THE EFFECT OF CADMIUM ON SOME ENZYMES IN THE DEVELOPING CHICK EMBRYO.

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by

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PART I

INTRODUCTION

I. 1. Properties of Cadmium

Cadmium (Gr. kadmeia - earth), a soft blue-white metal, is a member of Group II b of the Periodic Table, and lies between zinc and mercury.

Cadmium (Cd) possesses the following physical constants: Atomic number 48; atomic weight 112.4; valency 2 and specific gravity 8.7. The metal melts at 321^{o}C and boils at 765^{o}C, and is remarkably volatile for a heavy metal. Its volatility has constituted a special industrial hazard since its b.p. is below the melting point of copper (1083^{o}C), and copper-cadmium alloys were formerly difficult to prepare without gross losses of cadmium into the atmosphere.

There are eight stable isotopes, of which the consecutive isotopes Cd^{110} to Cd^{114} (Handbook of Physics and Chemistry 1969) occur in equal amounts in nature. As the electronic configuration of cadmium, $4\,d^{10}\,5\,s^{2}$, is very stable, the metal forms simple bipositive cations only.

The stereochemical behaviour of cadmium, which forms the basis of its interactions with enzymes and other compounds, is related to its atomic radius and

to the number and disposition of the electrons in the atom which are responsible for electrostatic and covalent binding forces.

Cadmium constitutes approximately 2×10^{-5}% of the earth's crust and is usually found together with zinc as the mixed carbonate ($ZnCO_3.CdCO_3$) and as the sulphide as a contaminant of zinc blende (ZnS). Industrial smelting of zinc blende provided a major source of sulphuric acid, zinc and cadmium. Cadmium is separable from zinc by differential volatilization. Cadmium condenses as a brown oxide which may be reduced with carbon.

A number of cadmium salts occur. As the chloride is easily soluble in water and was available in a high degree of purity (Analar reagent), it was employed in all the investigations described in this thesis. Cadmium chloride imparts an acid pH to the solution. The solubility drops markedly at alkaline pH owing to the formation of insoluble cadmium oxy-chloride.

I. 2. Industrial uses of cadmium

Industrial uses of cadmium vary widely. The

metal is the most efficient neutron - capture material known and is used extensively in nuclear reactors for the protection of workers. It is used in standard cells for a precise output of E.M.F.; in the paint industry, where the sulphide is an intense yellow pigment, and in the manufacture of bearings and electrical wires as a copper-cadmium alloy of low frictional resistance.

Cadmium oxide is employed in the manufacture of alkaline accumulators and was early recognised as a potent industrial irritant and antimetabolite.

I. 3. Cadmium poisoning in industry

Friberg (1950) noticed that workers exposed to cadmium in the alkaline battery industry suffered from ill health, and even died of exposure to cadmium fumes. In addition to the observations of Friberg (1950), Baader (1951) observed emphysema, proteinuria and weight-loss in a series of patients exposed to cadmium in a similar alkaline battery factory in Germany.

Cadmium poisoning symptoms usually assumed noticeable proportions only subsequent to a lengthy exposure

to the toxic metal, but the effects persisted for some time even if the patient was no longer exposed (Bonnell, Kazantzis and King, 1959).

Smith, Kench and Lane (1955) confirmed the findings of Friberg, and enlarged upon the biochemical evidence accompanying cadmium intoxication. Cadmium accumulation in body tissues of a cadmium worker and of three"normal" men was studied by Smith, Kench and Smith (1957). They conclusively demonstrated the deposition of cadmium in the soft tissues, particularly the liver, kidney, testes, pancreas, arteries and veins of the long-exposed body.

There are reported cases of a more rapid onset of the effects of cadmium; Lane and Campbell (1954) described 2 cases of emphysema, which proved fatal after 2 years exposure to the dust of copper-cadmium alloy workings.

Further investigations of the effects of cadmium on industrial employees, particularly those of Kench ,Smith and his colleagues (1955) and Bonnell (1955) laid the basis to what is now a well-established syndrome. The major clinical findings were identical with those described by Friberg (1950). Additional factors included dyspnoea, impairment of both tubular and glomerular renal function, and generalised proteinuria in 65% of patients.

In most cases of intoxication, cadmium entered the body via the lungs. The gastrointestinal tract is inefficient in absorbing cadmium. Most symptoms became apparent only after prolonged exposure.

I. 4. Experimental cadmium poisoning

There are many described cases of acute cadmium poisoning in laboratory animals, generally manifested by enzymatic and haematological changes.

The intoxication mechanism in such acute cases appears to be different from that in chronic poisoning, where a much lower dosage is administered for a much longer period of time, with an eventual accumulation of higher tissue concentrations of the metal. The syndrome of acute cadmium poisoning on the reproductive organs of laboratory animals is well documented. Cadmium exerts a very toxic effect on testicular tissue (Singh and Mathur, 1968; Parizek and Zahor, 1956).

The influence of the metal has been investigated as a hypertensive agent (Perry, Erlanger, Yunice and Perry, 1967; Schroeder, 1966), in cardiovascular

disease (Schroeder, 1967) and in the onset of amyloidosis after chronic poisoning of rabbits (Baum and Worthen, 1967).

It is widely recognised that zinc competes with cadmium (Schroeder, Nason and Mitchener, 1968; Barris, Pond, Walker and O'Connor, 1969) and zinc may be used to offset some of the more severe symptoms of cadmium-toxicity. Parizek and Zahor (1965) showed that symptoms induced by cadmium poisoning were similar to those caused by prolonged zinc deficiency and further that the destructive effect of Cd^{II} could be suppressed by a several-fold greater zinc dosage. It does appear, however, that zinc is of little importance in preventing testicular degeneration due to cadmium poisoning. Cameron and Foster (1963) concluded that the primary factor is the inhibition of thiol-containing enzymes by the formation of mercaptides with their sulphydryl groups. This leads to profound changes in spermatogenic epithelium and in the Leydig cells.

Cadmium has been added to the ever-increasing list of carcinogenic substances. There is a high incidence of malignancy in industrial workers associated with cadmium fume (Potts, 1965). Kazantzis, Fynn, Spowage and Trott (1963) induced tumours in rats by injecting them with cadmium salts. The carcinogenic nature of cadmium

has been confirmed by Cameron and Foster (1963), who detected a neoplasm in one of their experimental rabbits.

Neurological lesions have been reported in rats suffering from gross cadmium toxaemia (Gabbiani, 1966). The ganglion cells showed pycnosis of nuclei and ultimate lysis of cytoplasm. The present investigation on poisoned chicks showed similar lesions.

Acute cadmium poisoning decreases the circulating levels of all the protein components in rats (Lawford, 1961) with the exception of transferrin, which rises rapidly, possibly as a binding protein for free, circulating cadmium.

Metabolic disturbances which arise from dietary cadmium are dependent on the rate of transmural movement of the ion into the circulatory system. Experiments by Sahagian, Harding-Barlow and Perry (1967) have shown that the presence of ascorbate enhances Cd^{II} and Zn^{II} transport across the intestinal wall, and that iodoacetate augments the uptake of the two ions into the membrane cells, but decreases overall transport. They concluded that there was no active transport mechanism in the gut for cadmium, as no energy was expended in its uptake, nor was the ion capable of entering against a concentration gradient.

Significant anaemia does not result from chronic cadmium poisoning in man. An overt microcytic, hypochromic anaemia became manifest, however, in chicks poisoned by dietary cadmium over a period of three weeks (Hill et al, 1963). The overall effect of cadmium was one of marked depression of growth of chicks, loss of condition and weight, and increased mortality.

The intracellular distribution of cadmium as described by Cotzias et al (1961 b) indicates that approximately 15% of Cd^{109} absorbed by hepatic cells was bound to mitochondria. The effect of this mitochondrial-bound cadmium on the enzymes located in those organelles is important, since throughout the first 20 years of life the average young American accumulates significant amounts of cadmium in the liver and kidneys. Up to 4.5 mg cadmium in the kidneys of American youths and up to 20 mg in Japanese youths (Perry, Tipton, Schroeder, Steiner and Cook, 1961) has been reported.

I. 5. Interactions of cadmium with proteins

Cotzias, Borg and Selleck (1961 b) studied

the intracellular distribution of cadmium and concluded that the very low turnover of cadmium was due to the powerful binding of the metal to proteins. A specific metal-binding protein, metallothionein, containing a high percentage of thiol groups and a high percentage of cadmium has been isolated from horse kidney by Kagi and Vallee (1961). Cotzias et al (1961 a) claim that it is very probable that Cd^{II} is bound to a similar protein, possibly displacing zinc, the level of which rises rapidly in serum following cadmium administration. They further postulated that the body has no natural defence against this zinc anti-metabolite and that the cadmium continues to accumulate, even in the face of augmented zinc intake. Excretion of the metal did not relieve toxicity significantly. 20% was excreted within the first 3 days and thereafter the rate of elimination from the body fell rapidly. Their series of investigations supports the hypothesis of Gurd and Wilcox (1956) that Cd^{II} is more tightly bound to thiol groups than is zinc, and consequently can displace the zinc.

Kench and his colleagues in Manchester and Cape Town have made a systematic study of the interaction of cadmium with proteins in experimental cadmium poisoning. A low-molecular albumin has been observed to

arise during chronic poisoning of man and a number of other animals (Smith, Wells and Kench, 1961; Kench, Wells and Smith, 1962).

The turnover rate of minialbumin was more rapid than that of normal albumin molecules. The low-molecular albumin proved to be immunologically indistinguishable from normal serum albumin, relatively lacking in cystine and lysine, and devoid of tryptophan (Kench, Gain and Sutherland, 1965; Kench and Sutherland, 1966, 1967).

The study of the binding of heavy metals to proteins in biological systems is of utmost importance in understanding the various toxic properties of the metals.

Clarkson and Kench (1958) investigated the uptake of lead by human erythrocytes, and found that more than 95% is attached to the erythrocyte membranes as a lead phosphate complex. The lead was only very slowly removed by chelating agents such as E.D.T.A. Calculation revealed that there were sufficient binding sites on each erythrocyte to accommodate more than 10^6 lead ions. The figure is even greater for yeast cells, each of which is capable of binding 10^7 individual molecules (Rothstein and Larrabee, 1948). Investigations of erythrocyte uptake of cadmium are not well documented, and an attempt will be made in this study to determine whether or not it parallels lead in its behaviour.

Cadmium is bound to the soluble proteins in hepatic cells to a greater extent than it is to sub-cellular particulate bodies (Cotzias et al. 1961 b). Accumulation of iron in the liver results in the increased formation of the iron-binding protein, ferritin (Granick, 1943). It is therefore apparent that proteins offer metals a great variety of co-ordination centres. Different metals bind preferentially to different centres, the heavy metals Cd, Pb and Hg showing a great preference for sulphides; Fe, Co and Ni for N^{3-} and O^{2-}; and Cu and Zn for N^{3-} and S^{2-}. (Vallee and Williams, 1968)

1. 6. The effect of cadmium on enzymes

Metals are not universal components of enzymes, but virtually all classes of enzymes contain at least one which is metal-dependent. Thus the examination of both metalloenzymes and enzymes which are metal-activated is essential for the general understanding of the mechanics at the active site during substrate-enzyme interaction (Vallee and Williams, 1968).

Metal substitution at the active site is possible in many cases, and is an excellent technique for studying

the influence of the different substitute-metals on the enzyme, since it is a milder technique than many organic modifications, and usually leaves the enzyme with virtually unchanged activity. Carboxypeptidase A is a Zn-activated enzyme which can be actively substituted with cadmium to enhance the esterase activity and inhibit the peptidase activity. The overall effect of the exchange of metals was to induce a very minor alteration in the quarternary structure of the enzyme. The radical change in activity is indicative of the importance attached to the active site. Carboxypeptidase B undergoes a similar substitution with cadmium in place of Zn (Sarabhai, Stretton, Brenner and Bolle, 1964).

The now well-established binding of cadmium to thiol groups in the active sites of enzymes was investigated by Simon, Potts and Gérad (1947) in relation to succinic dehydrogenase, which was markedly inhibited in tissue brei incubated with 10^{-4}M cadmium. Simon *et al* maintain that tyramine oxidase, also a "sulphydryl" enzyme, is not inhibited by cadmium. They ascribe this to the protection afforded the active site by the conformational structure of the enzyme. Oxidative phosphorylation is uncoupled by very low concentrations of cadmium. The effect can be partially reversed, as

is the inhibition of succinic dehydrogenase, by chelation of Cd^{II} ions with ethylene diamine tetra-acetic acid (E.D.T.A.) or with dithiols (Simon et al, 1947; Fletcher, Fluharty and Sanadi, 1962).

