm. The procedure is described in Figure 5.3.

Treatment	ΔOD^{240}	% of control
Control	0.401	100.0
+ H_2O_2	0.216	53.9
+ H_2O_2 + catalase	0.381	94.9

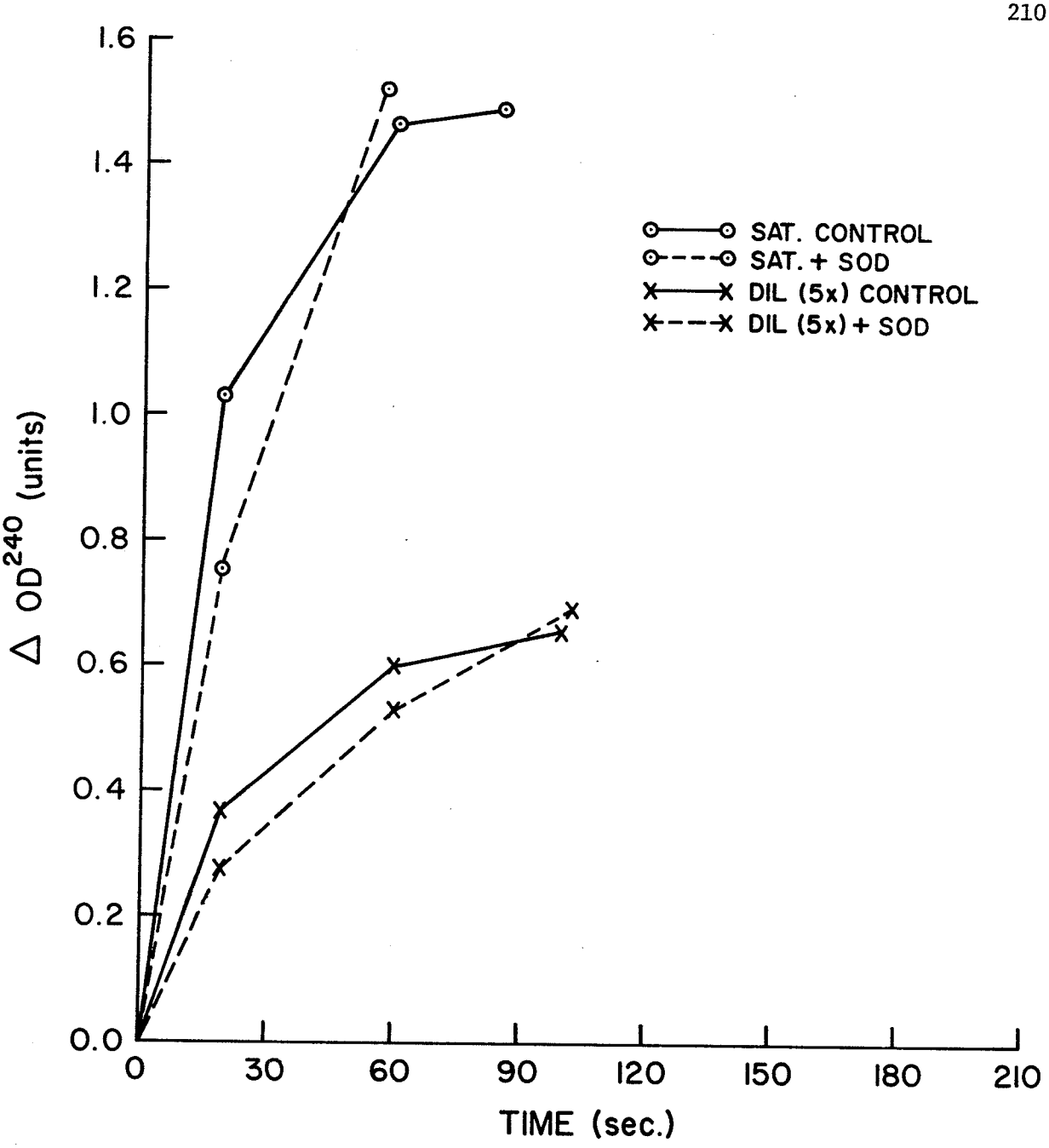
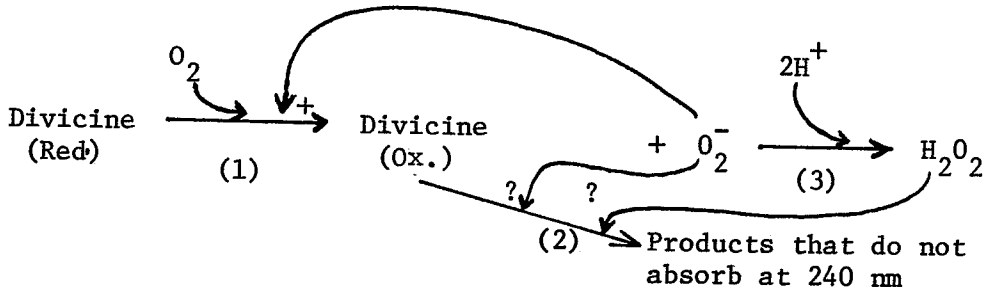


Fig. 5.4. Effect of SOD on the autoxidation of divicine at 240 nm. The experimental protocol is the same as that described in Figure 5.1, except that two levels of divicine were used and absorbance values were taken only until the maximum was reached. Fifty microliters of a solution of SOD in buffer (9.24 mg/ml) were added to 2.9 ml of the buffer and after determining the absorbance, 50 ul of divicine solution were added.

compounds the O_2^- produced will react further with the reduced form of the compound as shown below:

$QH_2 + O_2^- \rightarrow QH^\cdot + HO_2^-$; where QH_2 is the reduced form of the compound and $QH^\cdot$ is the partially oxidized form.

The HO_2^- reacts with H^+ to generate H_2O_2. The autoxidation of such strong reducing compounds will thus be inhibited by SOD and other competitive reactants for O_2^-. Therefore the effect of any compound that competes for O_2^- will depend on the extent to which O_2^- catalyses the autoxidation which is dictated by the relative redox potentials. Thus in the data reported so far it would seem that the initial inhibition by SOD and catalase could be explained by law of mass action by assuming that the autoxidation of reduced divicine follows the patter below:



In this scheme reaction 1 must be much faster than reaction 2.

Therefore catalase may inhibit the initial rate of autoxidation by removing H_2O_2 and hence O_2^- which supposedly catalyses the autoxidation of reduced divicine (reaction 1). The removal of H_2O_2 by catalase may also contribute to the stability of oxidised divicine (product of reaction 1) whose conversion to the non-absorbing products (reaction 2) may be catalysed by H_2O_2. However, there is speculation that catalase may react with O_2^- directly to form compound III as has been shown for

myeloperoxidase (Odajima and Yamazaki 1972). The inhibition of the initial rate of autoxidation of reduced divicine by SOD could then be explained on the basis that O_2^- enhances reaction 1, thus reducing the effective concentration of not only O_2^- but oxidized divicine. The enhancement of the final amount of oxidised divicine could be due to reduced breakdown of oxidized divicine (reaction 2) possibly by O_2^-. There is a possibility that the H_2O_2 produced by the SOD activity (reaction 3) could catalyse reaction 1 without affecting reaction 2, hence increasing the effective concentration of oxidized divicine that absorbs at 240 nm. Hence the initial inhibitory effect of catalase could also be due to removal of H_2O_2. However, data in Figure 5.3 and Table 5.1 seem to show that H_2O_2 may act as an end-product inhibitor or enhance further autoxidation and condensation of the oxidized divicine to the non-absorbing products (Bendich and Clements 1953). Its lack of effect on the initial rate of autoxidation (Figure 5.3) would indicate that it is not the first product of autoxidation and/or does not affect the first reaction in the sequence.

Figures 5.5 and 5.6 show that oxidized divicine strongly absorbs at 305 nm, but the absorption decreases with time (see control in both figures). This is similar to what happens at 240 and 250 nm, particularly in a sodium hydroxide solution without buffer. Both GSH and GSSG (Figures 5.5 and 5.6) hardly increased the initial absorbance at 305 nm; the effect of GSH was more perceptible. The effect of GSH was directly related to its concentration between 5 and 10 but not between 10 and 20 mM. The effects of GSH and GSSG on the latter part of the reaction were different. Addition of GSH (Figure 5.5) led to an increase in

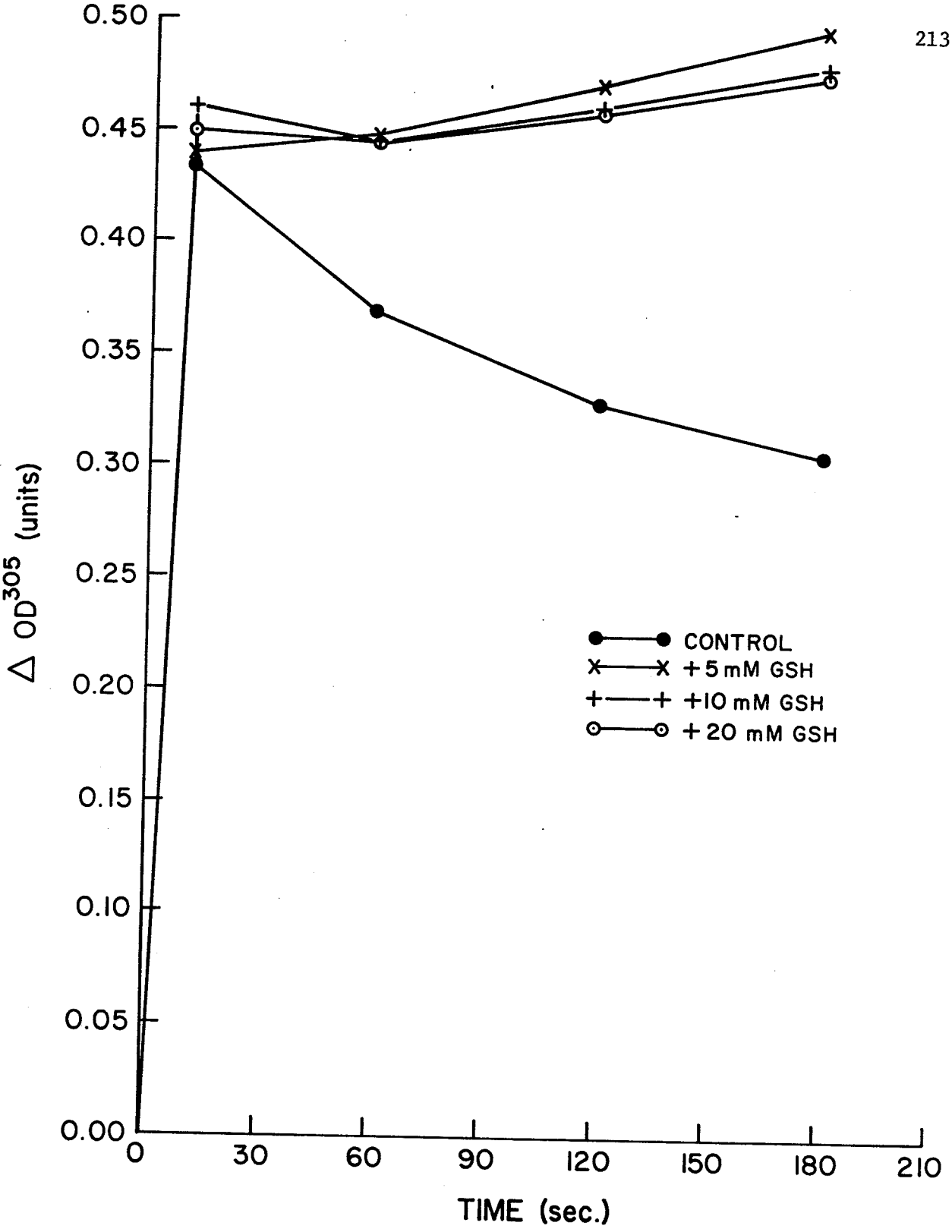


Fig. 5.5. Effect of GSH and its concentration on the autoxidation of divicine at 305 nm. The experimental protocol was similar to that of Figure 5.1. Fifty microliters of buffer and a solution of saturated divicine were added to 2.9 ml of buffer for the control and in the test preparation 50 ul of GSH solution replaced the buffer. A blank was run in each test. The pH of the buffer was adjusted to 7.5 as this was found optimum for the reaction between divicine and GSH at 305 nm (Lin 1963).