Lipoamide dehydrogenase was markedly inhibited in vitro by cadmium when the enzyme was in the purified form, and also when present in the complete 2-oxogluterate enzyme system (Sanadi, Langley and White, 1959). These observations are in agreement with reports by the Japanese workers Misaka and Nakanishi (1966).

In general, interactions of cadmium with the sulphydryl groups of many enzymes led to marked inhibition of the activities of those enzymes. There are however, some interesting variations as regards the response of certain enzymes to cadmium. Rifkin (1965) remarked on "a fair degree" of inhibition of Na^+, K^+ -stimulated adenosine triphosphatase activity and Schaub and Ermini (1969) reported that Cd^{II} initially stimulated Mg^{II} activated ATPase, when Mg^{II} was absent. However, addition of Mg^{II} initiated a competitive type of inhibition between the two metal-ions.

As has been previously mentioned, cadmium ions have a particularly interesting action on carboxypeptidase B. They elicit opposite effects with the two substrates of the enzyme. Esterase activity is markedly enhanced by

Cd^{II}, whilst peptidase activity is totally abolished. Co^{II} acts conversely (Folk and Gladner, 1961). The significance of these findings is that an activating ion may have a profound influence on the specificity of an enzyme.

Mitochondrial enzymes *in vitro* are extremely sensitive to inhibition by cadmium, and one of the intentions of this work is to determine to what extent *in vitro* observations can be extended to embrace the situation wherein cadmium acts *in vivo*. Under normal circumstances only approximately 10% of all the enzyme molecules present in the organelles are in an active state. Sonication or detergent-disruption increases the activity of the mitochondrial enzymes about ten-fold (Smoly, Kuylenstierna and Ernster, 1970). This fact must be borne in mind in attempting to compare the effects of cadmium on the activity of mitochondrial enzymes *in vivo* and *in vitro*. In mitochondrial enzymes the inhibitive mechanism is thought to be either through mercaptide formation or by displacement of any other cations essential for activation of enzymes.

The haem enzyme, tryptophan oxygenase, operative at the rate-controlling step in catabolism of tryptophan to nicotinamide has been observed, in the liver of cadmium-

poisoned rats, to be stimulated at low concentrations of Cd^{II} and inhibited at higher levels (Kench, Gubb and Sutherland, 1969).

Trypsin was activated 25% above control values by cadmium (Green and Neurath, 1953) in contrast to the powerful inhibitions by Hg^{II}, Ag^{I} and Cu^{II}. It is fallacious, therefore, to describe the action of cadmium in all cases as that of a "typical" heavy metal. Cadmium also stimulated prolinase, arginase and pyruvate decarboxylase (Dixon and Webb, 1965b).

Oxidation of 1-C^{14}-glucose to carbon dioxide is prevented in the presence of thiol inhibitors, including cadmium, although cadmium is not as efficient an inhibitor as is arsenite or p-chlormercuribenzoate (Dixit and Lazarrow, 1967).

Whether cadmium is innocuous or otherwise to individuals in the course of everyday life, whether it is an essential requirement for man, and how its direct actions on enzymes are modified within the cellular milieu are questions which appear worthy of investigation.

The purpose of the present study is to establish the disposition of cadmium within the liver of cadmium-intoxicated chicks and to attempt to relate intracellular concentrations of the metal to its <u>in vivo</u> action on

enzymes against a background of knowledge of the direct action of cadmium on the isolated, purified enzyme. It is hoped that conclusions may be drawn as to the localisation of enzymes within cellular structures; to establish whether the enzymes are exposed, hidden or protected by neighbouring compounds from the assault of cadmium ions. In short, to learn more, if possible, about the molecular structure of cellular organelles by employing cadmium as a metabolic probe.

I. 7. General Considerations

The work embodied in this thesis is in the nature of an exploratory investigation. It could not be a definitive one at this stage, as it was realised that there was insufficient information on many of the important factors operating *in vivo* to be able to set up adequate controls.

Factors recognised to be of obvious importance but whose role in any differences between *in vitro* and *in vivo* activities of enzymes cannot clearly be defined are as follows:-

(i) Temperature

Avian body temperature is 40°C, but the temperature chosen for the experiments was 37°C, this being the temperature of incubation of the eggs. This does not exclude the possibility that within the microenvironments of cells and tissues there could be variation of temperature associated with high metabolic energy, as witness the elevated temperature of the cytochrome-rich brown fat in the human neonate.

(ii) Time

For the *in vitro* experiments, pure enzymes or homogenates were incubated with appropriate concentrations of Cd^{II} for short periods (5 - 10 min). *In vivo*, the tissues were subjected to the action of cadmium for a number of days, probably in concentrations approximating those which were finally calculated from quantitative cadmium analysis in poisoned chicks.

The disposition of cadmium around the body following a single pulse dose is rapid.

The time factor is further complicated by the question of enzymic turnover, which is very rapid for liver enzymes. For example, the half-life of hepatic catalase is approximately 24 hrs. (Handbook of Biochemistry, 1968). The duration of exposure of individual enzyme molecules would

be limited by their physiological life span and would, therefore, be much shorter than the time that cadmium had resided in the cell. These parameters cannot be precisely assessed.

(iii) pH

A well-known characteristic of enzymes is their dependence on pH, a classic example being acid and alkaline phosphatases.

This dependence on pH stems from ionisation of key groups on the enzyme or substrate, and in particular in this study, on the ionisation of cadmium itself. The dependence on ionisation of the enzyme and substrate was studied by Apps (1968), using NAD-kinase; and by Clarkson and Kench (1958), who studied the binding of lead to the phosphate groups of erythrocyte membranes.

The optimal assay pH *in vitro* may not be that functional *in vivo* for a particular enzyme. The assay pH employed may also not be the one most effective in expediting the interaction of cadmium with the enzyme.

(iv) Distribution of cadmium

Cadmium may not be uniformly distributed even within an organelle such as the mitochondrion or microsome.

There could also be differences in the quantity of the metal bound by individual mitochondria, depending on age and metabolic activity.

The mean concentration of cadmium within the mitochondrion may thus be only a crude approximation of the concentration actually present in apposition to the enzyme assayed. The dehydrogenases are located in the inner matrix of the mitochondrion, and it is known that certain substances are not transported into that region of the subcellular particle (Smoly et al. 1970). Whether cadmium enters the inner matrix or not is still to be determined.

(v) Non-enzymic binding sites for cadmium

The fact that pro-enzymes and non-enzymic catabolic products could bind Cd^{II} more effectively than the holoenzyme itself has not been ignored, and, indeed, has been a matter for concern.

(vi) Inhibition of biosynthesis of enzymes

The biosynthetic site of the enzyme may be exposed to quite a different concentration of cadmium from that to which the enzyme is subjected in the mitochondrion. Evidence for biosynthesis of cytochromes within the mitochondrion is in a state of flux. Most enzymes, however, are formed within the ribosomes on the endoplasmic reticulum, and pass

subsequently to their cellular sites of action. As cadmium may act at the ribosomal level, biosynthesis may be vulnerable to alteration by the metal ions. This argument takes no account of variations in coenzymes, or derangements in transport of constituent amino acids.

With these considerations as a background, the activities of certain enzymes will be compared *in vitro* and *in vivo*, in the presence of similar concentrations of cadmium. Differences in time of exposure and other factors which could not be controlled will be considered in the analysis of the experimental findings.

I. 8. Objectives of the present study

The salient objectives of the study described in this thesis were as follows:-

(a) To obtain information on the action of cadmium on differentiating and developing tissues, as exemplified by the chick embryo, since although much is already known about the toxicology of cadmium in man and certain animals, very little is known of the effect of the metal on developing tissues.

(b) To determine whether there is a relationship between the degradation of ovalbumin and the synthesis of serum albumin, and whether a low-molecular albumin (mini-albumin) is formed.

(c) To broaden the knowledge of the biochemistry of cadmium in relation to its known biochemical actions and effects, and the influence it has both on various enzymes and on clinical disease; viz. emphysema, chronic renal failure, vascular necrosis, hypertension, proteinuria and anaemia.

(d) To explore inter-relationships of cadmium as a heavy metal with enzymes possessing active centres differing in chemical structure, namely:- thiol groups; both shielded,(malate dehydrogenase) and labile,(lipoamide dehydrogenase); enzymes with a haem prosthetic group (catalase, tryptophan oxygenase and cytochrome oxidase); flavo-enzymes (xanthine dehydrogenase); or metal activated enzymes (ATPase, xanthine dehydrogenase).

(e) To investigate the question of the location of enzymes intracellularly in relation to their accessibility to cadmium,and to determine whether it is possible to employ a metal-ion to localize an enzyme within cellular organelles, e.g. mitochondria, microsomes.

I. 9. Contributions of this study

The following findings emerged from the work on which this thesis is based:-

(a) Cadmium injected into the developing chick had profound, and often bizarre effects on the animals. The early chick embryo was extremely sensitive to very low doses of cadmium (5 µg would kill the chick if administered before the eighth day of incubation). Later poisoning (12 µg at day 13, 16 µg at day 15) elicited syndromes with a wide variety of lesions. The most common of those noted were: central nervous system disturbances (balance mechanisms impaired); muscular weakness; frequent hepatic necrosis; gross oedematous lesions on limbs, neck and head. Possibly the most bizarre of all, which also occurred in ostriches poisoned proportionately, was the failure of some of the chicks to absorb the yolk-sac into the peritoneal cavity, a mechanism usually timed to occur the day prior to hatching. The inability to accomplish this essential function usually resulted in the death of the newly hatched chick within 12 hours.

Many poisoned chicks could not summon the strength to hatch by themselves, and had to be helped. The poisoned chicks were often a day or two later in hatching than were normal chicks.

(b) Serum albumin and total protein concentrations

were slightly lower in the intoxicated chicks.

Albumin was prepared from the serum of both normal and cadmium poisoned birds. A low molecular weight protein was detected in the albumin preparations when they were chromatographed on a Sephadex G-75 column. There was no significant difference in the quantity of this protein recovered from either the normal or intoxicated chicks. The fraction of the albumin which constituted the low molecular protein varied from trace amounts to approximately 15% of the total preparation.

(c) Cadmium-poisoning distinctly depressed the level of circulating haemoglobin.

(d) The optimum time of preincubation of cadmium with the enzyme during the in vitro assays was determined as being fifteen minutes for the majority of enzymes. A greater length of time often led to non-specific enzyme denaturation due to factors not entirely associated with metal-enzyme interactions.

The precise period the enzyme was exposed to cadmium in its intracellular environment could not be estimated, but it appears from the results that the most probable length of time was the enzyme's physiological lifespan.

(e) Mitochondrial enzymes were assayed when in the intact organelles and when liberated by detergent-

disintegration. The activity in the latter case was invariably found to be 5 - 10 times higher, indicating that only 10 - 15% of the total enzyme activity in the mitochondria is functional at any time in the intracellular environment.

(f) The interactions of cadmium with purified enzymes, "crude" enzymes present *in vitro*, or the same enzyme *in vivo* in the cellular environment, were studied. Ten enzymes were selected and examined in detail. The salient facts and conclusions regarding the behaviour of these enzymes were such as to allow the enzymes to be classified into 5 groups, as shown below:

(i) Enzymes inhibited by cadmium both *in vitro* and *in vivo*:-

ALA synthetase, xanthine dehydrogenase and (to a much lesser extent *in vivo*) lipoamide dehydrogenase.

Assuming full ionisation, the number of Cd^{II} ions present *in vitro* for each lipoamide dehydrogenase molecule and responsible for 40% inhibition, was approximately 7 gm. atoms/molecule of enzyme. This value was much smaller than for the other two enzymes in this group (approximately 500 gm atoms Cd^{II} for each ALA synthetase or xanthine dehydrogenase molecule).

(ii) Enzymes inhibited *in vitro* but not *in vivo*:-

Succinic dehydrogenase and cytochrome oxidase.

The former was more sensitive to Cd^{II}, requiring approximately 10 g-atoms Cd^{II} per enzyme molecule for a 30 - 40% inhibition. Depression of cytochrome oxidase to the same degree required twice as much cadmium as this.

(iii) An enzyme resistant *in vitro*, but whose biosynthesis (or that of its prosthetic group, haem) was impaired by cadmium:-

Catalase.

The concentration of cadmium, approximately 6×10^{-5}M, inside the liver cells was capable of lowering catalase activity significantly, but had no effect on catalase *in vitro*.