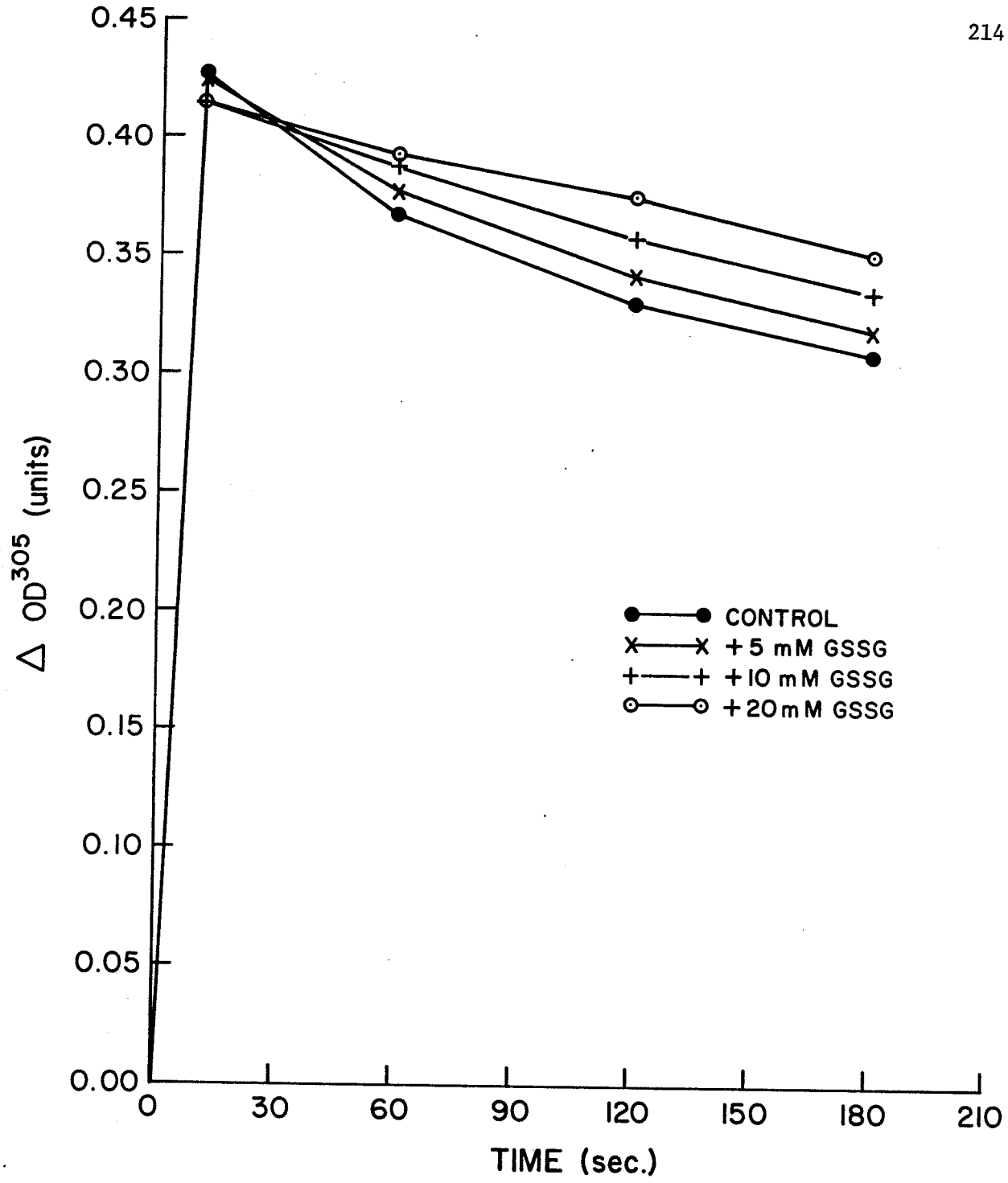


Fig. 5.6. Effect of GSSG and its concentration on the autoxidation of divicine at 305 nm. The experimental procedure was the same as in Figure 5.5.

absorbance above the initial value and was maintained above that of the control. This increase was negatively correlated with the GSH concentration. Though the initial absorbance changes tend to agree with those of Lin (1963), the latter observation is contradictory. This could be due to differences in times for which the experiments were run. Lin (1963) observed maximum changes in absorbance after about an hour while in studies in this chapter the reaction was monitored for only 3 minutes. In addition, the ratios of GSH to divicine may have been different. The quality and quantity of divicine prepared by the acid hydrolysis method are rather difficult to predict and therefore absolute ratios of GSH to divicine are impossible to accurately reproduce. This effect can be attributed to the highly unstable nature of divicine. Contrary to GSH, addition of GSSG (Figure 5.6) resulted in decreased absorbance with time similar to the control. The actual absorbance values, however, were higher than those of the control and they increased with increases in the GSSG levels.

It would seem from these results that GSH either reacts with oxidized divicine to form a new product, or by its reducing power, GSH protects oxidized divicine from further oxidation and condensation to form a non-UV absorbing material (Bendich and Clements 1953). The strong S-S bond in GSSG may prevent the formation of a condensation product with divicine, and since GSSG has no reducing power, the slight increase in absorbance in its presence over the control could be due to contamination with GSH or a limited condensation reaction with divicine.

The reactions between divicine and Hb or MetHb and the effects of bubbled oxygen are summarised in Tables 5.2 to 5.4 and Figure 5.7.

Additions of both Hb and MetHb (Tables 5.2 and 5.3) tended to increase the absorbance at 240 nm, thus indicating increased autoxidation of reduced divicine or a decreased rate of disappearance of oxidized divicine. In addition both tended to increase the absorbance at 577 nm, but not at 630 nm. Assuming that the absorbance at 577 and 630 nm were due to ferrous and ferric hemoglobin (Winterbourn et al. 1979) respectively, it would seem that reduced divicine and/or its products reduce MetHb to Hb and not vice versa. Therefore the decrease in absorbance at 630 nm observed with Hb would indicate a high contamination with methemoglobin.

However, Hb causes a greater increase than MetHb in the absorbance at 240 nm indicating that the haem group was not the major group reacting with reduced divicine or its products to cause an increase in the production of oxidized divicine. Hemoglobin, however does not seem to condense with divicine because the increases in absorbance at 240 nm would indicate free oxidised divicine. A complex of hemoglobin and divicine would more likely assume the optical characteristics of the large molecule hemoglobin. Therefore it seems that there is a transfer of electrons from reduced divicine to Hb or MetHb or the latter just catalyse divicine autoxidation. Winterbourn et al. (1979) reported that the reduction of MetHb to oxyHb by menadione, which involved O_2^- was inhibited by blocking the β-93 thiol group. Hence the denaturation of this group during preparation of MetHb may account for the differences observed between Hb and MetHb in these studies.

The influence of bubbling oxygen through the solutions on the

Table 5.2. Reaction between divicine and hemoglobin at 240, 577 and 630 nm. The experimental protocol is similar to that for Figure 5.1; a concentrated stock solution of Hb in buffer was diluted to the various concentrations and absorbance was monitored for 3 min. The ΔOD are derived from the 3 min. values.

Hb concentration (μm)	ΔOD		
	240 nm	577 nm	630 nm
0	0.389	0.000	0.000
3.3	0.431	0.0025	0.001
6.6	0.439	0.005	-0.001
13.2	0.462	0.012	-0.002

Table 5.3. Reaction between divicine and methemoglobin at 240, 577 and 630 nm. The experimental procedure is described in Figure 5.1; except that absorbance was monitored for 3 min.

MetHb concentration (μm)	ΔOD		
	240 nm	577 nm	630 nm
0	0.389	0.000	0.000
1.65	0.385	0.002	0.001
3.3	0.395	0.002	0.000
6.6	0.408	0.005	-0.001

Table 5.4. Effect of divicine on hemoglobin and methemoglobin and the influence of bubbled oxygen at 577 and 630 nm. The experimental protocol is described in Figure 5.1, except that absorbances were monitored for 11 min. due to the slow rate of change.

Treatment	ΔOD	
	577 nm	630 nm
Control	0.000	0.001
Hb	0.016	-0.005
Hb + O_2	NA[a]	NA
MetHb	0.009	-0.001
MetHb + O_2	0.012	-0.004

[a]NA = Not applicable, there was too much sedimentation in oxygen for absorbance to be meaningful.

reactions between divicine and Hb and MetHb are shown in Figure 5.7 and Table 5.4. The controls and other samples not bubbled with oxygen were equilibrated with atmospheric air. Figure 5.7 shows that the initial rate of autoxidation of divicine was not appreciably affected by any treatment. With time, MetHb like Hb enhanced the autoxidation of divicine. In both cases Hb was more effective. Oxygen caused a slight increase in autoxidation in the presence of both Hb and MetHb. Since 0_2 is required for the autoxidation of divicine these observations are logical. However, the same study should have been carried out with the control so as to allow a more definitive conclusion.

The changes at 577 and 630 nm (Table 5.4) were similar to those observed in Table 5.2 and 5.3. It is intriguing that oxygen increased the reduction of MetHb, which would indicate that oxygen was the electron donor, and therefore 0_2^- was the actual compound reducing the MetHb. Since oxyHb is actually a ferri-superoxy compound (Fridovich 1978), then reduction of MetHb by 0_2^- would be a reversal of the process described in Figure 1.6.

It may be concluded that 0_2^- and H_20_2 are the probable initial products of divicine autoxidation and high concentrations of H_20_2 enhance the conversion of oxidized divicine to products that do not absorb at 240 nm. Reduced glutathione either forms a stable condensation product with oxidised divicine or protects it from further degradation, while GSSG has neither of those properties. There is a slow reaction between reduced divicine or its product(s) with Hb or MetHb, leading to reduction of MetHb. If 0_2^- is the reducing agent, then this data would agree with that of Sutton et al. (1976) who observed that the

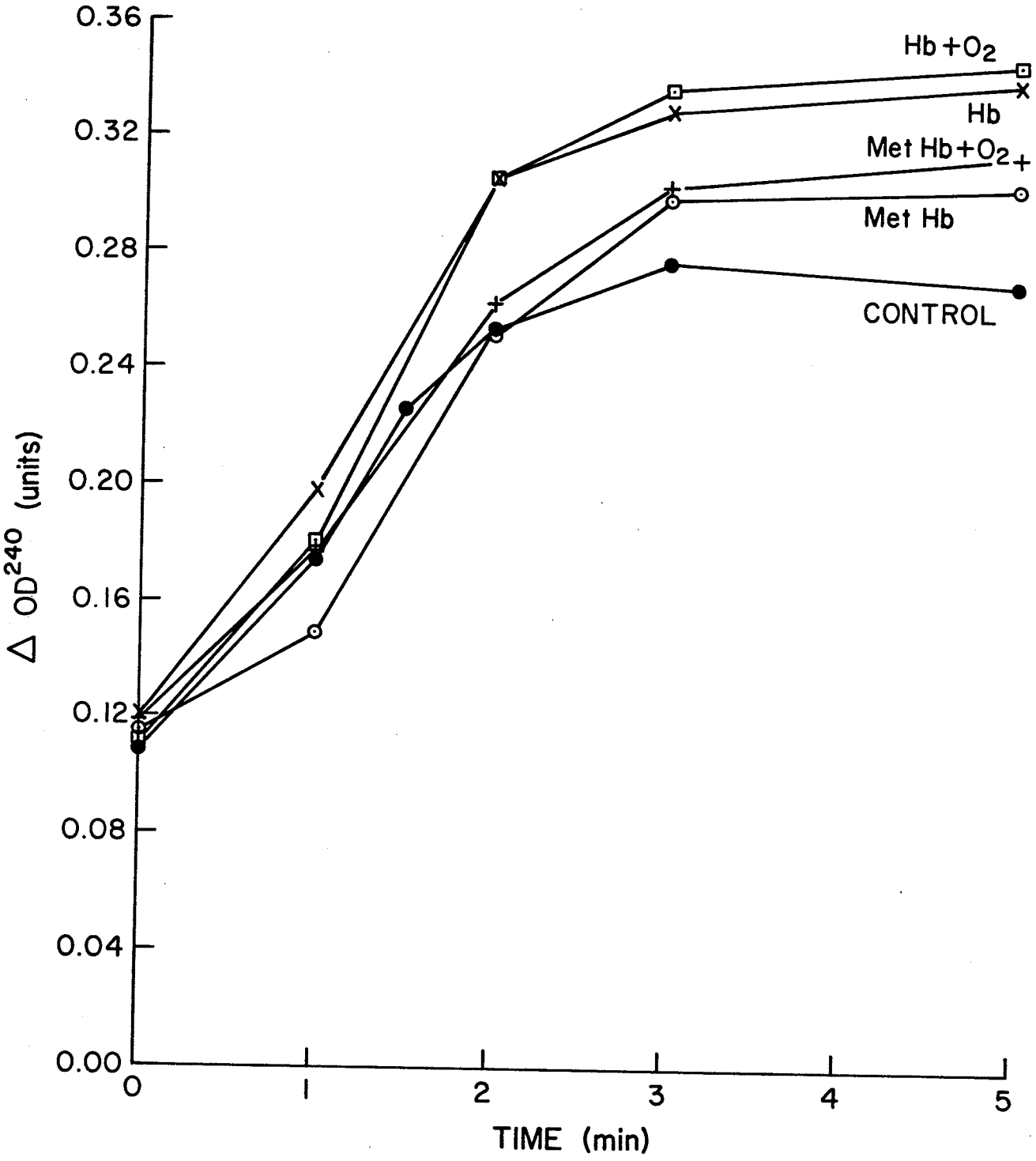


Fig. 5.7. Effect of hemoglobin and methemoglobin, and bubbled oxygen on the autoxidation of divicine at 240 nm. Experimental procedure is similar to that of Figure 5.1. The DIL (5x) solution and 6.6 uM solutions of Hb and MetHb were used and oxygen was bubbled through the solutions for 3 min. at a set pressure.

reaction between O_2^- and MetHb could not compete favourably with the spontaneous dismutation of O_2^-. However, the reaction between divicine and Hb does not seem to involve the haem group, and finally the reduction of MetHb seems to involve the superoxide anion.