(iv) Enzymes stimulated by cadmium, either *in vitro* or *in vivo*:-

Tryptophan oxygenase, soluble malate dehydrogenase, adenosine triphosphatase (erythrocytic) and, in certain cases, ALA-dehydrase.

The first three enzymes mentioned manifested elevated activity *in vivo* at cadmium concentrations in the range $2 - 7 \times 10^{-5}$M, while the tryptophan oxygenase was also stimulated *in vitro* by $0.5 - 2.0 \times 10^{-4}$M.

ALA-dehydrase was stimulated both *in vitro* and *in vivo* by a low concentration of Cd^{II} (2×10^{-5}M), but was rapidly

and progressively inhibited at higher concentrations in both environments.

(v) An enzyme insensitive to Cd^{II} both *in vivo* and *in vitro*:-

Mitochondrial malate dehydrogenase.

The concentration of Cd^{II} ranged from 1×10^{-6}M to 1×10^{-4}M *in vitro*, but elicited no change in enzymic activity. This was the only mitochondrial enzyme investigated to show this stability *in vitro*.

(g) Cadmium was assayed in four fractions, the nuclei and cell membranes, mitochondria, microsomes and the cytosol. After acid digestion, the concentration of the metal in each intracellular fraction was determined by atomic absorption spectrophotometry. The concentrations of Cd^{II} found in the various intracellular fractions were then used in *in vitro* assays of the applicable enzymes. In this way it was possible to relate the activity of the enzymes in the particular intracellular fraction with the concentration of cadmium that would be available in that region of the cell *in vivo*.

In general, cytoplasmic enzymes were more susceptible to cadmium than were the mitochondrially-located enzymes *in vivo*. There were, however, a number of anomalies which will be fully discussed in the following text.

PART II

EXPERIMENTAL AND RESULTS

(A) GENERAL STUDIES

II 1. Routine procedure for experiments with chicks.

(i) Incubation conditions

Fertilized eggs were obtained from Silversands Farm, Constantia, Cape Town, after 7 days of incubation.

The eggs were a cross between White Leghorn and Black Australorp. They were incubated at 36°C and 40% humidity, and were gently turned through 180° twice daily. There was a constant circulation of air through the incubator.

When the chicks hatched they were transferred to another incubator, and kept at 28°C, and 40% humidity. They were supplied with water and chick-meal ad libitum.

(ii) Method of intravenous inoculation of chick embryos

The procedure adopted was a modification of the method described by Beveridge & Burnet (1946).

A 12 - 15 day old egg was held against a strong light-box and a prominent blood vessel was mapped out on the shell. The direction of blood flow was ascertained by noting where the various arteries merged to form the umbilical artery. The vessels were usually most prominent at a junction.

The shell over the blood vessel was gently cut away, using a motor-driven dental drill fitted with a sharp rotary cutter. The cutter was used to incise just down to, but not through the outermost membrane. The area of shell bounded by the incision lines was gently removed with a scalpel.

Care had to be taken not to rupture the chorio-allantoic membrane, since this would cause the arteries under the membrane to collapse inwards, together with the whole egg contents. Such an occurrence would render the artery inaccessible, apart from the additional large risk of infection to the developing chick.

Using a glass rod, a small drop of sterile liquid paraffin was placed on the white, opaque membrane, to render it transparent. (Fig 1).

A No. 27 needle was then fitted to a tuberculin syringe held firmly in the injecting apparatus, and containing sterile cadmium chloride solution (100 or 200 µg Cd/ml) in isotonic saline. The needle was advanced very close to the membrane immediately above the artery, and, with a short, deliberate movement, was inserted into the artery, pointing in the direction of blood flow. (Fig 2). The screwtype injector was then manipulated to introduce 0.05 to 0.10 ml $CdCl_2$ (12 to 20 µg Cd) very slowly into the artery. When the required volume had been injected, the needle was carefully withdrawn, without additional trauma to the blood vessel. The exposed membrane was then covered with cellotape, and the eggs replaced in the incubator.

After innoculation, the exposed area of the eggs was kept uppermost as the egg lay in the incubator, in order to prevent allantoic fluid from dripping from any possible rupture in the membrane. The eggs were

Fig 1: Appearance of the blood vessels immediately under the shell on the 14th day of incubation.

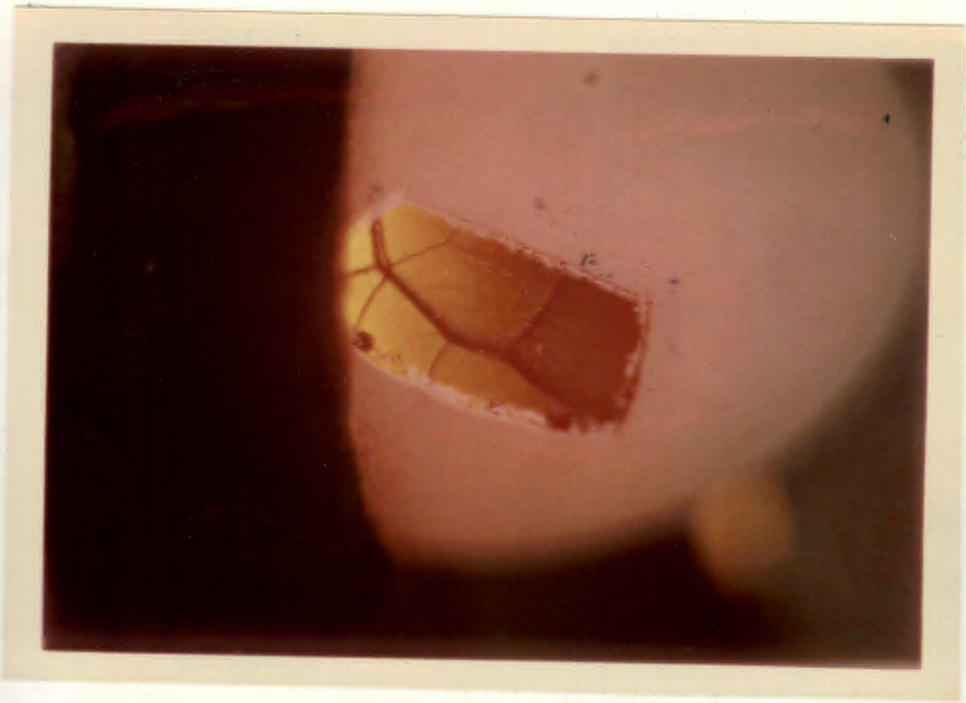
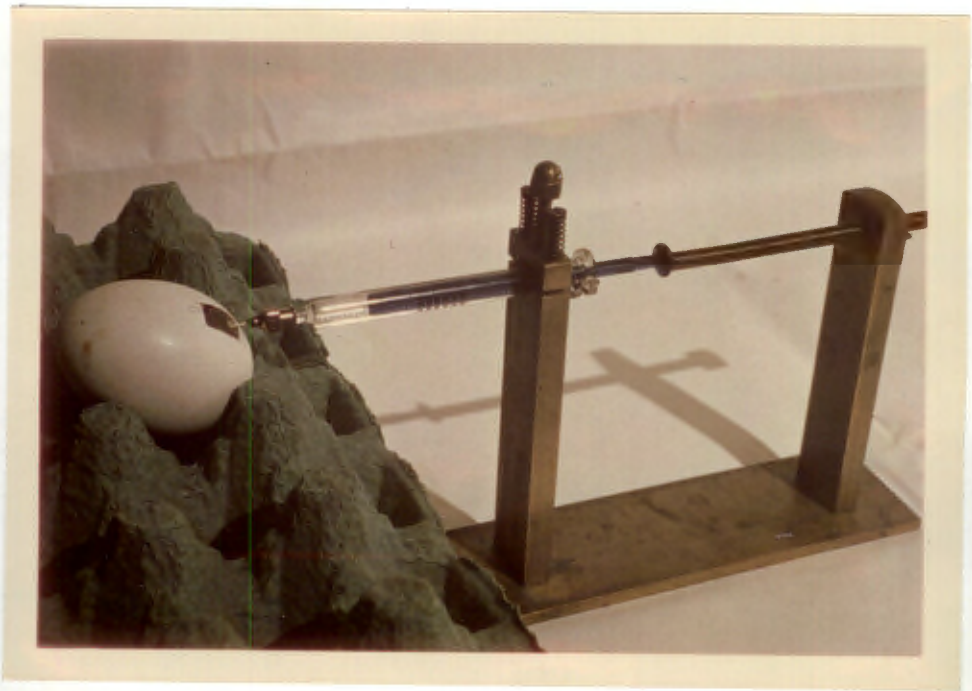


Fig 2: The apparatus used to inject exact quantities of sterile cadmium chloride into the arteries of the developing chick embryo.



gently turned through 90^{0} twice daily for the rest of the incubation period.

The quantity of cadmium injected depended very much on the age of the embryo. The optimal dose was taken as that quantity of cadmium which would kill approximately 50% of any particular sample of embryos at a given age. This was termed the "Lethal dose for 50% of sample," and abbreviated $L.D._{50}$ (Table 1 and Fig 3).

(iii) Method of collecting blood from chicks

A newly hatched day-old chick possesses a total volume of 3.0 - 3.5 ml. blood. As clotting takes place very rapidly in chicks it became necessary to devise a method for collecting the maximum amount of unclotted blood for use in enzyme-assays and for routine biochemical assays.

Heart-puncture, or withdrawal of blood from the aorta or vena cava vessels gave small yields which were either partially clotted or haemolysed. It was therefore necessary to sever a major vein or artery to obtain sufficient volume and to minimise clotting effects. The femoral vein or artery was used. The chick was stunned or anaethetised with ether, and the skin cut away to bare the groin. One very small drop

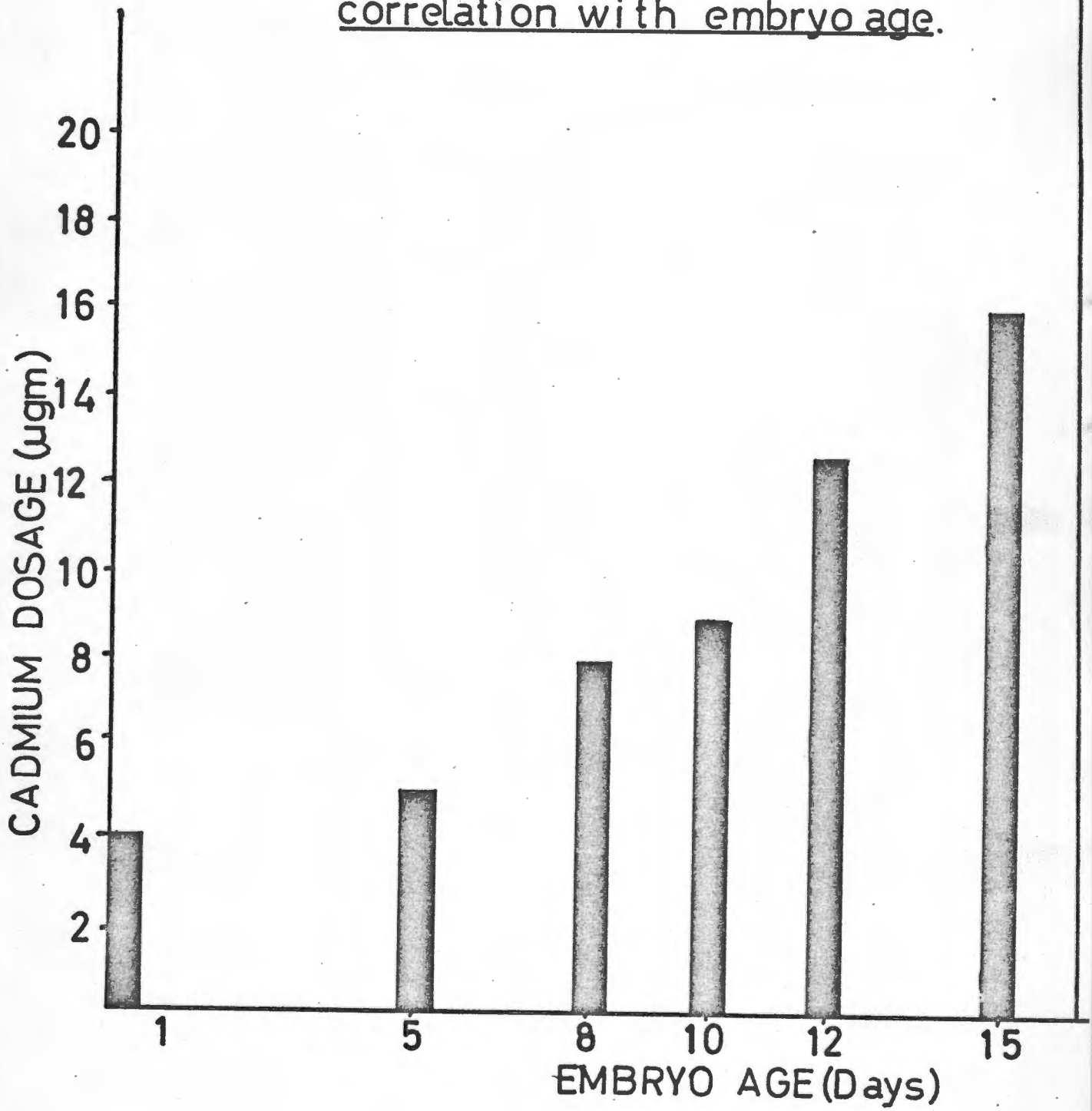
TABLE 1.