Data for section 5.2 are summarised in Tables 5.5 to 5.18 and Figures 5.8 to 5.14. In agreement with the spectral stability data in Chapter 2, vicine did not reduce cytochrome C (Table 5.5) while divicine did (Table 5.6). However, at the levels employed in Table 5.6 the increase in absorbance due to cyt. C reduction was affected more by the concentration of divicine than that of cytochrome C. This might have been due to the fact that the cytochrome C concentration was not adequate even with the lower concentration of divicine. This was confirmed in the data summarised in Figure 5.8. In fact, Figure 5.8 indicates that the concentration of divicine was less important than that of cytochrome C in determining the degree of cytochrome C reduction. It also shows that a level of cyt C between 0.04 and 0.08 mM was optimum for reduction by both levels of divicine. Below and above these levels there was less change in absorbance or even negative values. The decrease in cyt C reduction with increases in its concentration was greater with the SAT divicine than DIL (5x) divicine which did not register negative absorbances at the concentrations of cyt C employed. The maximum amount of cyt C reduced by either level of divicine was not very different (Figure 5.8). This lack of difference between levels of divicine in the ability to reduce cyt C at its optimum concentration may reflect a cyclic nature of the reaction where the oxidised divicine accepts electrons from the buffer and further reduces the cyt C and

Table 5.5. Reaction between vicine and cytochrome C at 550 nm (OD units). The procedure was similar to that described in Figure 5.1. The absorbance of the blank did not change therefore only single values are given at each cytc. concentration. Two minutes were chosen because preliminary tests with divicine indicated that maximum changes in absorbance were achieved then, and that they decreased thereafter.

	DIL (5x)		SAT.	
Treatment	0.01 mM cyt. C	0.02 mM cyt. C	0.01 mM cyt. C	0.02 mM cyt. C
Blank	0.093	0.181	0.090	0.177
Vicine at 11 secs.	0.093	0.184	0.092	0.180
2 min.	0.093	0.180	0.091	0.178

Table 5.6. Reaction between divicine and cytochrome C at 550 nm. The procedure is similar to that of Table 5.5 and Figure 5.1.

	DIL (5x)		SAT.	
Treatment	0.01 mM cyt. C	0.02 mM cyt. C	0.01 mM cyt. C	0.02 mM cyt C
Blank	0.084	0.182	0.084	0.176
Divicine at 11 secs.	0.095	0.193	0.128	0.241
2 min.	0.122	0.226	0.138	0.268
Divicine at 2 min. - Blank	0.038	0.044	0.054	0.092

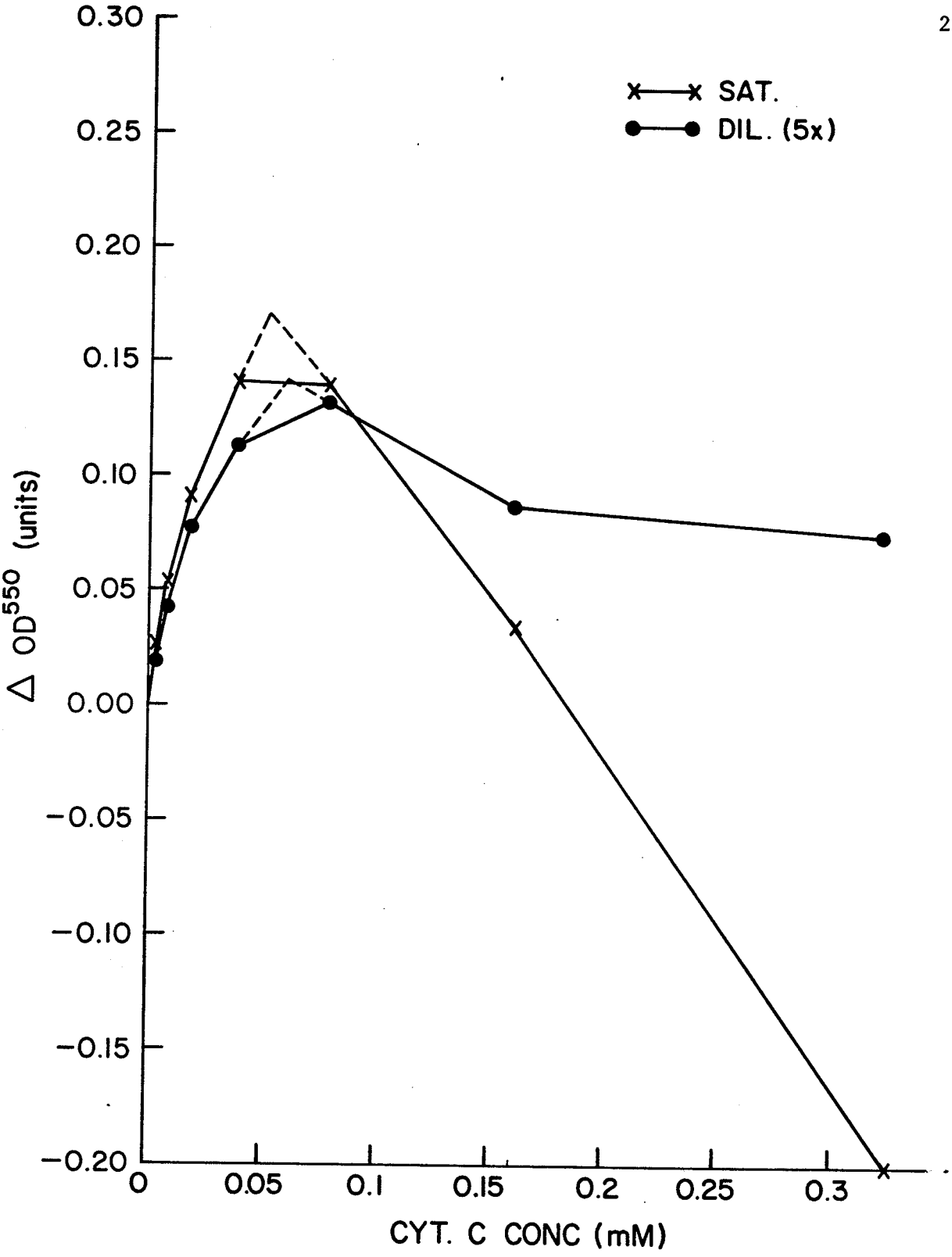


Fig. 5.8. Effect of cytochrome C concentration on its reduction by divicine. The procedure was similar to that of Table 5.5 and Figure 5.1 except that different concentrations of cyt C were used.

hence the lack of stoichiometry between the reactants. This lack of stoichiometry was observed between divicine and GSH (Lin 1963). The negative reduction of cyt C with increases in its concentration (Figure 5.8) would indicate that cyt C was initially partially reduced and that a product of divicine autoxidation oxidised cyt C. The product could be H_2O_2 from O_2^- dismutation or the oxidised divicine itself. A lower divicine concentration would not generate enough of the oxidising agent to cause negative reduction of cyt C. These results would suggest that the interpretation of data from reactions involving cyt C and divicine may be difficult and misleading depending on the relative levels of the reactants. With 0.04 mM cyt C it was demonstrated that the relationship between cyt C reduction and divicine concentration was hyperbolic (Figure 5.9) indicating that at a fixed level of cyt C the initial reaction appears to follow first order kinetics.

The question whether O_2^- was involved in the reduction of cyt C was investigated using SOD, catalase, H_2O_2 and other proteins. Table 5.7 shows that 154 µg/ml SOD had little effect on the reduction of cyt C, and that its effect increased with increases in cyt C concentration. However, the inhibition by SOD even at a level of 616 µg/ml was only 37 and 26% at one and three minutes, respectively (Figure 5.10). A preliminary study (Table 5.8) indicated that catalase also partially inhibited the reaction and that the effect was dependent on the relative concentrations of cyt C and catalase.

The effect of catalase on cyt C reduction by divicine was re-evaluated using levels of catalase comparable to those of SOD (Figure 5.10) when their molecular weights and active catalytic sites were

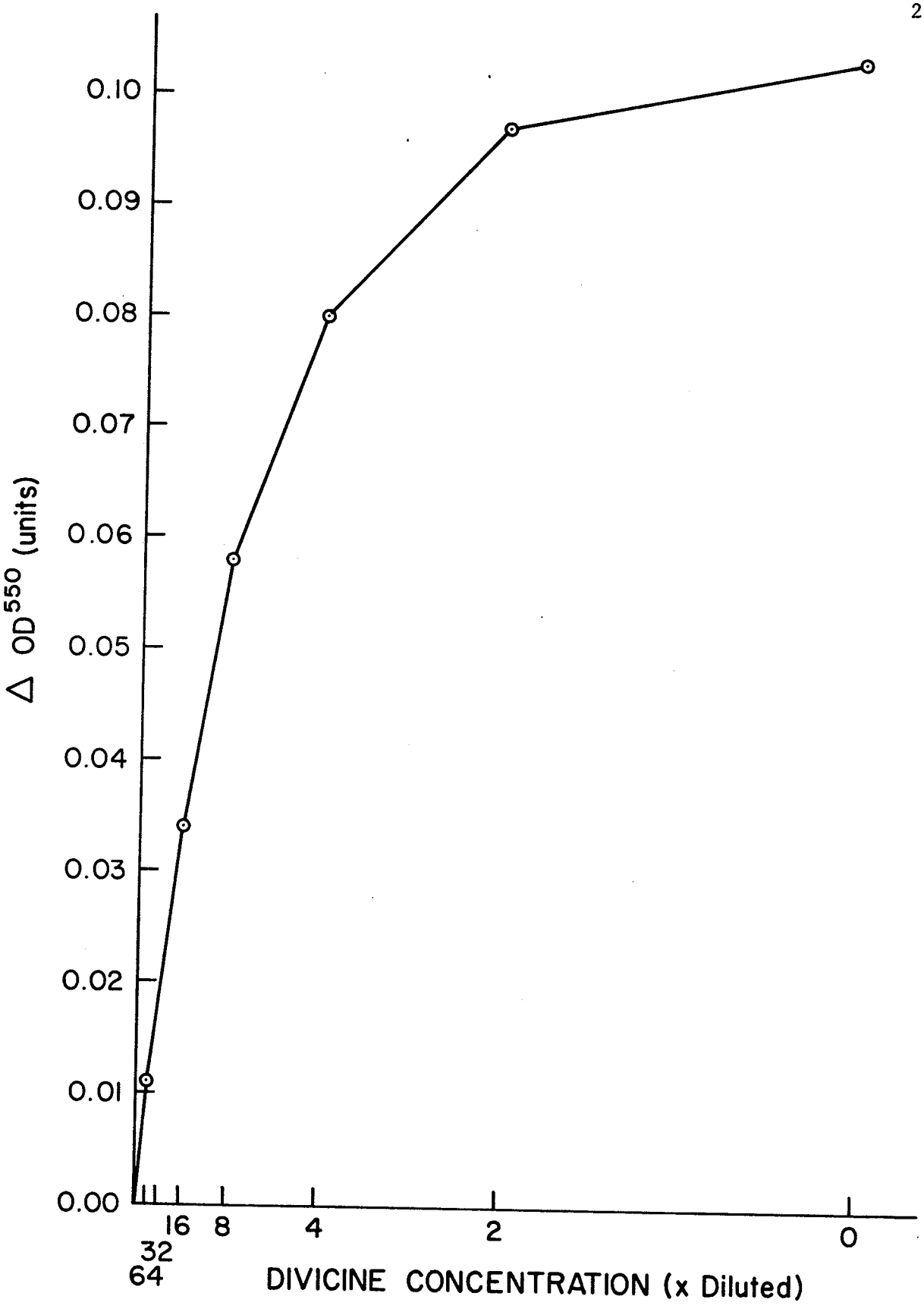


Fig. 5.9. Effect of divicine concentration on its reduction of cytochrome C. From Figure 5.8, a level of 0.04 mM cyt C was chosen and tested against various levels of divicine.

Table 5.7. Effect of SOD on the reduction of different concentrations of cytochrome C by divicine. Various concentrations of cytochrome C were prepared by diluting a stock solution in phosphate buffer. For the blank, 50 µl each of buffer and 0.1N HCl were successively added to 2.9 ml of cyt. C solution, while in the control the HCl contained divicine (SAT.) and in the test sample the buffer contained SOD prepared to give a concentration of 154 µg/ml in the reaction mixture. Absorbance was monitored at 550 nM for 3 min.

Cyt. C conc. (mM)	ΔOD^{550} (units)		% inhibition
	- SOD	+ SOD	
0.04	0.097	0.083	14.4
0.02	0.069	0.061	11.6
0.01	0.044	0.041	6.8

Table 5.8. Effect of catalase, cytochrome C and divicine concentration on the reduction of cytochrome C by divicine. The experimental procedure is same as described in Table 5.7. Values for the control are actual OD units, while those in presence of catalase are expressed as a percentage of the control.

Treatment	DIL (5x)		SAT.	
	0.01 mM cyt. C	0.02 mM cyt. C	0.01 mM cyt. C	0.02 mM cyt. C
Control	0.053	0.052	0.065	0.062
+ catalase 3.52 mg/ml	73.6	51.9	72.3	41.3
1.76 mg/ml	51.9	44.2	53.1	35.5
0.88 mg/ml	42.4	34.0	33.4	33.9

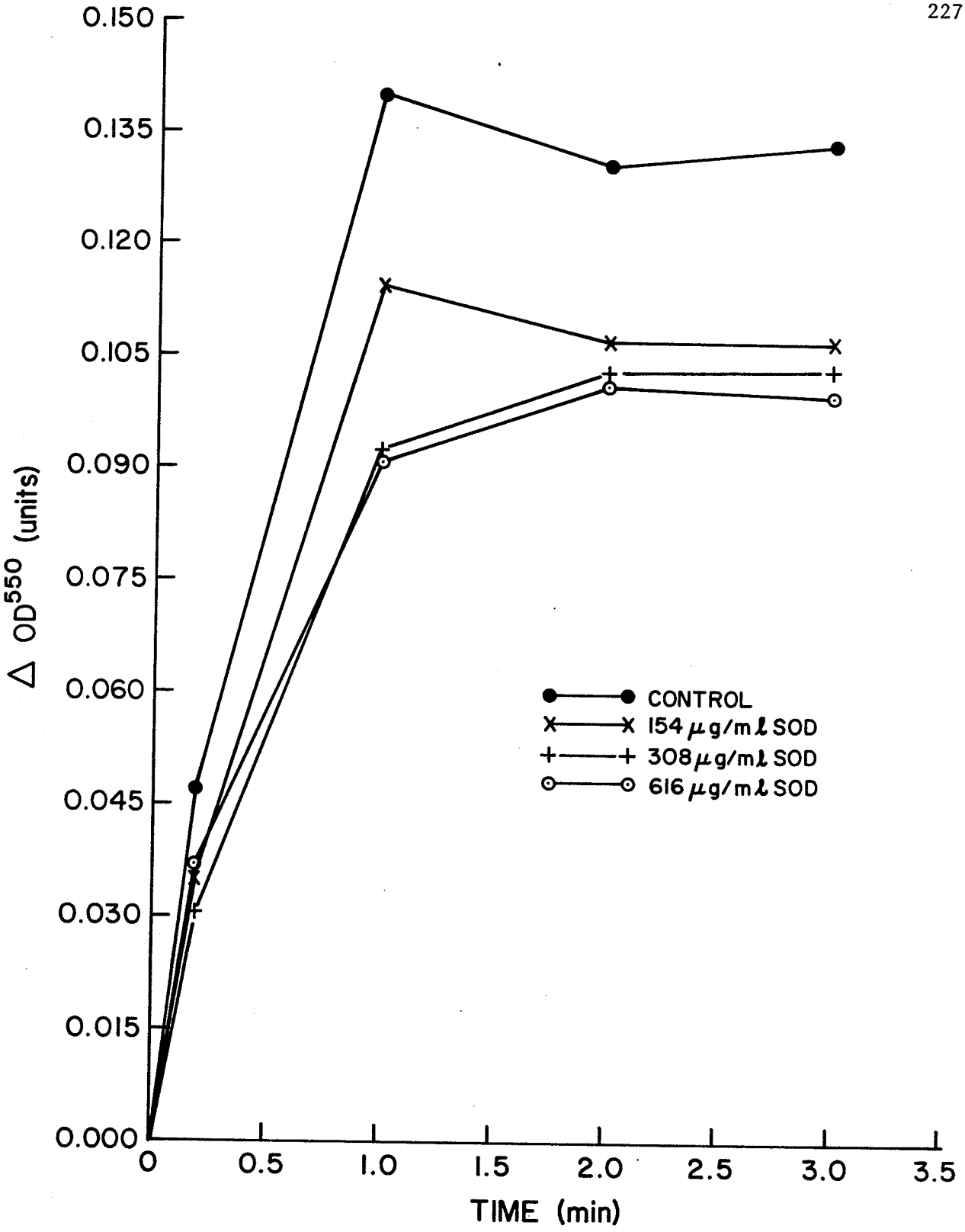


Fig. 5.10. Effect of SOD and SOD concentration on the reduction of cytochrome C by divicine. The experimental procedure was similar to that of Table 5.7. The control was checked periodically so as to insure that divicine had not decomposed.

considered (Figure 5.11). Although at the low levels the effect of catalase was similar to that of SOD, at the highest levels, catalase inhibited cyt C reduction more than SOD. The greater effect of catalase compared to SOD may be explained by its dual mode of action compared to the single mode of SOD. Catalase reduces H_2O_2 to water, and it may also react directly with O_2^- (Odajima and Yamazaki 1972) which as previously pointed out dismutates to $H_2O_2 + O_2$. Therefore removal of H_2O_2 decreases the effective concentration of O_2^- which results in a decrease in the reduction of cyt C. The conclusion from these studies was that the O_2^--dependent reduction of cyt C was a minor part of the reaction. However, it was found later that the inhibition of the reduction by catalase depended on the concentration of cyt C (Figure 5.12). Therefore the experiments reported here were performed at levels of low sensitivity to catalase inhibition, and the same could be true for SOD. However data in Figure 5.12 is contrary to that in Tables 5.7 and 5.8 and could be explained by the fact that the levels of cyt C employed in Tables 5.7 and 5.8 were inadequate for the divicine levels. Therefore stressing the magnitude of the error that results from the choice of the levels of the reactants. The results nevertheless demonstrate that catalase decreases the reduction of cyt C by divicine.

The effect of GSH on the reduction of cyt C by divicine was investigated at 240 (Table 5.9), 305 (Table 5.10) and 550 (Table 5.11) nm, and data in these tables are further summarised in Table 5.12. Reduced glutathione decreased the absorbance at 240 nm (Table 5.9). If this absorbance is assumed to be due to oxidised divicine, then GSH reduced its levels. This decrease in absorbance at 240 nm was associated with

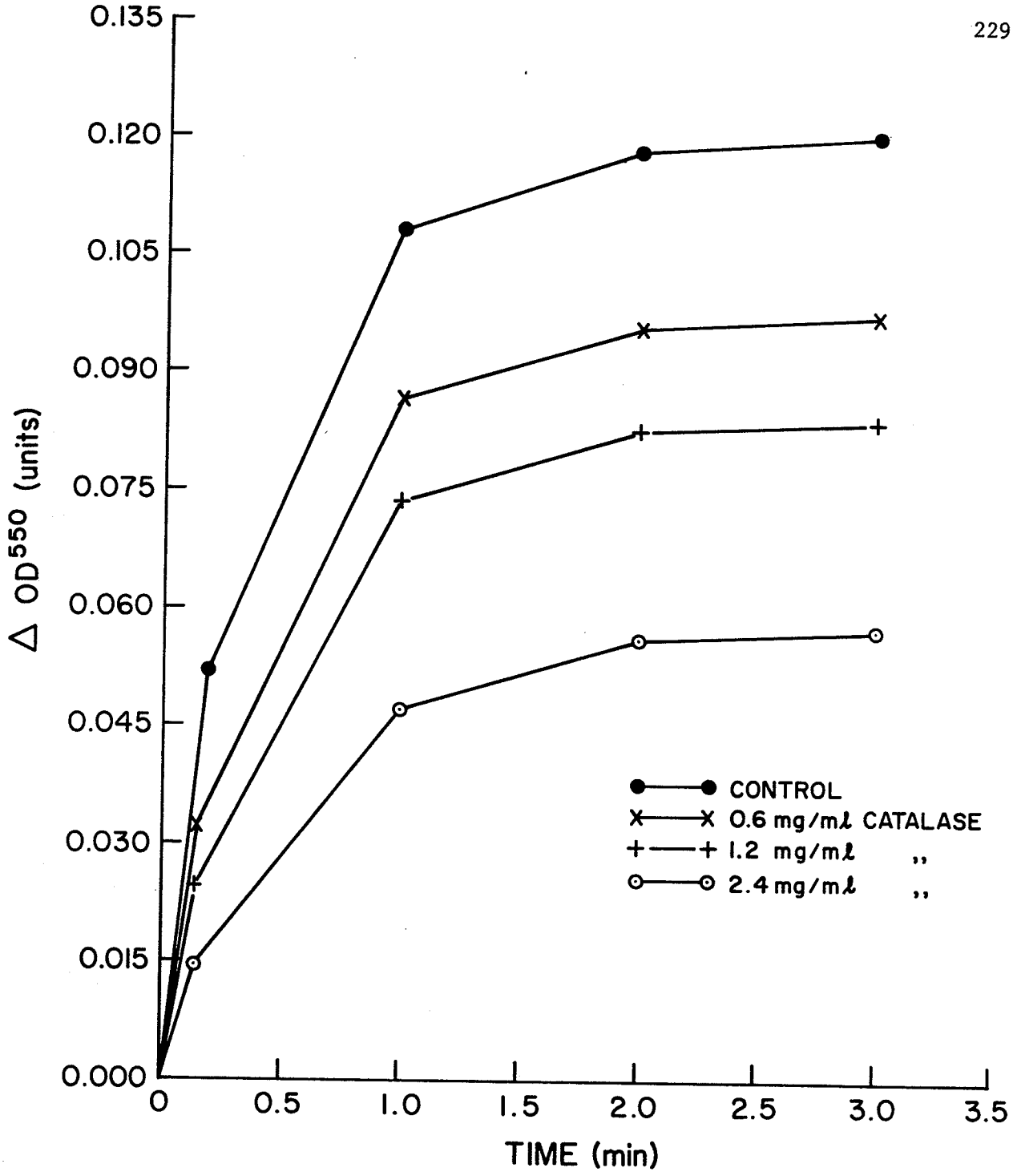


Fig. 5.11. Effect of catalase concentration on the reduction of cytochrome C by divicine. The experimental protocol is similar to that of Table 5.7 and Figure 5.10. The levels of catalase in this experiment are comparable to those of SOD used in Figure 5.10.

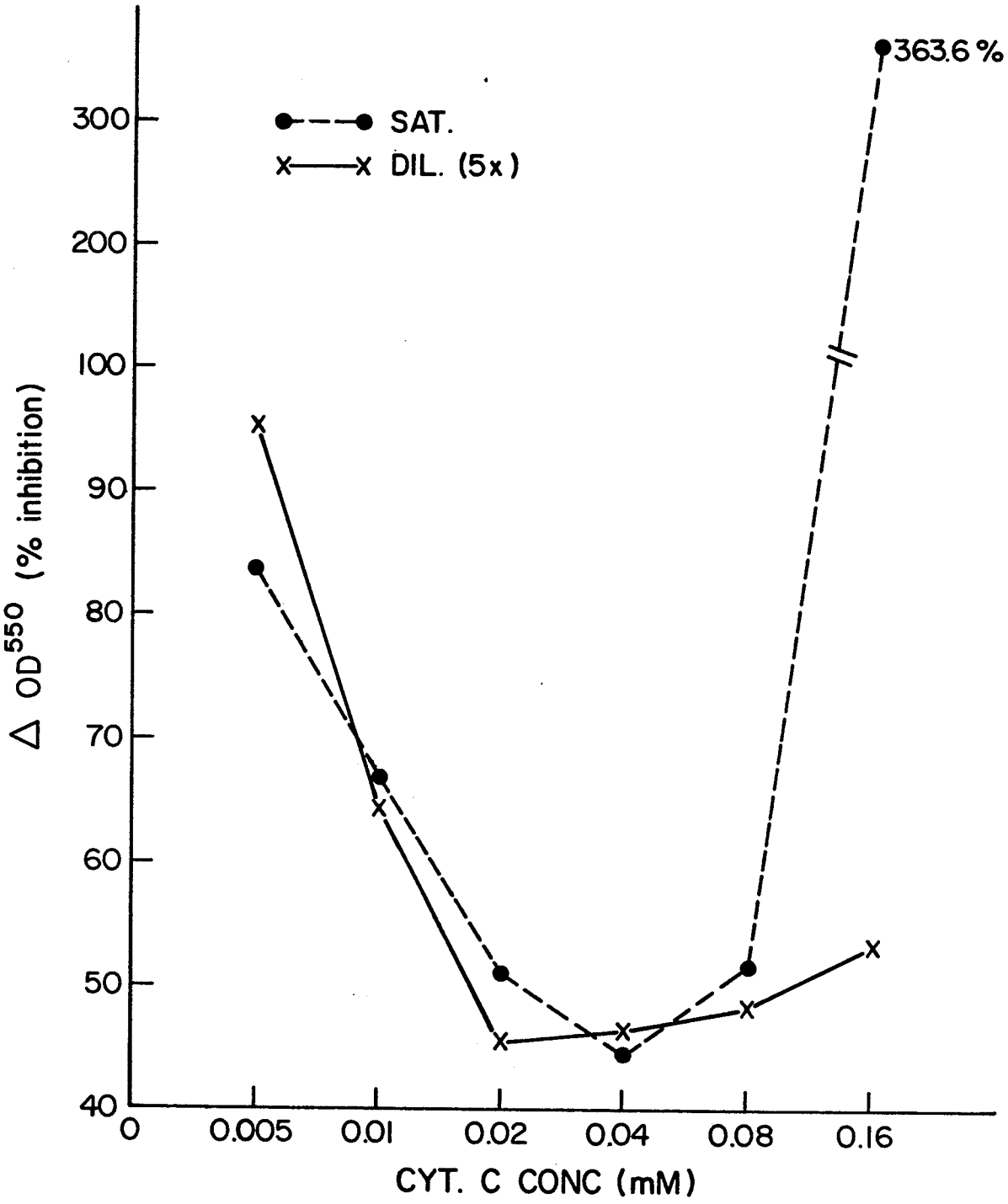


Fig. 5.12. Effect of cytochrome C concentration on the catalase effect on the reduction of cytochrome C by divicine. The experimental protocol was as described for Table 5.7 and Figure 5.10. A level of 1.76 mg/ml catalase was chosen from results in Table 5.9.

Table 5.9. Effect of GSH on the reduction of cytochrome C by divicine at 240 nM. Top values are for the control and the bottom ones are for the test sample containing GSH. The experimental protocol was similar to that of Figure 5.1 and 5.3; for the test sample 50 µl of 0.3M GSH in buffer and divicine (DIL (2x)) in 0.1N HCl were added concurrently to 2.9 ml of cyt. C in buffer. This was equivalent to adding 0.1 ml of 5 mM GSH or DIL (5x) divicine, respectively. For the control, buffer was added without GSH and for the blank buffer and HCl were added instead of GSH and divicine, respectively. This procedure was employed in Tables 5.11 and 5.12.