POISONING PROFILE

AGE OF EMBRYO (Days)	CADMIUM DOSAGE (μgm)	
	L.D. 35	L.D. 100
7	3	6
8–9	4	7
10	5–6	10
12	8	12–14
14	10–12	16
15	14	25
16	15	30

FIG. 3.

The $L.D_{50}$ intravenous Cd dosage correlation with embryo age.



of heparin was inserted into the joint, and the vein or artery severed with a pair of scissors.

In order to prevent haemolysis, blood was collected into a syringe without using a needle. The bleeding continued slowly for some time after the major volume of blood had been relatively rapidly removed. This "leaking" contained a high proportion of tissue fluid, lymph and cellular enzymes, particularly aldolase. It was thus discarded, as it would give erroneous results.

(iv) <u>The appearance of newly-hatched chicks poisoned by cadmium during embryological development</u>.

The morphological lesions of cadmium poisoning in the newly-hatched chick were numerous, and frequently bizarre (Figures 4, 8 and 9).

Among the commonest of the morphological abnormalities was the gross oedematous appearance of the neck, cranium and the limbs, particularly the feet. Nearly all chicks subjected to 10 μg Cd^{II} or more prior to day 14 exhibited this lesion. A generalised weakness accompanied the oedema, with the result that the young chick was often unable

Fig 4: The appearance of newly hatched chicks which had been poisoned on day 12.
(From l. to r.) 5 µg; 10 µg; normal; 7 µg Cd^{II} administered.

to break its way out of the shell on the twenty-first day. If these chicks were not manually assisted, they frequently died of exhaustion. The general development process of the gestation period was significantly retarded, so that the more severely poisoned chicks which did remain alive often made their first attempt at emerging from the shell 12 to 36 hours after the normal chicks had hatched.

The most bizarre of the immediately apparent effects which the poisoning had on the chicks was incomplete intra-abdominal absorption of the yolk-sac. This process usually occured during the last two days of embryological growth, and it appeared that the poisoned chicks were unable to carry out this essential function. Chicks which did hatch with incomplete yolk-sac absorption usually died within 24 hours.

The chick liver was often very friable, and appeared either greenish-yellow (bilirubin) or a dull off-white colour. The liver was almost invariably necrosed if the yolk-sac was not correctly absorbed. Histological examination of the liver from poisoned chicks shows a marked degree of bile-stasis.

Occasionally the chicks were born blind, or with a head-and-neck retraction, thought to be opisthotony.

The feet were often deformed, and the poisoned chicks showed a marked lethargy, even a day or two after hatching.

The cadmium treated birds usually slumped close to the surface on which they were standing and took some time to assume a normal walking posture. This "slumping" was occasionally extended to the head and neck. Balance was sometimes lacking, and the chicks stumbled rather than walked during the first two days after hatching.

The feathers of severely poisoned chicks were occasionally off-white in colour as compared to their normal yellow counterparts. Parallel to this, the yellow colour of the liver was usually absent in these cases, suggesting a decrease in the α - carotene content. The serum of cadmium-poisoned chicks was often lipaemic, indicating a disturbance in the absorption mechanism governing metabolism of the yolk-sac contents.

II 2. THE GENERAL BIOCHEMICAL MANIFESTATIONS OF CADMIUM-POISONING IN CHICK EMBRYOS.

Several different biochemical parameters were assayed in the sera of normal chicks and of those intoxicated with cadmium, in an attempt to assess the changes in overall metabolism brought about by the metal. The serum parameters measured were:

Urea, uric acid, lactic dehydrogenase, glutamate-oxalacetate transaminase, glucose, and electrolytes (Na^+, K^+, Cl^-).

The figures in the tables comprise the mean and standard deviation (where applicable). The number of individual chicks assayed is in brackets.

(i) Urea and Uric Acid

These two compounds were measured on the auto-analyser in the Chemical Pathology routine diagnostic laboratories, and were used primarily to obtain an index of renal function.

Methods

The urea was measured by the diacetyl monoxime method of Marsh, Fingerhut and Miller (1965).

Uric acid was determined by a method adapted from the manual procedure and involving reduction of a phosphotungstate complex. The standard Technicon methodology was employed.

TABLE 2 Serum urea and uric acid concentrations in normal and cadmium-poisoned chicks.

	Normal Chick	Cadmium-poisoned Chick	p
Urea (mg/100 ml)	16.0 ± 7.0 (8)	15.0 ± 7.0 (7)	NS
Uric acid (mg/100 ml)	10.7 ± 0.9 (8)	12.9 ± 1.3 (6)	NS

p = probability; and is considered significant if less than 0.05

NS = Not significant

(ii) Serum Enzymes: Lactic dehydrogenase (L.D.H.) and glutamate-oxaloacetate transaminase (G.O.T.)

These two enzymes are routinely assayed in the Chemical Pathology laboratory to detect heart or liver damage in patients. A series of normal and cadmium-poisoned chick sera were investigated to ascertain whether or not the metal intoxication exerted any influence on the above-mentioned tissue catabolism.

Method

L-aspartate and oxoglutarate were incubated with serum, and the rate of formation of oxaloacetate determined by the malate-

NADH system on the auto-analyser (Henry, Chiamori, Golub and Berkman, 1960).

TABLE 3 Serum L.D.H. and G.O.T. activities in normal and cadmium-poisoned chicks.

	Normal Chicks	Cadmium-poisoned Chicks	p
L.D.H. (I.U.)	565 ± 136 (8)	800 ± 135 (17)	0.05
G.O.T. (I.U.)	140 ± 35 (7)	165 ± 26 (8)	NS

The primary L.D.H. isoenzyme in serum was the slowest migrating muscle-type fraction. The raised level is not diagnostically significant of any particular lesion, and is probably due to non-specific tissue damage.

(iii) Serum Electrolytes

Two serum ions, Na^+ and K^+, were measured by flame photometry using a Technicon auto-analyser, and Cl^- was determined by electromeric titation (Cotlove, Trantham and Bowman, 1958).

The results give an index of renal function and were consequently used to monitor the effect of cadmium on the kidney in day-old chicks.

TABLE 4 Concentrations of electrolytes in the serum of normal and cadmium-poisoned chicks.

	Normal Chicks	Cadmium-poisoned Chicks	
		acute	chronic
Na^+ (meq/l)	152 (5)	150 (4)	165 (1)
K^+ (meq/l)	4.5 (5)	4.3 (4)	2.6 (1)
Cl^- (meq/l)	99 (5)	101 (4)	102 (1)

The results for acutely poisoned chicks are not significantly different from normal, but the chronically-poisoned chicks show marked changes. These deviations from the norm, particularly the low potassium, tend to indicate a renal malfunction.

Chronic cadmium poisoning is known to induce renal tubular malfunction in monkeys (Sutherland, 1967; Kench, Gain and Sutherland, 1965). Therefore, the picture that appeared was one of direct renal involvement in chronic poisoning. Acute cadmium-poisoning did not appear to cause discernible hepatic or renal failure.

(iv) Blood Glucose

This was measured in newly-hatched chicks to determine whether or not hepatic metabolism was affected in the cadmium-poisoned chicks.

The method of assay was the one routinely employed in the auto-analyser in these laboratories.

Method

Glucose in whole blood was determined by the method of Hoffman (1937), which utilises the potassium ferricyanide-ferrocyanide redox reaction. The method has been modified for automation, using Technicon auto-analyser equipment.

TABLE 5 Blood glucose levels in normal and cadmium-poisoned chicks.

	Normal Chicks	Cadmium-poisoned Chicks	p
Blood glucose (mg/100 ml)	211 (7)	162 (6)	<0.05

The lower level of blood glucose in cadmium-poisoned chicks may indicate a hold-up in the metabolism of the yolk-

sac contents, which are ingested 1 - 2 days prior to hatching and rapidly metabolised during the first few days after hatching. Apart from the fact that cadmium-poisoned chicks grow more slowly than their normal counterparts, there is no evidence for this.

(v) Haematology

Over an extended period of time many different chicks were examined for haematological differences which may have arisen due to cadmium intoxication. The total number of cells (P.C.V. = packed cell volume), the total haemoglobin concentration (Hb) and the mean corpuscular haemoglobin count (M.C.H.C.) were studied. The results are shown below.

TABLE 6 Haematological results of investigations on normal and cadmium-poisoned chicks.

	Normal Chicks	Cadmium-poisoned Chicks	p
Haemoglobin (Hb)(gm./100 ml.)	10.1 ± 0.7 (16)	8.0 ± 1.01(8)	<0.05
P.C.V. (percent)	32.0 ± 1.8 (14)	25.5 ± 2.0 (9)	<0.05
M.C.H.C. (percent)	32.5 ± 1.4 (14)	28.5 ± 1.3 (9)	<0.05

The fall in Hb could be due to a single factor becoming affected by the cadmium, e.g. a lowered M.C.H.C. would indicate the same rate of cellular synthesis but less Hb found in each cell. Alternatively, a low P.C.V. and normal M.C.H.C. would correspond to poor erythrocyte synthesis, but normal Hb formation. Cadmium-poisoned chicks appear to suffer from a combination of both disturbances.

II. 3. (i) DETERMINATION OF TOTAL SERUM PROTEINS IN NORMAL AND IN CADMIUM-POISONED CHICKS.

Routine examination of the serum proteins of chicks was performed throughout the course of the experiments. Total protein concentrations were measured by the microbiuret method of Lane and Mavrides (1969).

The pattern of the constituent proteins was established by the Beckman "microzone" electrophoresis technique (Beckman Instruction Manual, RM - IM 3, 1965).

The procedure was standardised as regards buffer pH and ionic strength, potential gradient, application, sample size and duration of electrophoresis. The microzone cell consisted of two buffer compartments connected by a bridge

on which the supporting inert cellulose acetate membrane was rested. The ends of the membrane extended into the buffer system. A cover over the suspended membrane maintained a stable environmental humidity.

Membranes were impregnated with buffer prior to application of the sample by means of the standard Beckman Micro-applicator. The buffer was a 0.075 barbital-barbitone solution pH 8.6, and electrophoresis was performed at 250V for 20 min. The electrophoretically separated proteins were fixed and stained for 10 min. in a solution containing 0.2% (w/v) Ponceau S in 3.0% (w/v) TCA and 3.0% (w/v) salicylsulphonic acid. After rinsing in 5% (v/v) acetic acid, the strip was rendered transparent by immersion in an acetic acid/ethanol mixture and then dried.

Membranes were stored in protective clear plastic envelopes, and passed through a scanning device of the Beckman Analytrol. The serum protein pattern was assessed quantitatively in terms of colour intensity and peak width, computed on an integrator.

Comparison of serum proteins of normal and cadmium-poisoned chicks.

Serum proteins of normal and cadmium-poisoned chicks as a whole did not differ greatly, but an occasional pattern

from a cadmium-poisoned chick appeared grossly abnormal. The serum albumin concentrations tended to be decreased in the cadmium-poisoned chicks.

Albumin also constituted a smaller proportion of total serum protein in the poisoned birds than it did in normal chicks. The appearance of a pre-albumin peak in some of the grossly poisoned animals on hatching was one significant difference observed between normal and intoxicated birds (Fig. 5). The protein was present in younger embryos, but usually disappeared by the time the chicks hatched, or was present in such low concentration as to be undetectable by cellulose acetate electrophoresis (Polson, 1970).

Comparison of human and chick serum proteins.

The much lower concentration of both total protein and serum albumin as compared with human serum is readily evident from Fig. 6. Albumin from chicks migrated more slowly, and γ-globulins slightly more rapidly than did their human counterparts. However, adequate separation was achieved for normal scanning. Chick serum proteins separated into 4 peaks, namely, albumin, α1, α2 and γ-globulins. Avian serum contains no protein fraction corresponding to β-globulins (Spector, 1956).