	ΔOD^{240} (units)			
	Time (min.)			
Cyt. C conc. (mM)	0.3	1	2	3
0.01	0.280	0.441	0.555	0.581
	0.242	0.222	0.181	0.184
0.02	0.265	0.413	0.509	0.522
	0.255	0.206	0.134	0.111
0.04	0.302	0.401	0.438	0.485
	0.233	0.074	0.054	0.025

Table 5.10. Effect of GSH on the reduction of cytochrome C by divicine at 305 nM. See Table 5.9 for the procedure.

	ΔOD^{305} (units)			
	Time (min.)			
Cyt. C conc. (nM)	0.3	1	2	3
0.01	0.116	0.155	0.169	0.159
	0.112	0.158	0.185	0.188
0.02	0.139	0.233	0.277	0.270
	0.128	0.231	0.277	0.277
0.04	0.223	0.373	0.417	0.423
	0.161	0.352	0.440	0.440

slight increases and decreases in absorbance at 305 (Table 5.10) and 550 nm (Table 5.11), respectively. Thus indicating a new product that absorbs at 305 nm which partially inhibits cyt C reduction. This data complements that of Lin (1963) and in addition it appears that GSH reacts with the reduced divicine and hence prevents it from donating electrons to cytochrome C.

A number of hemolytic compounds have been shown to produce O_2^- when they react with Hb or MetHb (Goldberg and Stern 1975; Jain and Hochstein 1979; Winterbourn et al. 1979). Therefore it was thought that the susceptibility of cyt C reduction to SOD or catalase would be altered by the presence of Hb or MetHb (Table 5.13). It appears from Table 5.13 that both Hb and MetHb increased the reduction of cyt. C. These results would suggest that instead of competing for electrons with cyt C, Hb and MetHb promote the autoxidation of divicine whose products then reduced cyt C. This could be achieved by the iron in their haem group since heavy metals are known to catalyse the autoxidation of divicine (Bendich and Clements 1953; Mager et al. 1969). Therefore even the increase in absorbance at 240 nm (Tables 5.2 and 5.3, and Figure 5.7) in their presence might be mainly explained on catalytic rather than on the basis of mass action. In addition the autodismutation of O_2^- is fast (Fridovich 1978) and it is possible that cyt C does not compete favourably for electrons from the superoxide anion. Thus the method might be a very inefficient one for detecting O_2^-. Also, the presence of Hb or MetHb did not increase the proportion of cyt C reduction susceptible to SOD indicating that the increased autoxidation of divicine did not

Table 5.11. Effect of GSH on the reduction of cytochrome C by divicine at 550 nM. See Table 5.9 for the procedure.

Cyt. C conc. (nM)	ΔOD^{550} (units) Time (min.)			
	0.3	1	2	3
0.01	0.016	0.034	0.042	0.044
	0.009	0.027	0.037	0.037
0.02	0.030	0.057	0.066	0.068
	0.020	0.050	0.060	0.058
0.04	0.046	0.085	0.096	0.097
	0.035	0.078	0.078	0.065

Table 5.12. Summary of data in Tables 5.9 to 5.11. [a]Absorbance at 3 minutes was used. These values generally represent the trend at all time periods. Where they did not, the maximum values of each group were employed.

Cyt. C conc. (nM)	ΔOD (GSH-containing samples as % of control)[a]		
	240 nM	305 nM	550 nM
0.01	5.2	104.0	80.4
0.02	21.5	102.6	88.2
0.04	31.7	111.2	84.1

Table 5.13. The effect of SOD and catalase on the effect of Hb and MetHb on the reduction of cytochrome C by divicine at 550 nM. The experimental protocol was similar to that in Figure 5.1 and 5.3, except that Hb or MetHb and cyt C were added to buffer at the desired concentrations, and 2.9 ml of the solution used in the assay. Fifty microliters of enzyme and divicine were then added in that order.

	ΔOD^{550} (Test sample as % of control)			
Treatment	- SOD	+ SOD	- catalase	+ catalase
Control	100	81.8	100	95.5
+ Hb	116.7	106.3	117.1	107.6
+ MetHb	116.4	97.5	117.3	103.0

Table 5.14. Effect of divicine on the activity of SOD and catalase. SOD (154 µg/ml), catalase (0.6 mg/ml) and divicine (DIL (5x)) were used in the assay of Beauchamp and Fridovich (1971). The assay for the control contained 2.9 ml of NBT cocktail, 50 µl of buffer, and 50 µl 0.1N HCl. In the assay that contained divicine, 50 µl of divicine replaced that of 0.1N HCl in the control, while in samples for SOD or catalase alone, 50 µl of buffer containing the enzyme replaced the buffer. When divicine and SOD or catalase were combined, they replaced HCl and buffer, respectively in the control, and were added concurrently, but separately.

	ΔOD^{550} (Test sample as % of control)	
Treatment	- Divicine	+ Divicine
Control	100	135.2
+ SOD	10.8	10.0
+ catalase	32.1	48.5

involve production of O_2^- but a transfer of electrons by other means. However, the proportion of the reaction susceptible to catalase was increased. The reason for this is not clear, but may depend on the relative redox potentials of these compounds, or may indicate that more H_2O_2 was generated in presence of Hb or MetHb. Finally it was found that divicine did not inhibit the activity of SOD or catalase in vitro (Table 5.14) and therefore was not the reason for the low inhibition of cyt C reduction by these enzymes.

It was thus concluded from the section on cyt C that divicine reduces cyt C, but a minor part of the reduction was due to O_2^-. However, this seems to depend on the relative proportions of divicine and cyt C. Divicine complexes with GSH but not GSSG, forming a complex that absorbs at 305 nm, and divicine reacts slowly with MetHb but no evidence of that with hemoglobin. These latter two compounds also seem to promote the reduction of cyt C by divicine. Divicine did not inhibit the activity of SOD or catalase in vitro.

Since data with cyt C showed very little effect of SOD and catalase on its reduction, it was decided to investigate the presence of O_2^- by the NBT reduction method (Tables 5.15 to 5.18 and Figures 5.13 and 5.14). Similar to cyt C, NBT was reduced by divicine in both phosphate (pH 8.0) and carbonate (pH 10.2) buffers, therefore the effects of SOD and catalase at equal molar and catalytic site concentrations were investigated (Table 5.15 and Figure 5.13). Both SOD and catalase virtually completely inhibited the reduction in phosphate but not carbonate buffers; SOD was more effective than catalase or catalase and SOD combined. In addition SOD reduced the initial rate of NBT reduction while catalase did not

Table 5.15. Reaction of divicine with NBT and the effect of SOD and catalase and type of buffer. Nitroblue tetrazolium (5.6×10^{-4} M), EDTA (10 µM for carbonate, 100 µM for phosphate), were prepared in the buffer and 2.9 ml of the solution used in the assay. Sodium carbonate buffer (0.5M, pH 10.2) and the phosphate buffer used in section 5.2.1 were tested. In the control, 50 µl of buffer and that of divicine (in 0.1N HCl) were successively added to the NBT solution and after stirring absorbance was monitored until a maximum was recorded. For SOD and catalase tests, 50 µl of buffer was replaced with buffer containing the respective enzyme, and then divicine was added. When both enzymes were added, more concentrated solutions were made and less volumes used.

	Max ΔOD^{560} (test as % of control)[a]	
Treatment	Phosphate buffer (pH 8.0)	Carbonate buffer (pH 10.2)
+ SOD (154 µg/ml)	5.3	91.0
	5.4	84.2
+ Catalase (0.6 mg/ml)	23.3	90.7
	21.6	79.2
+ SOD + catalase	7.8	NR[b]
	10.6	NR

[a] Top values are for divicine (DIL 2x), and the bottom ones are for DIL 5x.

[b] NR - not run because there was no noticeable effect with SOD and catalase alone.

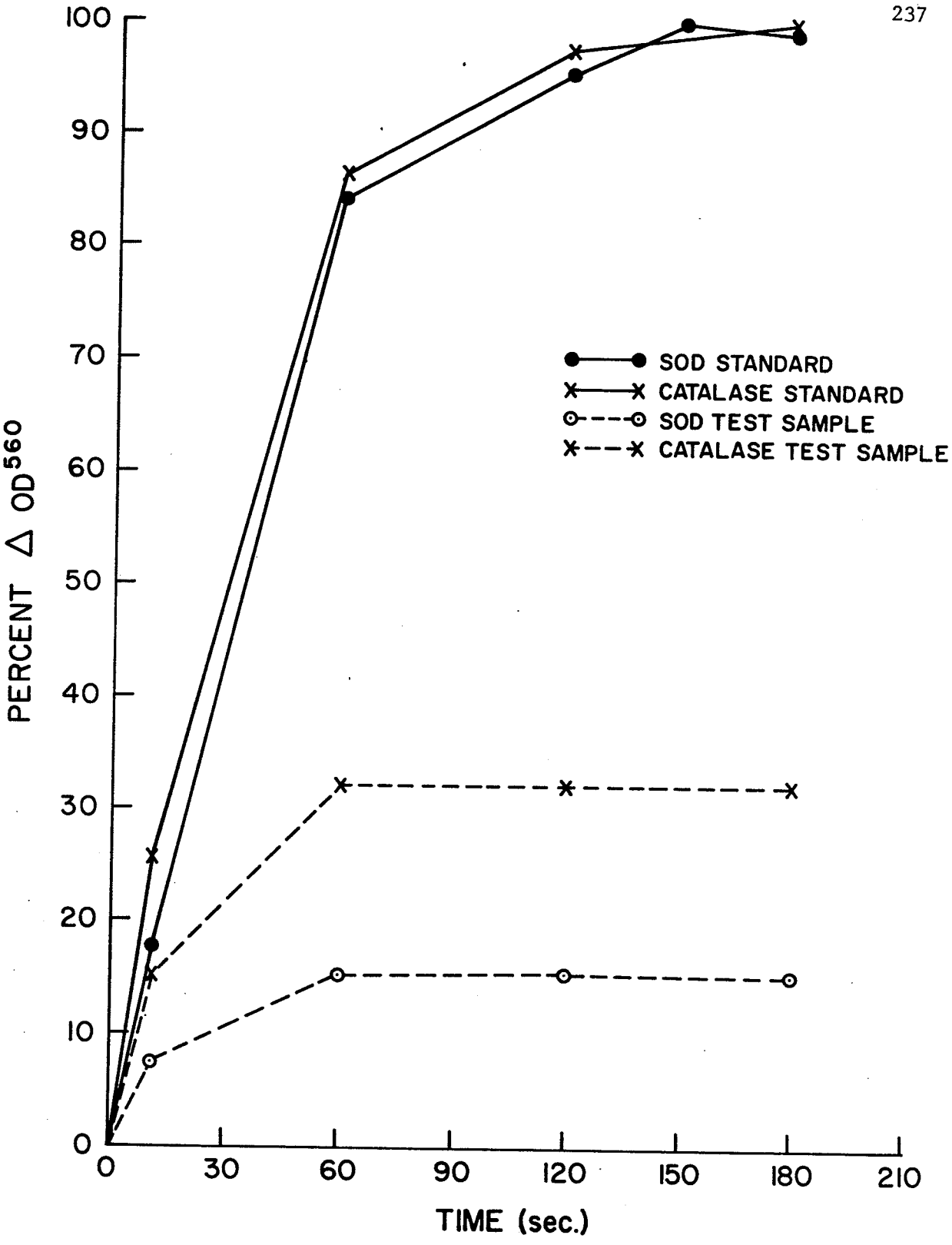


Fig. 5.13. Effect of SOD and catalase on the reduction of NBT by divicine. Procedure was similar to that of Table 5.15. The Δ OD values for the SOD and catalase controls were different, therefore the data was normalised by considering the Δ OD value at 3 minutes as unity, and expressing other values relatively.

(Figure 5.13) indicating that the two enzymes may act at different stages. This data would indicate that O_2^- is the first product of divicine autoxidation and that NBT is mainly reduced by O_2^-. The lack of activity of the enzymes in carbonate buffer could be due to the high pH outside their optimum range, particularly that of catalase (4.1 to 8.8) (Siegel and Siegel 1958); but that of SOD lies between 5.5 and 10.0 (Fridovich 1975) therefore pH could not have inactivated it. However, Marklund and Marklund (1974) showed that while SOD inhibited the autoxidation of pyrogallol by 93 to 99% between pH 7.9 and 9.0, the inhibition fell to 15% at pH 10.6. It is also possible that at the high pH the reduction of NBT by divicine does not involve O_2^-.

Two questions remained to be answered. The first was whether SOD and catalase were acting catalytically or as substrates for O_2^-, and second whether they were the only proteins that could affect the reduction of NBT. It was found that the denatured enzymes were less inhibitory compared to the normal ones (Figure 5.14). Thus Figure 5.14 shows that without the normal enzymes NBT was reduced by divicine as indicated by the purple colour (4a and 4b), and the formation of the purple colour was inhibited by both normal SOD and catalase (2a and 2b), but not so much by the denatured enzymes (3a and 3b). In fact the blank of the denatured enzymes (did not contain divicine) showed a colour change (1a and 1b) and this may account for part of the colour change in the test sample of the denatured enzymes (3a and 3b). It would have been better if quantitative data could be obtained, but this was not possible with presently available technique. In addition, the blanks for the normal enzymes (2a and 2b) and those for the controls (4a and 4b) were

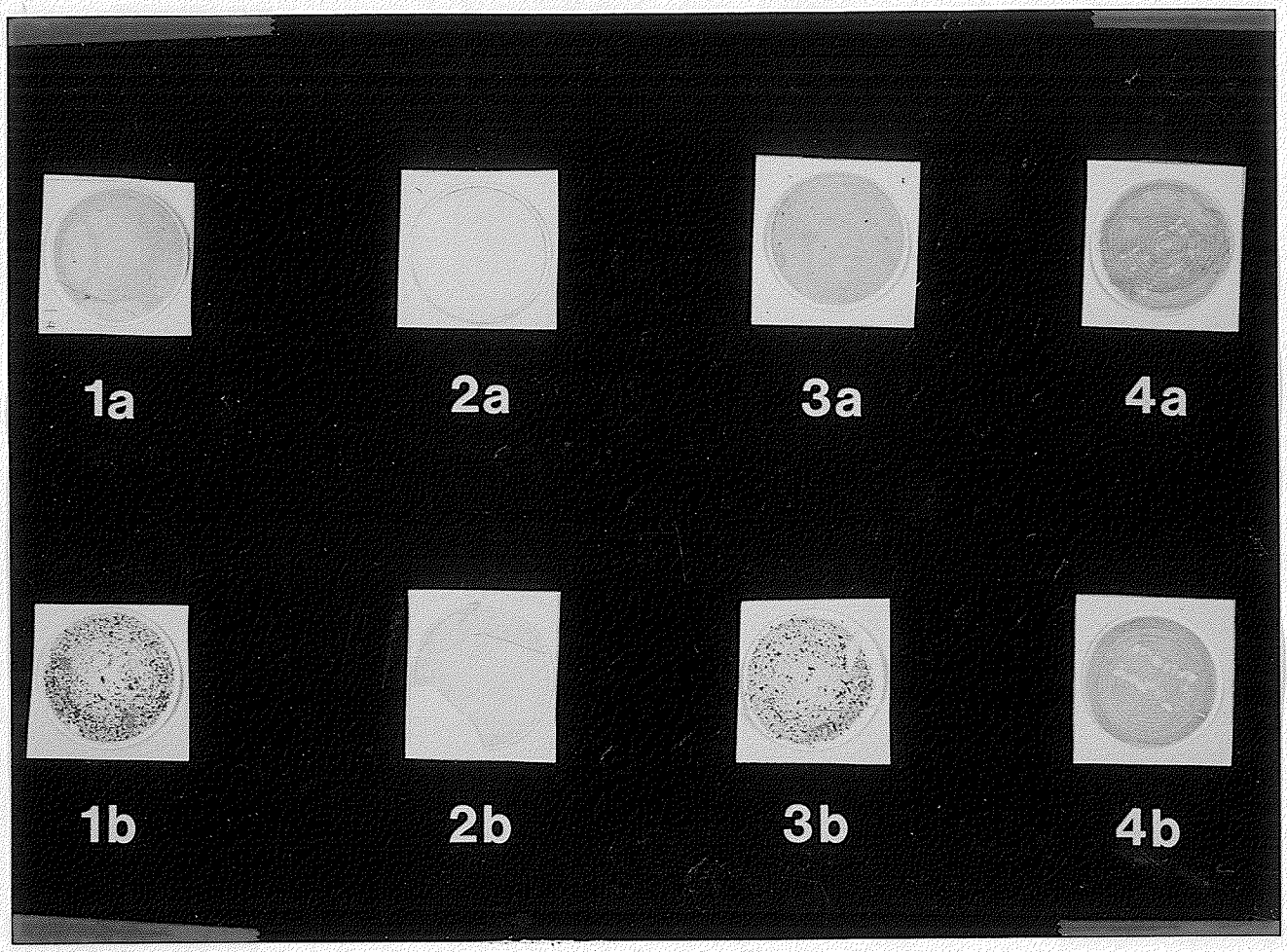


Fig. 5.14. Effect of denatured SOD and catalase on the reduction of NBT by divicine. See Figure 5.13 for the procedure.
1 = NBT + HCl + heated enzyme
2 = NBT + divicine + normal enzyme
3 = NBT + divicine + heated enzyme
4 = NBT + divicine
a = SOD
b = catalase

not included in the figure but they were lighter in colour than the blanks for the denatured enzymes (1a and 1b). This seems to indicate that the activity of normal SOD and catalase in inhibiting NBT reduction was catalytic; although any denatured protein may behave differently after denaturation. Also, the levels employed would strongly suggest that the enzymes acted catalytically.

It was found that at comparable molar concentrations SOD, BSA, Hb, MetHb, alcohol dehydrogenase and ovalbumin inhibited the reduction of NBT by divicine in solutions equilibrated with atmospheric air (Tables 5.16 and 5.18). Therefore the effect of SOD and BSA were tested on a reaction that is known to be carried out by O_2^- (Table 5.17). Thus BSA at even much higher molar concentrations than SOD inhibited the reaction by only 22% while much lower concentrations of SOD virtually eliminated the reaction. These results indicate that the highly charged BSA is capable of acting as an electron and/or proton sink even in small concentrations. However, the dismutation reaction of O_2^- is too fast for it to compete for the electrons from O_2^-. Data in Table 5.19 shows that these proteins did not inhibit the reduction of NBT in an atmosphere of nitrogen as opposed to that of air. This would seem to indicate that these proteins affect the O_2-dependent reduction of NBT hence dismutate the superoxide anion. This also suggests that divicine reacts with O_2 to produce O_2^- which then reacts with the compounds tested. Indirect data suggests that hemoglobin may also have some SOD activity (Buettner et al. 1978). It is also possible that the proteins were reduced by nitrogen and therefore could not accept electrons from divicine. The control indicated that NBT was reduced more in an

Table 5.16. Effect of SOD and BSA on the reduction of NBT by divicine. See Table 5.15 for the procedure.

	ΔOD^{560}	
Treatment	OD units	% inhibition
Control	0.190	-
BSA		
35.2 µg/ml	0.112	53.9
352.0 µg/ml	0.088	41.3
SOD (154 µg/ml)	0.091	52.3

Table 5.17. Effect of SOD and BSA on the NBT cocktail reaction of Beauchamp and Fridovich (1971). One-tenth of a milliliter of the enzyme (test sample) or buffer (control) were added to 2.9 ml of the cocktail mixture and then illuminated as described in the original reference.

	ΔOD^{560}	
Treatment	OD units	% inhibition
Control	0.151	-
BSA		
35.2 µg/ml	0.112	26.2
352.0 µg/ml	0.117	22.3
SOD (15.4 µg/ml)	0.007	95.6

Table 5.18. Effect of gas medium and some proteins on the reduction of NBT by divicine. All compounds were added at equal molar concentrations (5.1×10^{-7} M) and divicine (DIL 2x) was used.

Treatment	Max ΔOD^{560} (Test as % of control)	
	Air	Nitrogen
Control[a]	0.199	1.137
Hb	48.0	100.8
MetHb	42.2	101.0
Alcohol dehydrogenase	37.1	121.1
Ovalbumin	45.7	108.9

[a]Actual ΔOD^{560} units are given.

atmosphere of nitrogen than that of air, hence implying that NBT was inefficient in scavenging electrons from O_2^- and a poor competitor compared to oxygen in abstracting electrons directly from the reduced divicine. Similar results were reported for dialuric acid (Fee et al. 1975). The slight increases in reduction of NBT in the presence of nitrogen due to presence of these proteins are difficult to rationalise, however, it could be that the proteins were partially reduced under nitrogen and the increased absorbance was not only due to NBT reduction. However this data in different gas media should be treated as preliminary. More work is also needed to establish the effects of different normal and denatured proteins and the metabolism of O_2^- in general. It would seem difficult to imagine that SOD, an enzyme inside the RBC would be the first line of defence against O_2^- which could be generated in plasma once its parent compounds reach the basic medium. Therefore plasma proteins would be a logical first line of defence and hence SOD would be effective if the radical were generated intracellularly.

Efforts with e.s.r. spectroscopy did not show any signals for free radicals. This could have been due to the high temperatures at which the investigations were performed. It is known that some free radicals, particularly O_2^- are virtually impossible to detect even at temperatures below freezing (Bray 1961). It is also possible that O_2^- in this case does not accumulate to detectable levels because it further oxidises the reduced divicine. Fee et al. (1975) reported that they could not observe any signal with dialuric acid even with a rapid freeze-quench method except when the concentration of dialuric acid was much higher than that of oxygen; even then the signal was weak. They concluded

that under normal conditions the concentration of free radicals from dialuric acid did not exceed 0.1 μM which was the sensitivity of their machine. The low solubility of divicine in acid does not permit its use in very high concentrations, and yet it cannot be prepared in base except under nitrogen because it will autoxidise immediately. Therefore, special equipment is necessary to directly confirm the generation of O_2^- by e.s.r. spectroscopy.

It may be concluded from data in this chapter that divicine reacts with GSH to form a complex that absorbs at 305 nm. Divicine slowly reduces MetHb to Hb, and both Hb and MetHb seem to catalyse the autoxidation of divicine. Divicine reduces both cyt C and NBT. However, while the reduction of NBT is more dependent on O_2^- than that of cyt C and therefore is a better indicator of O_2^- levels than cytochrome C. Preliminary data also indicates that in addition to SOD, other proteins may have the capacity to dismutate O_2^- or just abstract an electron from it. Therefore further work is needed to establish the products of divicine autoxidation and the metabolism of the superoxide anion and other related products.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

6.1. Vicine and convicine can be extracted from FBPC with acetone/water mixtures preferably in the ratio of approximately 2.5:1 or less so long as the amount of acetone is enough to denature the protein, maintain the quality of the product and the total volumes of the extraction mixture is enough for the convenient processing of the sample. Vicine and convicine form crystals when the extract is concentrated to 0.07-0.08 volumes (based on weight of FBPC), the pH of the solution lowered to neutrality (pH 7.2) and the sample allowed to stand for a period greater than 12 hours either at room temperature (27^{o}C) or in the refrigerator (0-4^{o}C). The crystals can be harvested by centrifugation and further cleaned by washing with water, ethanol and acetone. Vicine can then be selectively crystallised out at pH of approximately 9.0. Adjustment of the residual supernatant to pH 7.2 yields a convicine-rich crystallite. Washing and recrystallisation yields products that are "pure" vicine (97% vicine and 3% convicine) and convicine (91% convicine and 9% vicine). Highly pure compounds could be obtained by one or two additional recrystallisations. The yield of crude vicine is 40 to 50% of that in FBPC depending on the method used. This yield can be increased to some extent by increasing the volume of the extraction solvent, particularly water, but the yield per unit volume of extraction solvent would be reduced. This may not be desirable as the most expensive and time consuming part of the isolation is the concentration step (Stage 2). In the isolation it is important that the pH of the extract

be increased so as to improve the extractability of vicine and convicine; however, at pH levels above 9.5 there is a decrease in yield of crude vicine. This effect has not been examined during the subsequent recrystallisations to obtain the pure components, but it does not seem to affect their yield.

Most of the properties determined confirm that the compounds isolated from FBPC by either methods 1 or 2 are vicine and convicine. Some of the characteristics deviate from the literature values and reasons for these differences were discussed previously, in addition some characteristics have not been updated for many years. Acid hydrolysis of the "vicine" produces a sample with spectral and stability properties similar to those of reduced divicine which further supports the conclusion that the parent compound was vicine.

6.2. Dietary crude vicine increases RBC hemolysis, and at levels exceeding 1% retards the growth rate of chicks. Dietary levels of vicine exceeding 1% would be attained in diets that contained over 80% fababeans, or approximately 60% FBPC. Although the reduced growth rate could in part result from reduced feed intake, the latter seems to be minor and perhaps a consequence of some biophysiological effect of vicine (e.g. elevated plasma lipids or amino acids) that are involved in the control of feed intake. It may be concluded that heat-resistant vicine and convicine are another factor in fababeans that retard chick growth.

In agreement with earlier data with laying hens (Olaboro 1978) it was confirmed that vicine depresses egg and yolk masses by decreasing mainly their weights, and to a lesser degree, production rate. In

addition vicine increases the fragility of the yolk membrane and the incidence of yolk blood spots, and reduces fertility and hatchability of the eggs. Plasma constituents are also affected. Dietary crude vicine results in elevated plasma lipid and lipid peroxides, and depressed the ratio of plasma vitamin E/lipid levels; these were associated with low hematocrits and an increased degree of erythrocyte hemolysis in vitro. Dietary vicine reduced the levels of Hb, but increased those of GSH and the activity of SOD in RBC. Conversely, dietary vicine reduced the activities of liver SOD, catalase and GSH-px, with hardly any effect on protein, but slightly elevated GSH levels.

Dietary vitamin E at levels 40 times higher than those recommended by NRC for the laying hen, improved the egg mass, fertility and hatchability, lowered liver weight and lipid levels, and increased the activities of GSH-px and SOD, but not catalase or the other parameters examined in the study. Perhaps much higher levels of vitamin E are required to provide protection with regard to the latter parameters, but lower levels may be adequate for protection against reduced egg fertility and hatchability. Glucose in the incubation medium inhibited hemolysis in vitro of RBC from vicine-fed birds.