The pre-albumin constitutes a fifth peak, but only in embryos of 12 - 18 days.

FIG. 5.
SERUM ELECTROPHORESIS.

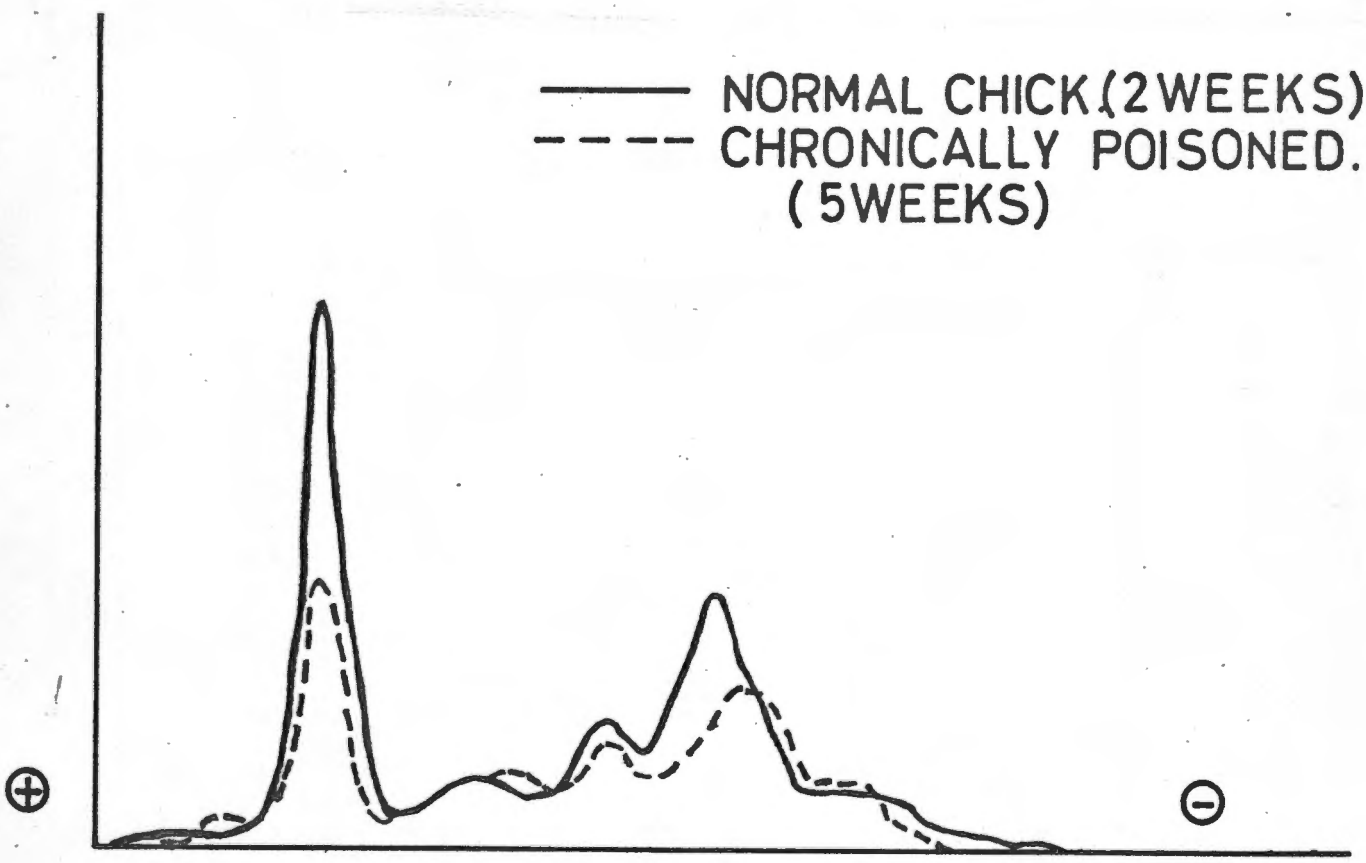
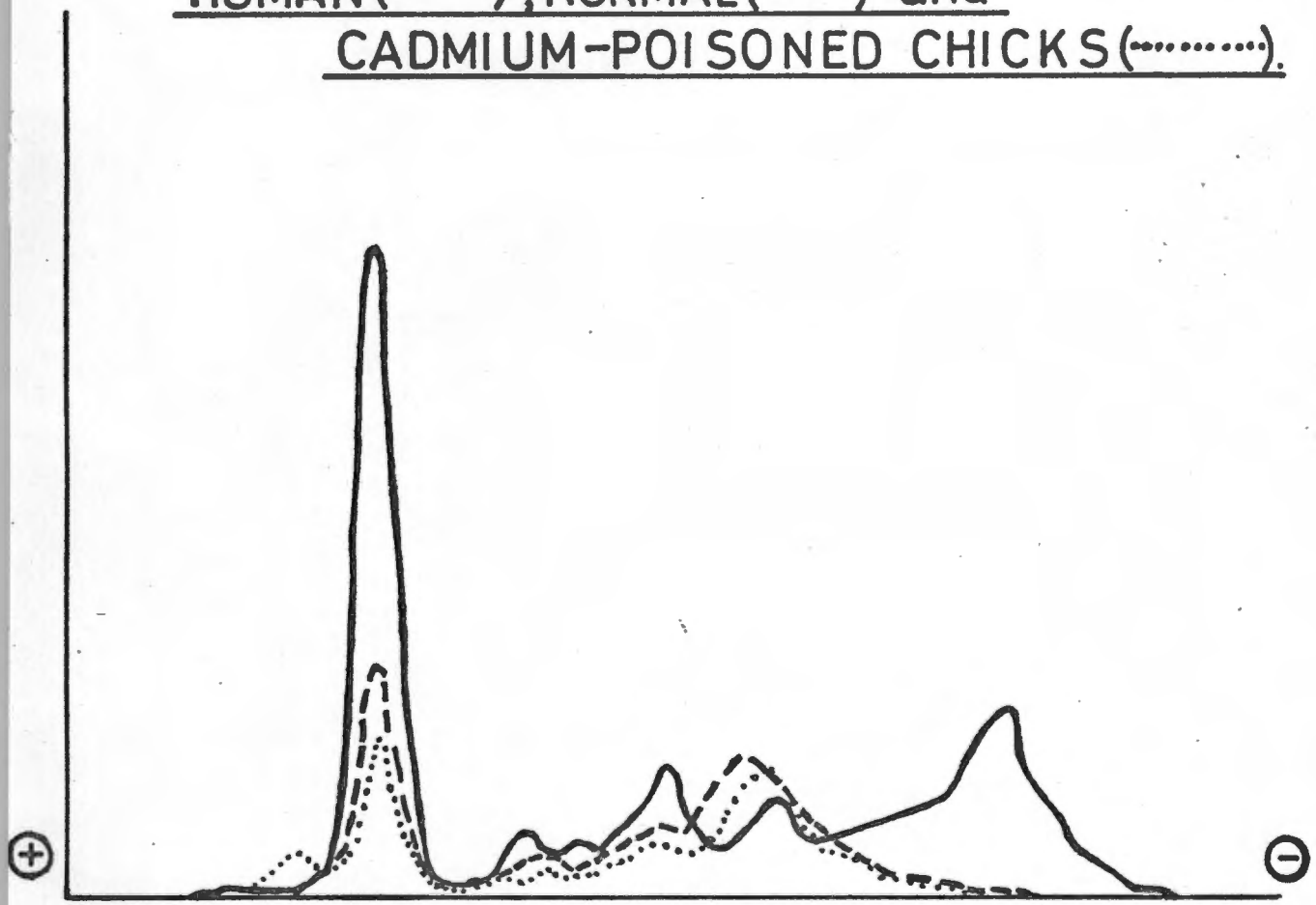


FIG. 6.

SERUM ELECTROPHORESIS.

HUMAN (——), NORMAL (----) and
CADMIUM-POISONED CHICKS (·····).



II. 3. (ii) THE EFFECT OF CHRONIC CADMIUM-POISONING ON CHICK SERUM PROTEINS.

Blood was taken from a two-week old chick prior to the initiation of a two-week period of intensive cadmium intoxication. The chick was again bled after the fortnight, during which time it received up to 100 µg cadmium by injection into the crop every two days. The total amount of cadmium administered in 9 separate injections was 5.5 mg. Serum was collected for electrophoresis and for total protein estimation.

The results of the control (before cadmium injection) and test sera (a week after completion of the poisoning schedule) are shown in Table 7.

TABLE 7 The quantitative change in the serum proteins of a chronically cadmium-poisoned chick.

	Age: 2 weeks		Age: 5 weeks		Change	
	mg/ml	% of Total	mg/ml	% of Total	mg/ml	% decrease
Total Protein	35	100	22.5	100	-12.5	35.7
Albumin	13	39	5.5	27	-7.5	57
α_1-globulin	2.5	7	2.0	9	-0.5	20
α_2-globulin	5	14	4.5	20	-0.5	10
γ-globulin	14	41	10.0	44	-4.0	28

The serum albumin suffered most severely from the massive dose of cadmium injected into the chick, contributing 60% to the overall decrease in protein concentration.

The chick lost weight dramatically during the poisoning. Although it was fed and watered ad libitum during the experiment, the weight loss (78.7 - 67.4 gm) was nearly 15%.

The liver weighed 3.5 gm, and the Cd^{II} concentration present in the liver was 118.5 µg/gm, constituting an overall 4% of the injected cadmium.

II. 4. PREPARATION AND MOLECULAR EXCLUSION CHROMATOGRAPHY OF PURIFIED CHICK SERUM ALBUMIN

Albumin was prepared from the chick serum by the method of Vallance-Owen and McMaster, (1968). The albumin was electrophoretically homogeneous, and was subsequently subjected to molecular exclusion on a cross-linked dextran gel (Sephadex G-75) to determine whether all the albumin molecules were the same size.

The purpose of these experiments was to determine whether the cadmium produced the low-molecular albumin in chicks that it does in chronically poisoned animals and man. (Smith, Wells and Kench, 1961; Kench, Wells

and Smith, 1962; Kench, Gain and Sutherland, 1965). It has been computed that approximately 50% of circulating albumin molecules in workmen chronically poisoned with cadmium were aggregated minialbumin (Kench et al. 1965; Kench and Sutherland, 1966) whilst it constituted up to 25% of the total albumin in chronically poisoned monkeys (Sutherland, 1967).

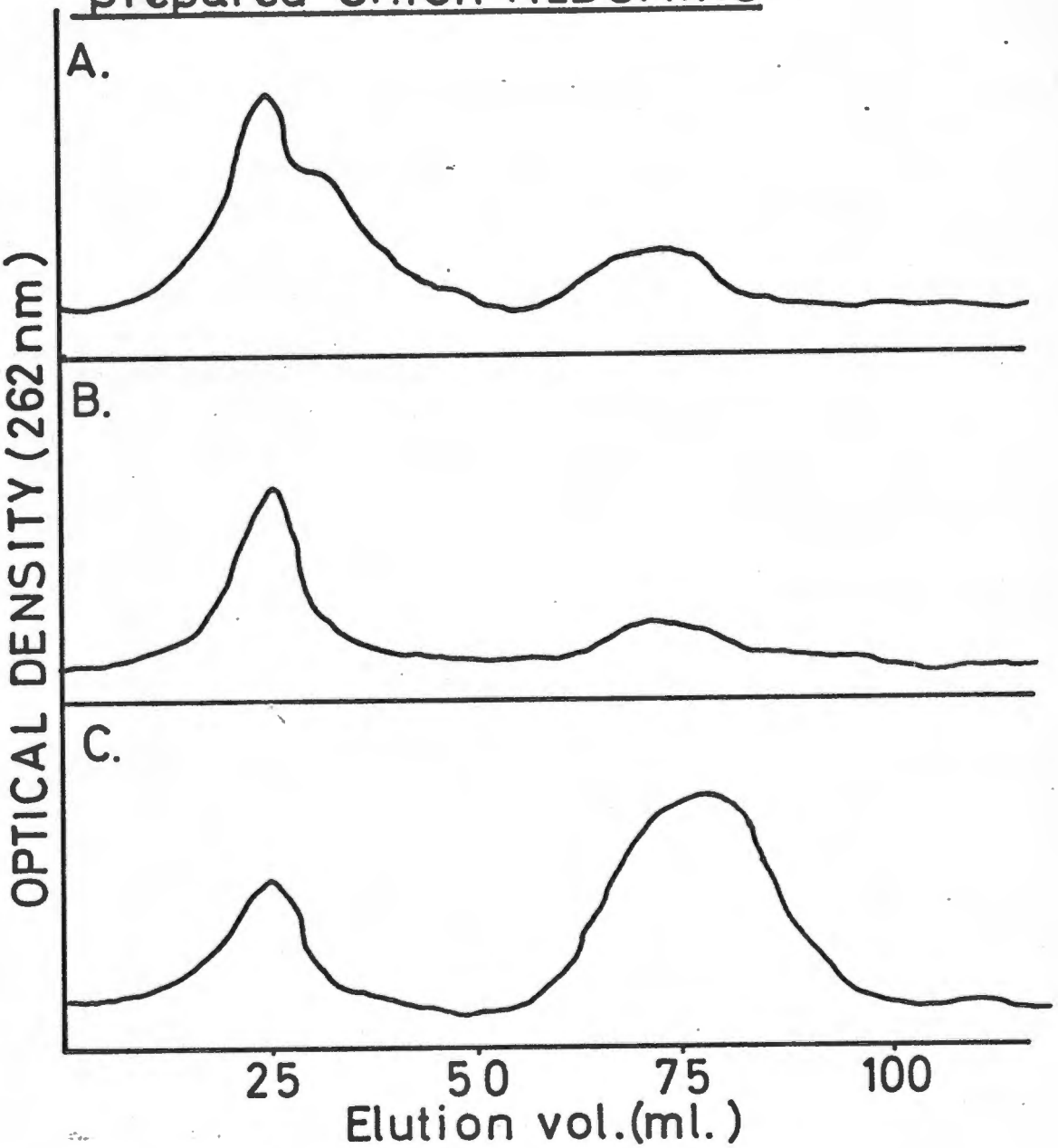
Sephadex G-75 efficiently separates the minialbumin (M.Wt. 5,000 - 15,000) from albumin of conventional molecular size (M.Wt. 67,000). The smaller molecules have a marked tendency to aggregate, especially in low ionic strength solutions (buffers), and in earlier work in this Department, NaCl and urea were added to the elution buffers to prevent this phenomenon.

Separation of the two albumin species was conducted on a Sephadex G-75 column (25 x 2 cm), developed with 0.2 M phosphate buffer, pH 7.4. The flow-rate was maintained by pump or gravity-feed at 18 ml/hr, and the eluate passed through a Beckman Uvicord recorder which monitored the optical density at 262 nm.

The eluted peaks were collected separately, desalted, (using Sephadex G-10 or dialysis in boiled cellophane tubing) and lyophilised. It may be observed from Fig. 7 that there was no increase in the production of minialbumin due to poisoning by cadmium. There was a trace of low molecular

FIG.7.

SEPHADEX G75 traces of prepared CHICK ALBUMINS.



A = Normal chick serum.
B = Cadmium-poisoned chick serum.
C = Normal chick Liver

albumin present in both normal and cadmium-poisoned day-old chicks, but the quantity varied with each preparation. The project was not pursued further, as it was decided that the single pulse-dose of administered cadmium was insufficient to produce the chronically poisoned state necessary for an increased production of the low-molecular weight protein (Kench, Gain and Sutherland, 1965; Kench and Sutherland, 1966).

Hepatic albumin preparation.

Albumin was prepared from the liver of normal and of cadmium-poisoned neonatal chicks. It had a very sticky texture, and when chromatographed on a column of Sephadex G-75, exhibited a large, disperse peak over the molecular weight range of minialbumin (Fig. 7). On further examination this peak was found to be low in protein, but relatively rich in α-carotene. This contaminant had been extracted with serum albumin into the ethanol - HCl extract. α-Carotene is a precursor of vitamin A, and has an intense yellow colour. The absorption maxima occurred at 485 nm and 454 nm. The yellow colour of neonatal chick liver may thus be ascribed to α-carotene. The presence of the pigment completely prevented quantitation of any minialbumin possibly present in the albumin preparation (Fig. 7) as

the chromophore was eluted by the same volume as a low molecular protein, and completely masked the quantitation by U.V. absorption of any protein component that may possibly have been present.

The protein concentration in the large peak was very low (0.02 mg/ml), which indicated that very little minialbumin, if any, was present.

II. 5. THE EFFECT OF CADMIUM INTOXICATION ON THE DEVELOPMENT OF THE OSTRICH EMBRYO.

A second avian species, the ostrich, was investigated in parallel with the chicks, as these larger birds would provide much more biological material for the numerous biochemical tests to be performed.

Fresh, fertilised eggs were obtained from Mr. Potgieter, Calitzdorp, Cape and Mr. Lipschitz, "Safari Ranch", Oudtshoorn.

Ostrich eggs are acknowledged to be very difficult to incubate successfully, and altogether 9 embryos died before hatching. Only two chicks, 1 normal and 1 cadmium-poisoned, having survived the full 6 weeks incubation period, were sufficiently developed to permit investigations to be conducted on them.

Methods

The eggs were incubated at 37°C in a humidified cabinet, and gently rotated through 180° twice daily. The full incubation period was six weeks.

After three weeks those chicks selected for poisoning had a small hole drilled through the shell into the air sac. 75 µg cadmium (as the chloride)in 0.9% (w/v) NaCl was carefully injected through the hole into the allantoic sac. Sterile equipment was used. The hole was sealed with cellotape (Beveridge and Burnet, 1946).

Intravenous cadmium administration was unsuccessful because of the difficulty in locating blood vessels owing to the opacity and thickness of the shell. Removal of a small piece of the shell resulted in the death of the embryo within a few days. The technique was abandoned after several attempts.

Two eggs, 1 normal and 1 poisoned, were opened with a rotary cutter two days after the end of the full incubation period, as neither showed any sign of spontaneous hatching. The normal ostrich had absorbed most of its yolk-sac, and the unabsorbed portion appeared healthy. The yolk-sac was tightly enclosed in a well-vasculated membrane. The chick appeared to be 1 - 2 days premature. It had died very shortly before the shell was opened, probably due to the excessive handling during the previous two days. (Figs. 8 and 9)

Fig 8: Normal (left) and cadmium-poisoned ostriches. The failure of the latter to absorb its yolk-sac may be observed.

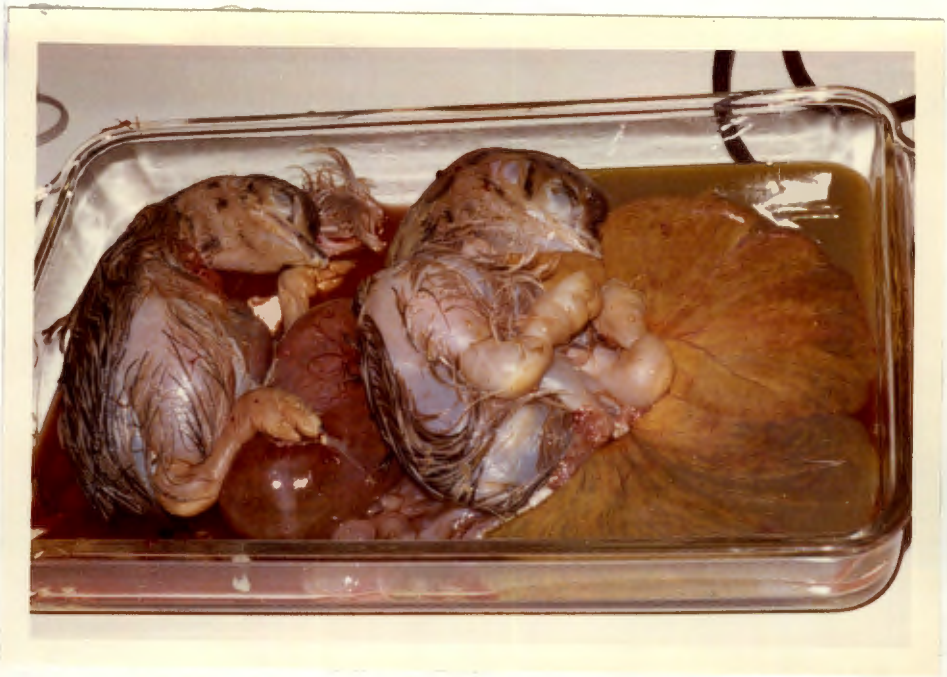


Fig 9: Leg and liver of cadmium-poisoned ostrich (left) as compared with normal. The bile stasis of the leg and pulpy oedema of the limb are direct toxic effects of cadmium.

Sufficient unclotted blood was obtained via heart puncture to conduct various diagnostic investigations and to prepare serum albumin.

The cadmium-poisoned embryo presented with several gross anatomical lesions - the most obvious of which were:-

(a) Extreme weakness. No spontaneous movement was observable, even with the shell opened at the air sac.

(b) A large, unabsorbed and very fluid yolk sac, insufficiently perfused with blood vessels.

(c) Gross oedema of the limbs, particularly the legs.

(d) An extremely friable, small liver, greenish in colour, a discoloration thought to be due to biliverdin.

Although still alive, the poisoned chick was obviously moribund, and doubtless would have been too weak to hatch by itself.

Blood was obtained from a superficial vessel immediately under the shell in which situation oxygenation of the blood takes place. The serum was heavily lipaemic, probably

as a result of mobilisation of yolk constituents. Serum protein electrophoresis patterns are shown in figure 10.

TABLE 8 Biochemical parameters assayed in normal and cadmium-poisoned ostriches.

	Normal	Poisoned
Uric acid	22 mg/100 ml	83 mg/100 ml
Urea	57 mg/100 ml	40 mg/100 ml
Total protein	28 mg/ml	24 mg/ml
Albumin	8.3 mg/ml	9.5 mg/ml
Total globulin	20 mg/ml	13 mg/ml

Preparation of albumin and subsequent gel filtration.

Albumin was prepared from the serum of the normal and cadmium-poisoned ostriches by the method of Vallance-Owen and McMaster (1968).

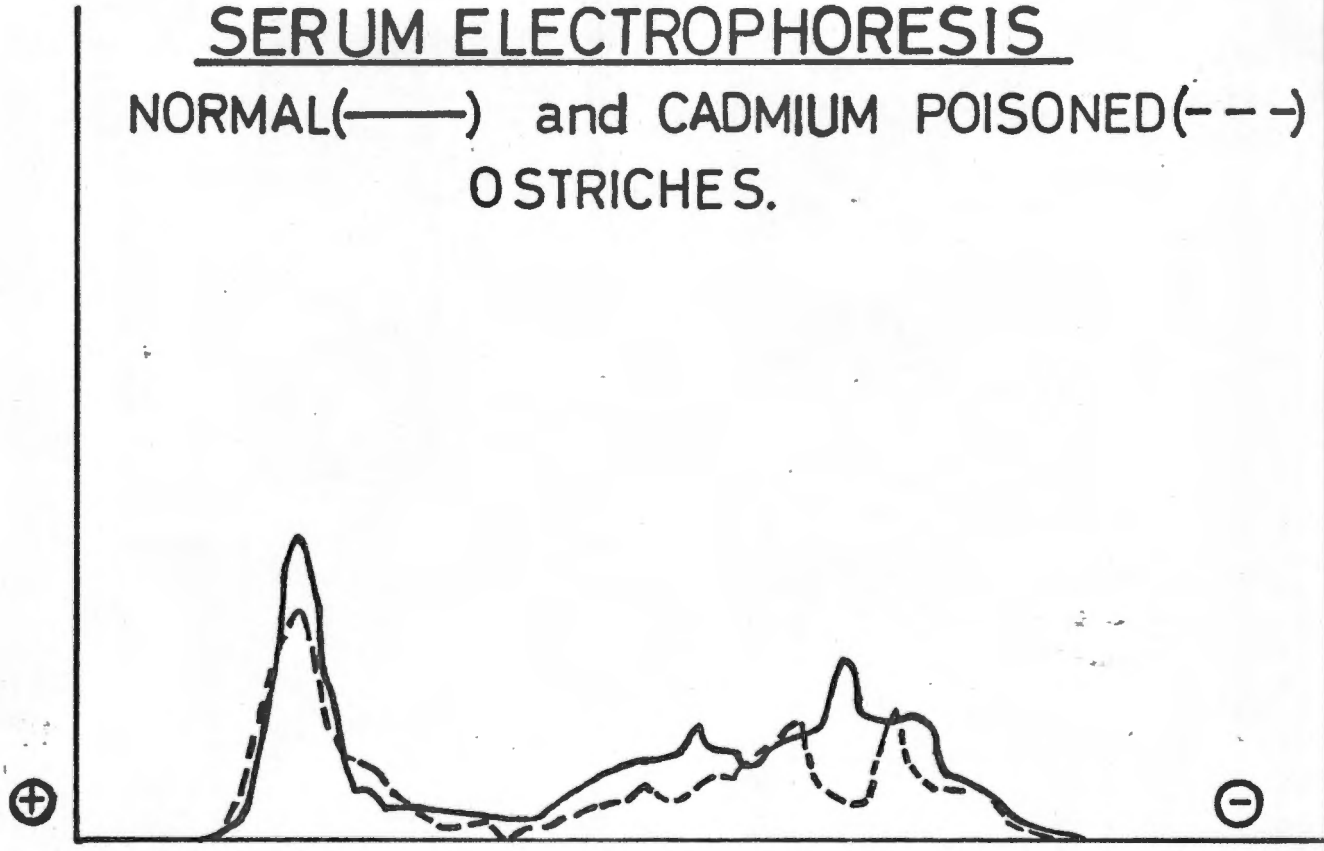
After removal of lipid from the isolated albumin with Bloor's reagent (ethanol : ether, 3 : 1) it was redissolved in buffer and subjected to gel filtration on a Sephadex G-75 column (2 x 25 cm). No minialbumin was detected in either the normal or the cadmium-poisoned bird.