Vicine and convicine are unreactive and therefore must be converted to some oxidising metabolites that cause lipid peroxidation, hemolysis and the other effects that are characteristic of oxidising compounds. The initial reactive metabolic products of these glycosides are their aglycones (divicine and isouramil) which could be produced by β-glucosidase of the intestinal flora (Luisada 1941) and/or serum (Flohe et al. 1971). However, this enzyme has not yet been isolated from either source.

The lipid peroxidation and hemolysis observed with birds fed vicine supports data reported by Flohe et al. (1971) on the effect of divicine on normal human RBC in vitro, and that reported for dialuric acid on RBC from vitamin E-deficient rats (Fee et al. 1975). The latter observed that dialuric acid led to hemolysis in three distinct stages: the RBC were modified in an unknown manner within the first 2 minutes, this was followed by lipid peroxidation, and when the latter reached 75% of its maximal value, hemolysis was observed. Fee et al. (1975) further observed that catalase or a mixture of catalase and SOD protected the cells against hemolysis, only if added during the initial stage, indicating that once lipid peroxidation started, hemolysis was inevitable. However, as cited earlier in the literature, events leading to hemolysis are controversial.

The protective effect of glucose against hemolysis in vitro indicates that in vicine fed birds glucose is not either transported into the RBC or metabolised into its reducing products, particularly NADPH. A similar effect in vitro was observed with normal human RBC challenged with divicine, but not cells from individuals deficient in G-6-PD (Mager et al. 1965, 1969). Therefore vicine and/or its metabolites seem to compete for utilisation of reducing equivalents, and inhibit the enzymes of the pentose phosphate pathway or the entry of glucose into the cells. Vicine has been reported to inhibit G-6-PD in vitro (Lin and Ling 1962b).

The failure of vitamin E to protect RBC from hemolysis as opposed to the embryo may reflect differences in membrane structural composition, particularly the lipid levels, and the proximity of the RBC to the area of divicine autoxidation and therefore high levels of prooxidant concen-

tration. The high concentration of the prooxidants may oxidise vitamin E before it is effective, and may also alter the membrane structure necessary for vitamin E to bind before it could offer protection (Lucy 1972). There could be a blood barrier between the circulation and embryo, similar to the brain-blood barrier, which restricts the amount of vicine or divicine that can reach the embryo. In addition divicine may prevent regeneration of active vitamin E by ascorbic acid; Borg et al. (1978) showed that a rapidly autoxidising compound catalyses the autoxidation of an otherwise weak autoxidising compound like ascorbic acid. If ascorbic acid were irreversibly oxidised, it would not be able to regenerate reduced vitamin E (see literature review), therefore making the demand for vitamin E excessive.

The inhibition of liver catalase and GSH-px activity by dietary vicine predisposes the organ to H_2O_2 and other potential oxidants. The low GSH-px activity would account for the elevated levels of GSH in both the liver and erythrocytes, but would indicate either a normal or elevated activity of GSH-reductase. The low GSH-px activity may reflect inhibition of its synthesis, lack of selenium or instability of the enzyme itself.

6.3. Data from Chapter 5 demonstrated that reduced divicine produces O_2^- during its autoxidation. The mechanisms of the reaction are not known but from the data in Chapter 5 and available literature (Borg et al. 1978) the postulated sequence is shown in Figure 6.1. Reaction 2 would compete with the dismutation reaction, the latter could be quite fast particularly in presence of EDTA-Fe^{2+} complex. The proposed scheme

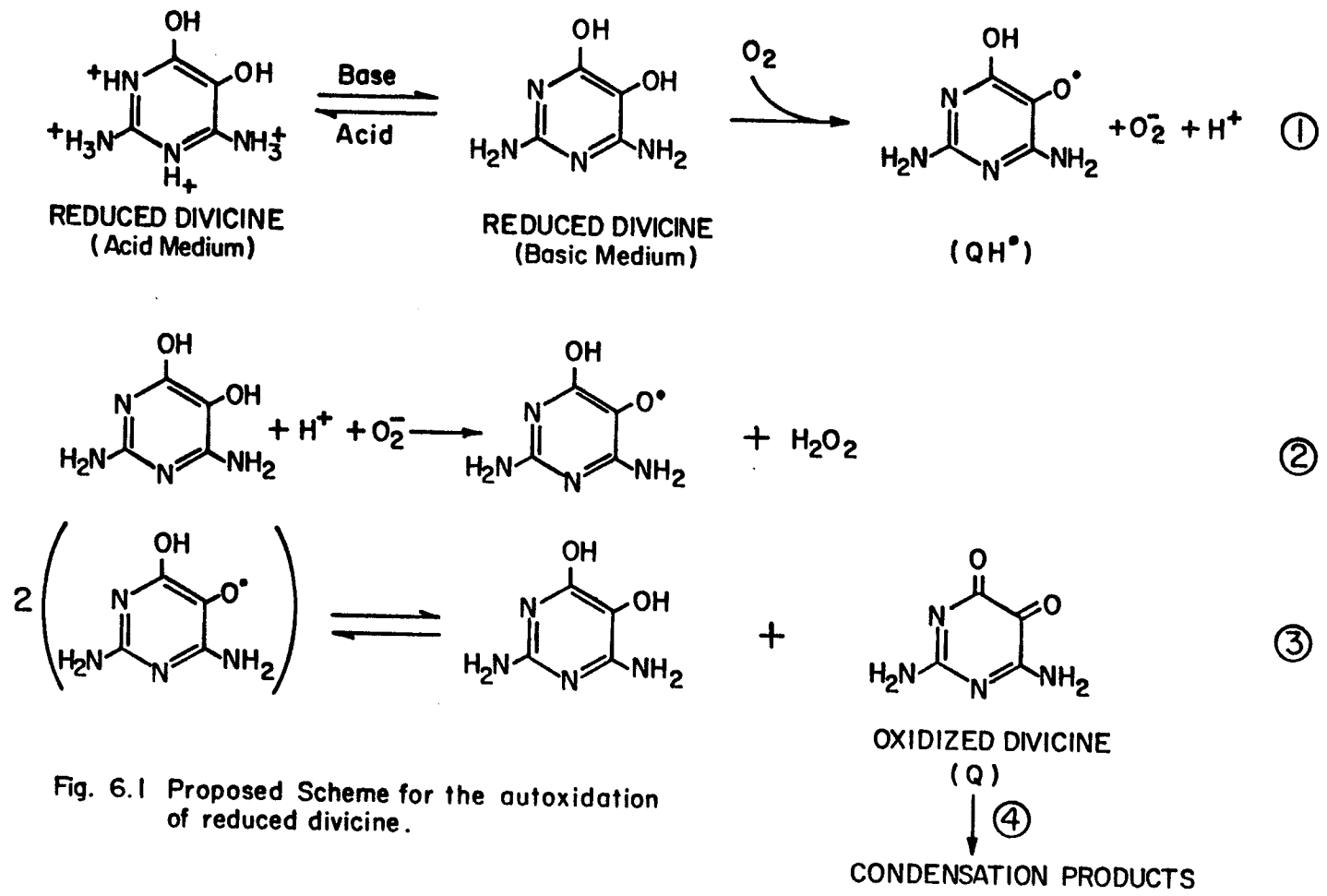


Fig. 6.1 Proposed Scheme for the autoxidation of reduced divicine.

would fit that of a rapidly autoxidising compound (Borg et al. 1978; see Table 6.1) and account for the effects of SOD and catalase. This scheme would also account for the similarity between the reaction kinetics of the autoxidation of divicine and that of dialuric acid (Richardson 1932; Borg et al. 1978). However, the alternative, but unlikely pathways suggested by other workers (Borg et al. 1978) could also occur. Extensive studies are needed to identify the various intermediates to confirm that proposed sequence. In addition the reaction in acid may follow the same sequence but at a slower rate and the groups other than 4 and 5 would remain the same. The data in Chapter 5 also shows that many biological compounds other than SOD have the capacity to scavenge O_2^-, but their mode of action is not known.

The dismutation of O_2^- produces H_2O_2 and O_2, and O_2^- reacts with H_2O_2 to form $^{\cdot}OH$ which is known to be the most potent oxidant radical. This radical causes lipid peroxidation, hemolysis and destruction of other reducing compounds like GSH, vitamin E or other free radical scavengers; the latter class of compounds protect cells from the effects of $^{\cdot}OH$ (see Literature Review). The macerated eggs and the increased fragility of yolk membranes of eggs from vicine-fed birds would further indicate membrane destruction.

The reaction between divicine and GSH showed that in addition to producing free radicals, divicine forms condensation products with some biomolecules. However, contrary to the fast reaction between divicine and GSH, those between the former and Hb or MetHb were slow. Thus Hb and MetHb do not seem to play a defensive role compared to GSH and also other plasma proteins such as albumin. This data is similar to that

reported by Sutton et al. (1976) who observed that O_2^- oxidised and reduced OxyHb and MetHb, respectively, but the reaction was perceptible only after 30 minutes, which is 3 times longer than the time for which the same reactions were monitored in the current study. In addition the presence of EDTA-Fe^{2+} complex could have contributed to the low rate of the reaction in this and other studies; Bendich and Clements (1953) reported that EDTA (0.5×10^{-5} - 0.5×10^{-4} M) stabilised alkaline solutions of divicine and related pyrimidines. The addition of divicine to solutions of Hb or MetHb in both cases resulted in a slight decrease and increase in absorbance at 630 and 577 nm, respectively. This unexpected observation with Hb solutions would indicate that the commercial Hb was highly contaminated with methemoglobin. The slow reaction with the heme groups would indicate that both Hb and MetHb either just catalyse the autoxidation of divicine, the O_2^- reacts with another group (e.g. the β-93 thiol group) or the dismutation reaction was more efficient at this pH than the reaction with MetHb. The reaction between O_2^- and Fe^{2+} and Fe^{3+} of OxyHb and MetHb, respectively is shown in reactions 1 and 2 (Sutton et al. 1976; Winterbourn et al. 1976).

$$O_2^- + 2H^+ + \text{heme} - Fe^{2+(+)} - O_2^{(-)} \rightarrow \text{heme} - Fe^{3+} + H_2O_2 + O_2 \qquad (1)$$

$$O_2^- + \text{heme} - Fe^{3+} \rightarrow \text{heme} - Fe^{2+(+)} - O_2^{(-)} \qquad (2)$$

$$2\ O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (3)$$

Thus reactions 1 and 2 compete with 3 for O_2^-. Reaction 3 can be catalysed by EDTA-Fe^{2+} complex. In addition reactions 1 and 2 are favourable at pH 7.0, and decrease with higher pH, which could have occurred at the

pH (8.0) employed in studies reported in this thesis.

6.4. The link between rapidly autoxidising compounds, lipid peroxidation and hemolysis are rather complex and not clearly known. However, Borg et al. (1978) have reviewed the subject and its apparent that there are two schools of thought as to the mode of action of rapidly autoxidising compounds in causing the biochemical and physiological effects. One school supposes that the rapidly autoxidising compounds condense with essential biomolecules like GSH that protect the cell from the oxidant stress. The complexing with a structural molecule would lead to structural disruption. This theory is supported by such data as the reaction between GSH and divicine, but if this was the only mode of action, high levels of the toxic compounds would be required to cause harm.

Since most autoxidising compounds are toxic in small amounts, it would appear that they set off a chain reaction and/or they and their products are recycled. Many autoxidising compounds have been shown to produce O_2^- and other radicals whose effects have been already discussed. Therefore the link between rapidly autoxidising compounds and their biochemical effects can be summarised in a set of equations (Table 6.1). These are the likely reactions from kinetic considerations and the other possible reactions that are not likely to occur can be found in the paper by Borg et al. (1978). It is hypothesised that vicine and convicine when converted to their aglycones also have a similar mode of action.

Table 6.1. Relationship between strong and weak autoxidising compounds and their biochemical effects. QH_2, strong autoxidisers; DH_2, weak autoxidisers.

Autoxidation of QH_2:

For QH_2 = 6-OHDA, 6-ADA, DA, etc.

Likely Reactions

$$QH_2 + O_2 \xrightarrow{k_1} QH + O_2^{\cdot-} + H^+ \quad (1)$$

$$QH_2 + O_2^{\cdot-} + H^+ \rightarrow QH^{\cdot} + H_2O_2 \quad (2)$$

$$2QH^{\cdot} \longleftrightarrow QH_2 + Q \quad (3)$$

$\Sigma 1,2,3$: $$QH_2 + O_2 \rightarrow Q + H_2O_2 \quad (4)$$

$$Q \rightarrow P \text{ (dihydroxyindoles, etc.)} \quad (4a)$$

Dismutation of $O_2^{\cdot-}$:

$$O_2^{\cdot-} + H^+ \longleftrightarrow HO_2^{\cdot} \ (pK_a = 4.75) \quad (5)$$

$$O_2^{\cdot-} + HO_2^{\cdot} + H^+ \xrightarrow{k_6} O_2 + H_2O_2 \quad (6)$$

$\Sigma 5,6$: $$2O_2^{\cdot-} + 2H^+ \xrightarrow{k_7} O_2 + H_2O_2 \quad (7)$$

$\Sigma 1,1,5,6,3$: $$QH_2 + O_2 \rightarrow Q + H_2O_2 \quad (8) = (4)$$

Catalysis of DH_2 autoxidation by QH_2's:

For QH_2 = 6-OHDA, 6-ADA, DA, etc. and DH_2 = ascorbate, reductate or other net electron donors ....

Likely Reactions

$$DH_2 + QH \xrightarrow{k_{12}} DH^{\cdot} + QH_2 \quad (12)$$

Continued .....