FIG. 10.

SERUM ELECTROPHORESIS

NORMAL(——) and CADMIUM POISONED(- - -)

OSTRICHES.



Discussion

The results of the investigations on the ostriches are in accordance with both the accepted lesions attributable to cadmium-poisoning in many different animals, and with those manifested in the chicks.

The raised uric acid indicates renal damage, and the lowered serum proteins either a chronic infection or, more likely, an impairment in one of the normal biosynthetic mechanisms of embryological development. The liver of the cadmium-poisoned ostrich presented evidence of marked cellular damage, including bile stasis (Plate 3).

Had more success been achieved with breeding and intravenous poisoning of this species, it is certain that a clearer picture of the whole cadmium-poisoning syndrome as regards birds would have emerged.

II. 6. INTRACELLULAR DISTRIBUTION OF CADMIUM IN THE LIVER OF ACUTELY OR CHRONICALLY POISONED CHICKS.

This experiment was carried out in order to determine whether changes in activity of enzymes studied *in vivo* and *in vitro* in the presence of cadmium could be correlated with the distribution and concentration of cadmium in the various subcellular fragments.

1. Isolation of subcellular fractions.

The mannitol-sucrose-EDTA (MSE) medium described by Tyler and Gonze (1967) was used routinely. Preparation and centrifugation of the liver homogenate was performed exactly as described by Johnson and Lardy (1967).

The liver was removed and immersed immediately in ice-cold MSE medium and homogenised in a Potter-Elvjhem glass homogeniser, using a loose-fitting motor-driven rotary Teflon plunger. The liver tissue of the chicks was found to be more friable than human liver, and after six strokes the plunger moved freely through the homogenate. The process was not carried past this stage, as excessive homogenisation damaged subcellular particles. The loose-fitting Teflon plunger was considered to be the least damaging of the many types available, ground-glass and low-clearance homogenisers caused excessive rupture of mitochondria.

Differential centrifugation in a Sorvall RC-2B at $0^{o}C$ was employed to separate the subcellular fractions into cell debris (including nuclei, cell walls and erythrocytes), mitochondria, microsomes and the soluble components (cytosol). (Schneider, 1948).

Prior to centrifugation the homogenate was a distinct yellow, due to the high proportion of unmetabolised yolk constituents present in the liver immediately after hatching. The first centrifugation at 600 x g removed most of the solid matter, by sedimentation of the nuclei and cellular membranes, and flotation of most of the lipid into a firm pad 1 - 2 mm thick at the top of each tube.

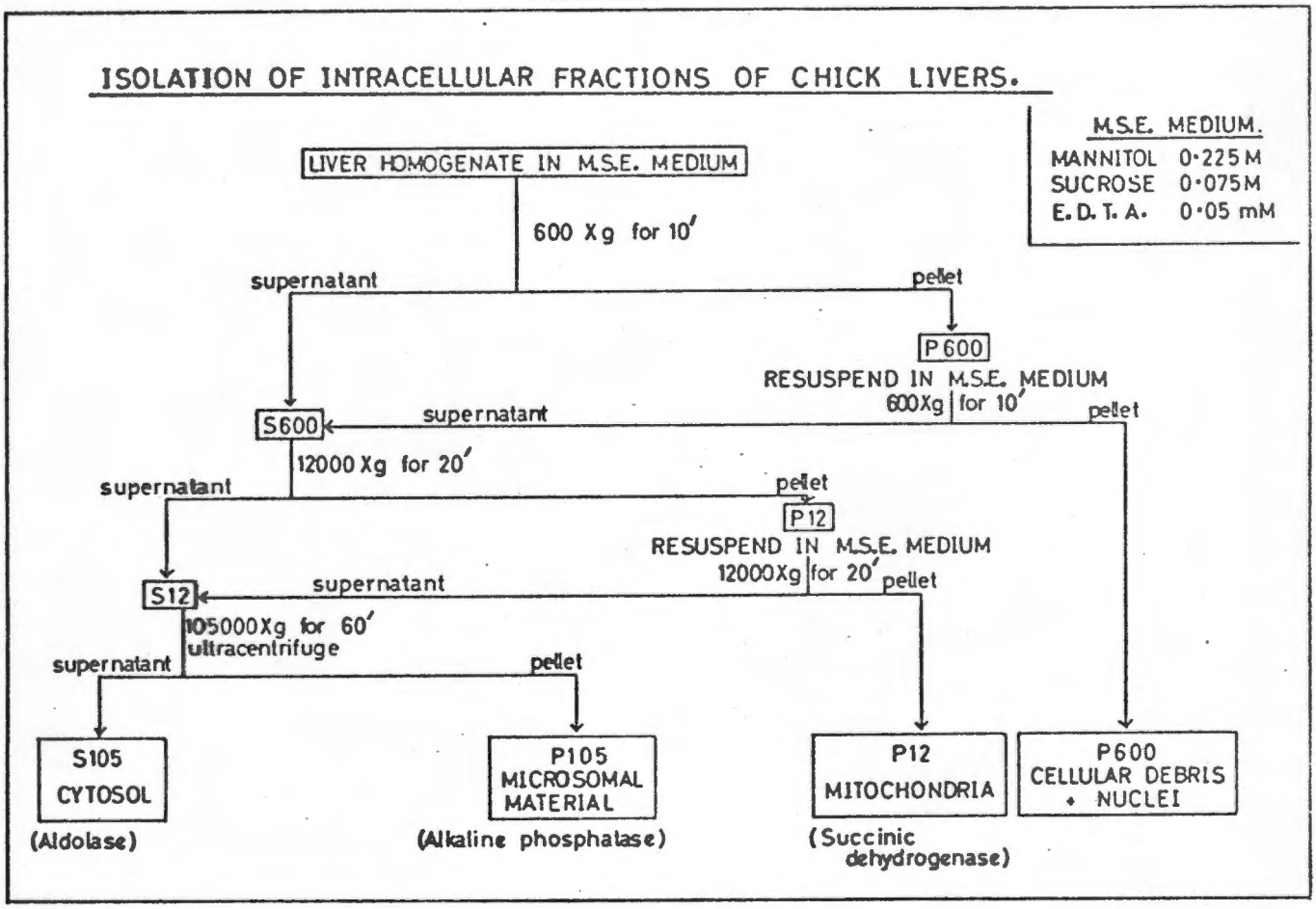
Subsequent centrifugations at 10,000 x g in the Sorvall RC-2B and at 105,000 x g in a Beckman preparative ultracentrifuge model L (35,000 r.p.m. for 60 min.) progressively cleared the initially opaque solution. The final cytosol was only faintly yellow-white in colour. The mitochondrial and microsomal pellets were pale brown. There was very little haemoglobin apparent in the homogenate at this stage in chick development.

The flow sheet for the preparation of these fractions is shown in Fig. 11.

2. Assay of homogeneity of subcellular fractions.

The purity of the isolated microsomes, mitochondria and cytosol was assessed in terms of their characteristic enzymes.

FIG.11.


ISOLATION OF INTRACELLULAR FRACTIONS OF CHICK LIVERS.
M.S.E. MEDIUM.
MANNITOL 0·225 M
SUCROSE 0·075M
E.D.T.A. 0·05 mM
LIVER HOMOGENATE IN M.S.E. MEDIUM
600 Xg for 10′
supernatant
pellet
P600
RESUSPEND IN M.S.E. MEDIUM
600Xg for 10′
S600
supernatant
pellet
12000 Xg for 20′
supernatant
pellet
P12
RESUSPEND IN M.S.E. MEDIUM
12000Xg for 20′
S12
supernatant
pellet
105000Xg for 60′
ultracentrifuge
supernatant
pellet
S105
CYTOSOL
(Aldolase)
P105
MICROSOMAL
MATERIAL
(Alkaline phosphatase)
P12
MITOCHONDRIA
(Succinic dehydrogenase)
P600
CELLULAR DEBRIS
+ NUCLEI

Alkaline phosphatase occurs predominantly in microsomes, although traces of the enzyme may be detected in the nuclei and mitochondria. Aldolase is located only in the cytosol and succinic dehydrogenase is confined to mitochondria (Dixon and Webb, 1964 c). The activity of each of these enzymes was assayed in all fractions. The relative specific activities of the enzymes in each fraction were then taken as an index of the purity of the fraction (Table 9).

This procedure was applied to a pooled sample of 4 livers.

The dry weights of the fractions after correcting for the weight of the medium were found to be:-

Fat + debris	0.40 gm.	) in a wet weight of 3.2 gm. liver
Mitochondria	0.19 gm.	
Microsomes	0.10 gm.	
Cytosol	0.28 gm.	

TABLE 9 Purity of subcellular fractions as assayed by site-specific enzymes

Cellular fraction	Protein	Succinic Dehydrogenase		Alkaline Phosphatase		Aldolase	
	(mg/ml)	S.A.	% of Total Activity	S.A.	% of Total Activity	S.A.	% of Total Activity
Cell debris	8.6	0.019	6	5.2	13	0.027	5
Mitochondria	1.7	0.247	78	6.2	16	0.032	6
Microsomes	3.0	0.005	2	26.3	69	0.023	4
Cytosol	4.8	0.042	14	0.7	2	0.458	85

Discussion

Disruption of mitochondria during homogenisation was a possible reason for the appearance of 14% of the mitochondrial enzyme, succinic dehydrogenase, in the cytosol.

Alkaline phosphatase activity in cellular debris could be ascribed to microsomal components retained by the cell walls. Endoplasmic reticulum is, to a certain extent, attached to the cytoplasmic membrane to which it lends rigidity and thereby adds support to the structure. This may explain why there was a residual contamination of microsomal-specific enzyme in the cellular debris fraction. Small quantities of the enzyme are also present in the nuclei and mitochondria in rat liver (Dixon and Webb, 1964 c). Avian liver may well have significant quantities of alkaline phosphatase bound to the large intracellular particles, so that the alkaline phosphatase activity in these fractions as shown in Table 9 does not necessarily indicate contamination.

3. Determination of cadmium in the subcellular fractions of chick liver.

The isolated components of the liver cells were air-dried in an oven at 100^{0} overnight, then weighed, and subjected to a "wet" oxidative digestion procedure originally

described by Smith, Kench and Lane (1955).

The digestion flasks were washed several times in redistilled water. The tissue was covered with conc. nitric acid and warmed gently to 70^{o}- 80^{o}. Brown fumes of nitrogen dioxide (N_2O_4) were evolved as the digestion proceeded. When all solid matter had disappeared, 1-2 ml of pure sulphuric acid was added to the HNO_3 and the solution heated further until fumes were no longer emitted. The flask was allowed to cool and approximately 0.5 ml aliquots of H_2O_2 (30% v/v) cautiously added. The contents of the digestion mixture were heated again and the process repeated until fumes appear only following addition of H_2O_2. The solution was heated strongly until no more fumes escaped and the digest was quite clear and colourless. The flask was allowed to cool and its contents made up to an exact volume with distilled water.

The acidity of the digestion mixture was kept as low as possible to prevent undue corrosion of the absorption spectrophotometer.

Cadmium concentration in the final solution was determined using a Beckman Atomic Absorption Spectrophotometer fitted with a cadmium lamp.

The wavelength for the determinations was 2288 Å and the voltage 6 mV. Standard solutions of cadmium varying in concentration from 0.2 to 10 µg/ml were employed to

construct a standard calibration curve. Since at higher concentrations of cadmium, atomic absorption and concentration were no longer linearly related, it was essential to obtain a concentration in the sample within the range of linearity (Fig. 12).