Table 6.1 (Continued)

	$2DH^{\bullet} \xrightarrow{k_d} DH_2 + D$	(13)
Σ1,2,12,12,13:	$DH_2 + O_2 \rightarrow D + H_2O_2$	(14)
Σ1,1,12,12,13,5,6:	$QH_2 + O_2 \rightarrow Q + H_2O_2$	(14a)
	$DH_2 + O_2^{\bullet-} + H^+ \xrightarrow{k_{15}} DH^{\bullet} + H_2O_2$	(15)
Σ1,15,13,13:	$DH_2 + O_2 \rightarrow D + H_2O_2$	(16) = (14)

Catalysis of DH_2 autoxidation by Q's:

	$Q + DH_2 \rightarrow QH^{\bullet} + DH^{\bullet}$	(20)
	$Q + DH_2 \rightarrow QDH_2$	(20a)
Σ20,12,13:	$Q + DH_2 \rightarrow QH_2 + D$	(22)
Σ22,4 = Σ20,12,13,1,2,3:	$DH_2 + O_2 \rightarrow D + H_2O_2$	(23) = (14) = (16)

Autoxidation of lipids (LH = polyunsaturated lipid):

Initiation:

	$LH + RH^{\bullet} \rightarrow L^{\bullet} + RH_2$	(24)
	$LH + QH^{\bullet} \rightarrow L^{\bullet} + QH_2$	(24a)

Propagation:

	$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$	(25)
	$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$	(26)

Continued .....

Table 6.1 (Continued)

Σ25,26: $LH + O_2 \rightarrow LOOH$ (27)

Reactivation:

$LOOH + M^{n+} + H^+ \rightarrow LO^\cdot + M^{(n+1)+} + H_2O$ (28)

$LOOH + O_2^{\cdot-} + H^+ \rightarrow LO^\cdot + O_2 + H_2O$ (28a)

$LO^\cdot + LH \rightarrow L^\cdot + LOH$ (28b)

Termination:

Polymerization:

$2LOO^\cdot \rightarrow LOOL + O_2$ (29)

$2L^\cdot \rightarrow LL$ (29a)

$L^\cdot + LOO^\cdot \rightarrow LOOL$ (29b)

Reaction with "target" (TH):

$L^\cdot + TH \rightarrow T^\cdot + LH$, $THL^\cdot$, etc. (30)

$LO^\cdot + TH \rightarrow T^\cdot + LOH$, etc. (31a)

Generation of Hydroxyl Radicals:

Fenton's Reaction:

$Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + OH^\cdot + H_2O$ (34)

Propagation (physiological conditions):

$Fe(II)Chelate + H_2O_2 + H^+ \rightarrow Fe(III)Chelate + OH^\cdot + H_2O$ (35)

$Fe(III)Chelate + O_2^{\cdot-} \rightarrow Fe(II)Chelate + O_2$ (36)

Continued .....

Table 6.1 (Continued)

Σ35,36: $$H_2O_2 + O_2^{\cdot-} + H^+ \rightarrow OH^{\cdot} + O_2 + H_2O \quad (37)$$

Coupling to target molecule (TH):

Direct reaction (radiomimetic):

$$OH^{\cdot} + TH \rightarrow T^{\cdot} + H_2O, \text{ HTOH}^{\cdot}, \text{ etc.} \quad (38)$$

Initiation of lipid chain peroxidation:

$$OH^{\cdot} + LH \rightarrow L^{\cdot} + H_2O \quad (39)$$

6.5. From this research it is suggested that a number of areas be investigated:

A. The effect of various nutrients e.g. Zn, Se, Cu, Fe and others that catalyse the autoxidation of divicine, or are components of molecules (particularly enzymes) that are involved in the metabolism of the products of oxygen reduction. The type and level of dietary fat should be investigated; Pryor et al. (1976) reported that most of the TBA-reactive material was only from PUFA containing 3 or more double bonds. In addition the MDA produced itself can cross-link with proteins.

B. The accumulation of vicine and convicine in eggs and tissues and the off flavours and keeping quality of the latter from birds fed crude vicine should be evaluated in view of its effects on lipids and its corresponding effect on membranes.

C. The effect of vicine on the fertility of cockerels should be established.

D. The effect of vicine on hormone levels and their metabolism in both cockerels and laying hens. Synthesis of phosvitin (yolk protein) is controlled by oestrogen (McIndoe 1971), and therefore production of small yolks by vicine fed birds necessitates such studies.

E. The type of plasma lipids and the levels of lipid-transporting proteins should be established in order to appreciate the mechanism by which vicine causes elevated plasma lipid levels.

F. More work should be carried out to establish the biochemical effects of dietary vicine on chicks. These could be a convenient animal model for the study of favism.

G. Finally, it would be worth demonstrating by esr that a free radical is produced during the autoxidation of divicine. In addition research should be carried out to determine the effect of various types of free radical scavengers on the hemolytic effects of divicine in vitro.

A P P E N D I X

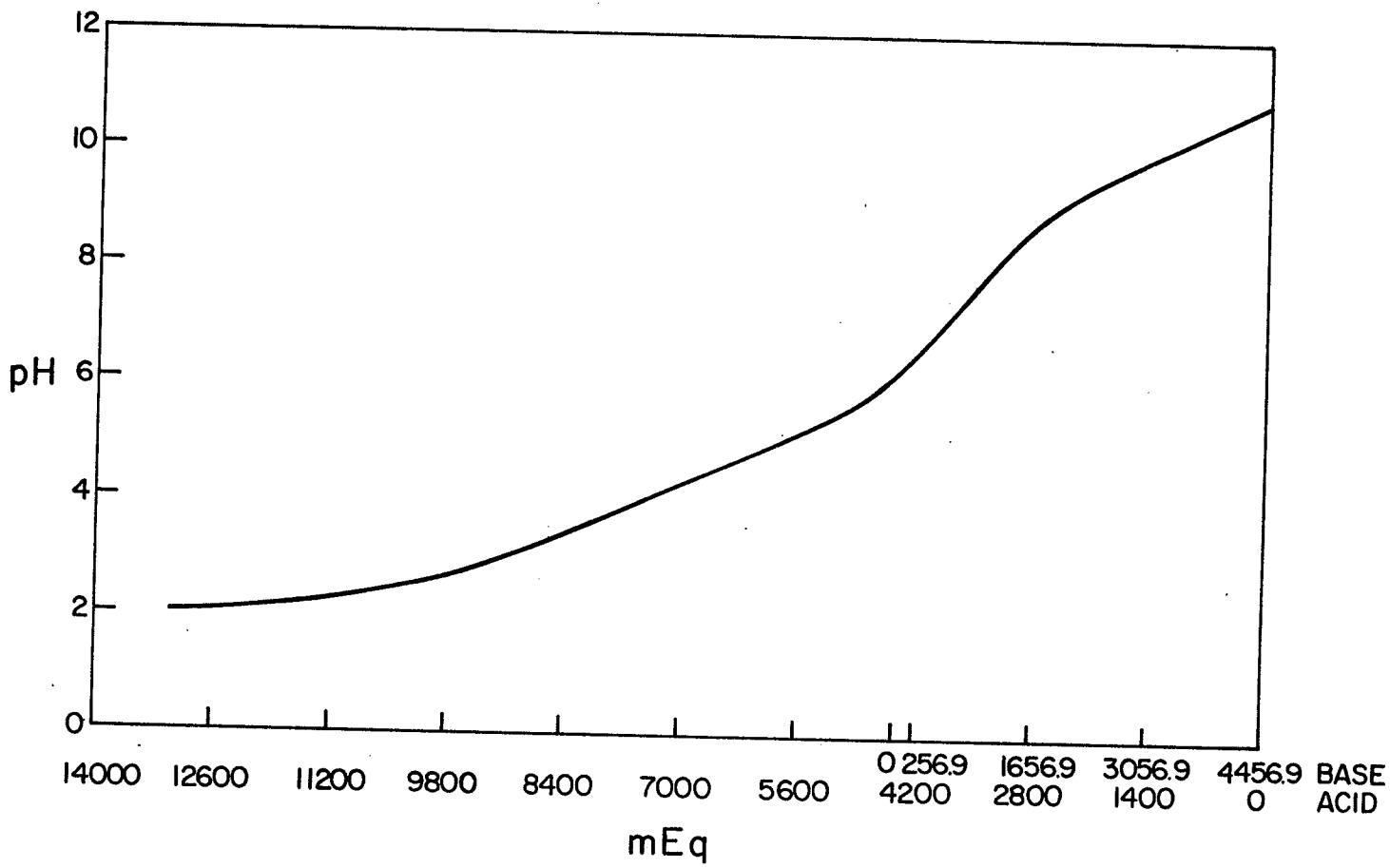


Fig. 2.1 A Titration Curve of FBPC. See section 2.1.2 for the procedure.

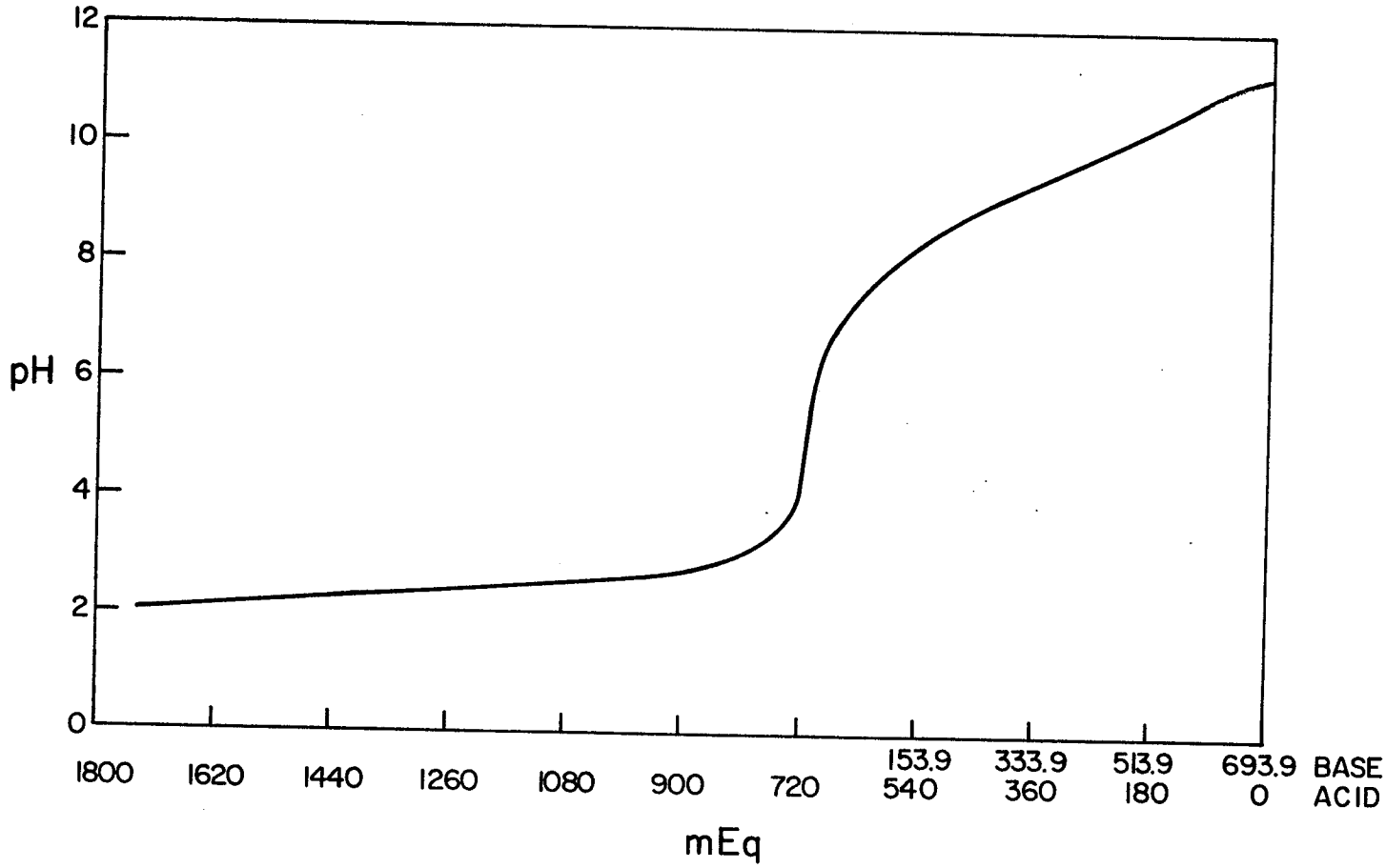


Fig. 2.2 A Titration Curve of Crude Vicine. See section 2.1.10 (k) for the procedure.

Table 2.1A. Reagents and voltage control for isoelectric focusing

Reagents (A) Acrylamide solution: 10.0 g acrylamide
0.33 g Bis-acrylamide
Make up to 50.0 ml with distilled deionised water.

(B) Concentrated mixture (for 6 gels):
1.87 ml distilled deionised H_2O
7.0 ml reagent (A)
0.7 ml 40% ampholites (LKB) (pH 3.5-10.0)

(C) Mixture for 1 gel:
1.5 ml concentrated mixture B
0.5 ml 40% sucrose
2.3 ml of vicine solution
20.0 ml ammonium sulphate (100 mg/ml)

Voltage control:

Time run (hr)	Voltage applied
0.5	50
1.0	100
overnight	150
0.5	300
2nd overnight	150

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