Cadmium concentrations were read from the standard curve, and the appropriate dilution factors were applied to calculate the exact concentration of cadmium in each fraction (Fig. 13).

Table 10 gives corrected and uncorrected values of cadmium concentrations in the various isolated subcellular components.

The corrected values make allowance for contamination of the individual fractions by certain of the other cellular components which may have a different cadmium concentration.

TABLE 10 Content of cadmium in intracellular liver fractions, corrected for impurity, as calculated from distribution of enzyme activities.

Fraction	Uncorrected value (ppm)		Corrected value (ppm)	
	Acute	Chronic	Acute	Chronic
Debris	1.6	6.0	1.4	5.1
Mitochondria	3.1	13.5	3.1	13.5
Microsomes	4.3	9.6	4.3	9.6
Cytosol	7.5	52.0	8.4	58.0

FIG. 12.

STANDARD CURVE: CADMIUM.

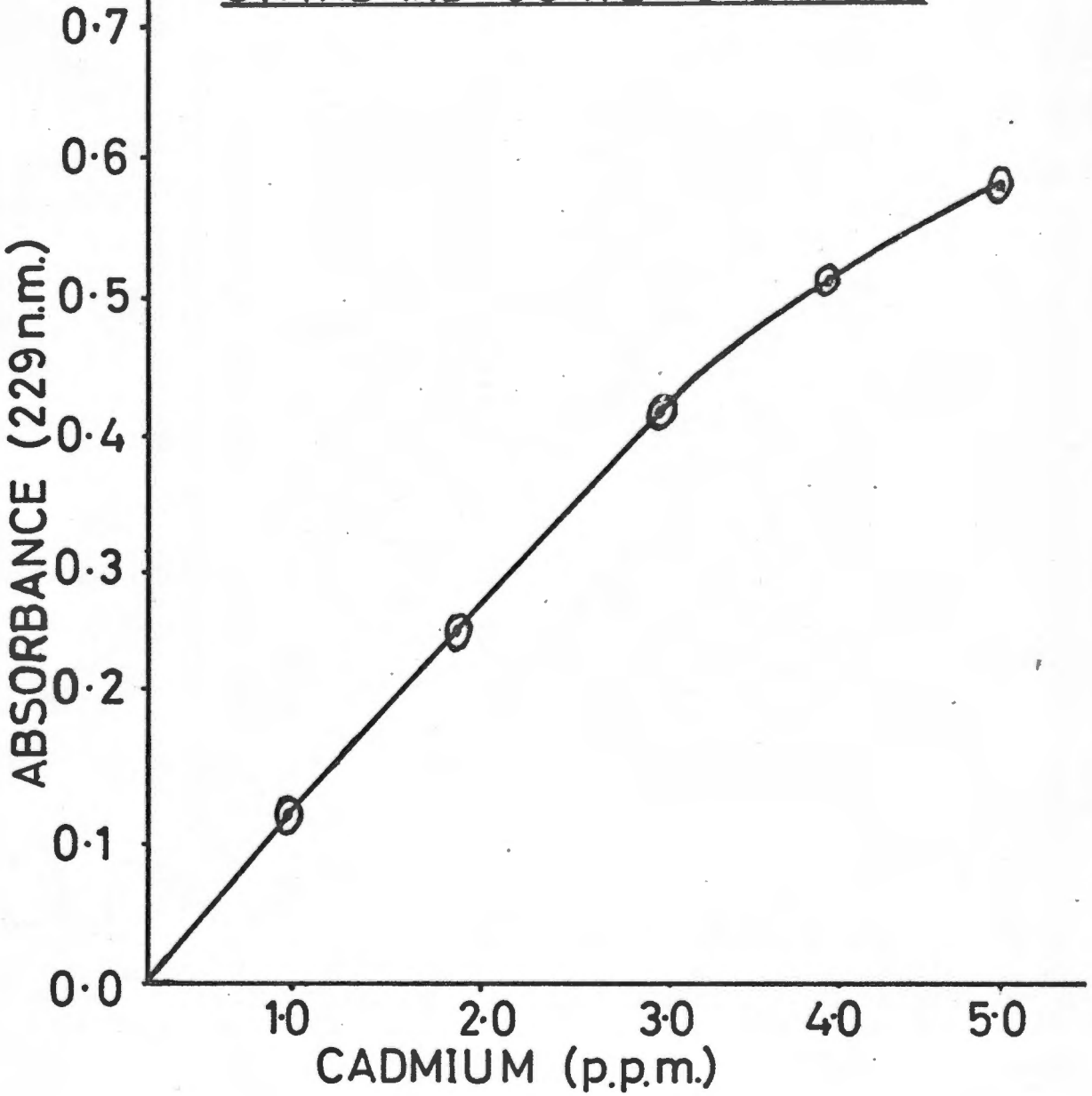
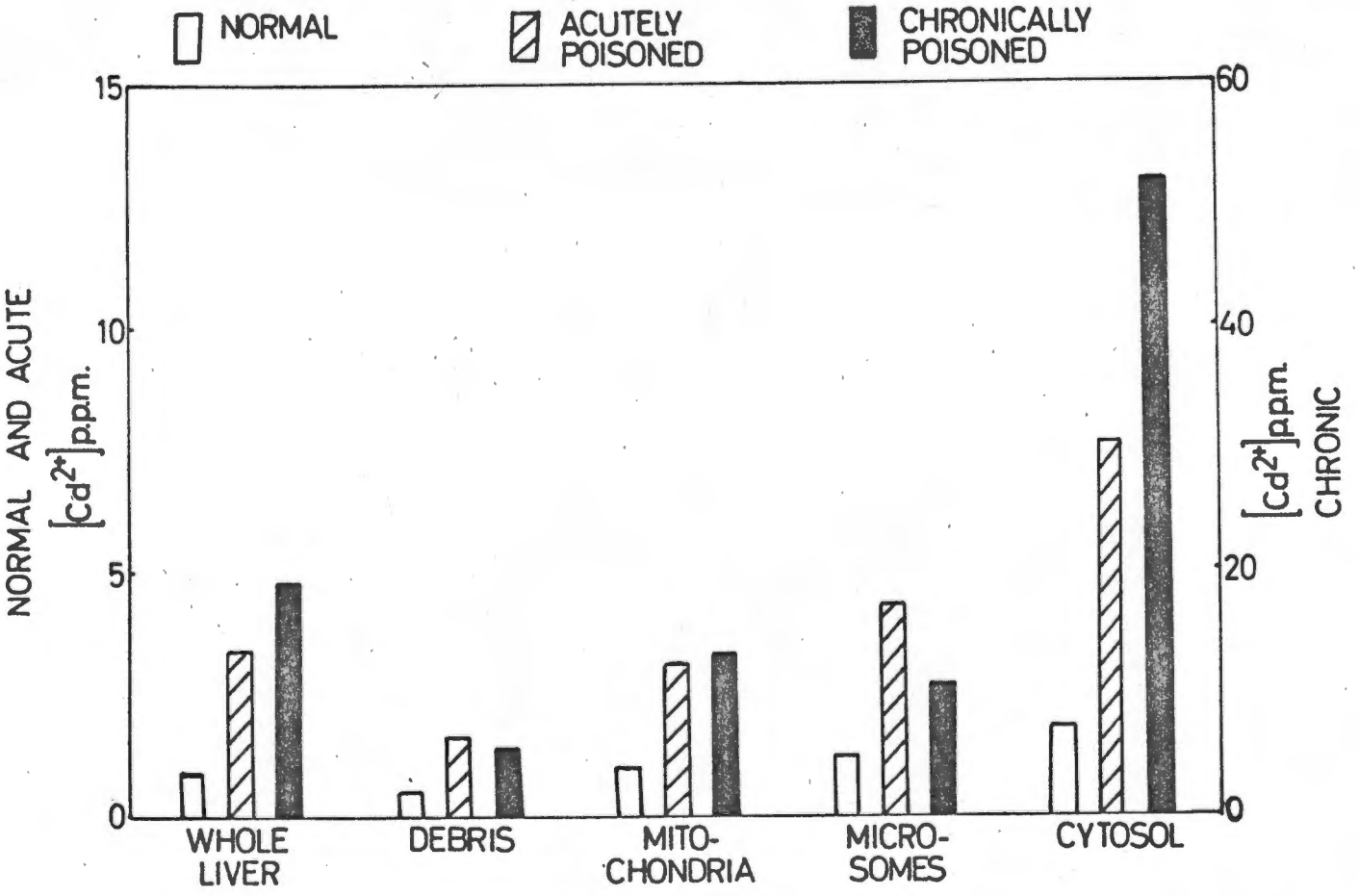


FIG.13.

DISTRIBUTION OF CADMIUM IN LIVER CELLULAR FRACTIONS IN NORMAL, ACUTELY AND CHRONICALLY POISONED CHICKS.



Concentrations of cadmium in whole blood and serum were also measured in normal and cadmium-poisoned chicks. The values obtained are given below (administered dose - 18 µg cadmium at day 15). Such data were deemed of special relevance with respect to:-

(a) *in vivo* activities of enzymes such as catalase and ATPase.

(b) total undeposited, or "circulating" cadmium.

(c) changes brought about in parameters such as erythrocyte PCV.

TABLE 11 Cadmium in the blood of normal and cadmium-poisoned chicks

	Normal	Cadmium-poisoned
Serum	-	0.8 - 1.0 ppm
Erythrocytes	-	0.75 ppm

Clarkson and Kench (1958) showed that 95% of blood lead was bound to the erythrocyte membranes. The values in Table 11 indicate a very different adaptation of the body to cadmium as opposed to lead.

PART III

EXPERIMENTAL AND RESULTS

(B) ENZYME STUDIES

III. 1. ADENOSINE TRIPHOSPHATASE

(ATP: phosphohydrolase)

(E.C. 3.6.1.4.)

Adenosine triphosphatase (ATPase) catalyses the hydrolysis of the terminal phosphate ester link in ATP,

$$ATP \longrightarrow ADP + Pi$$

and, to a lesser extent

$$ADP \longrightarrow AMP + Pi$$

The enzyme was studied in erythrocytes to ascertain whether the effect of cadmium on ATPase could wholly or partially explain the differences observed in the serum electrolytes of cadmium-poisoned chicks, the enzyme being a controlling factor in the Na^{I}/K^{I} balance of the blood (Dunham and Glynn, 1961). The ATPase present in erythrocytes has the same properties as the myosin ATPase found in muscle. It is activated by Mg^{II}, and, in the presence of Mg^{II}, inhibited by Ca^{II} (Kielley and Meyerhoff, 1948; Caffrey, Tremblay, Gabrio and Huennekens, 1956).

ATPase of actomyosin, normally activated by Ca^{II}, has been shown to be more strongly activated by several divalent cations, including Cd^{II}, at optimal concentrations of 2 - 5 mM (Schaub and Ermini, 1969).

Early in the present work, packed cell volume (P.C.V.) and haemoglobin concentration were observed to be depressed

in Cd^{II}-poisoned chicks. Conceivably many of the erythrocyte enzymes may have been similarly affected and ATPase, as a key enzyme, was chosen for investigation. In order to ensure that a low P.C.V. would not, in itself, contribute to a misleading value for the level of ATPase, the results were calculated per ml. of packed cells. The ATPase of erythrocytes is situated virtually entirely in the membranes (Clarkson and Maisels, 1952). Enzyme activity is measured in terms of inorganic phosphate liberated following hydrolysis of ATP, the Pi being complexed with molybdate ion (Fiske and Subbarow, 1925).

Reagents

(a) The Elon Reagent:

1 gm. Elon (monomethyl-p-aminophenyl sulphate) +

3 gm. $NaHSO_3$ dissolved in water and diluted to 100 ml.

(b) Acid molybdate solution:

50 ml. 5% (w/v) sodium molybdate ($NaMoO_4$)

25 ml. 10 N. H_2SO_4

25 ml. distilled water

(c) ATP - 20 mg. (Seravac Ltd.)

Methods

(i) Collection of blood

A procedure whereby the maximum quantity of whole blood could be obtained rapidly and cleanly had to be evolved, as day-old chicks have a blood volume of only about 3 - 4 ml.

The chicks were stunned, and the skin directly over the crutch was rapidly divided to expose both the femoral vein and artery. A tiny drop of fresh heparin (approximately 20 µl containing 500 units heparin Na) was placed directly over the two blood vessels, and an incision was made through them into the hip joint.

Blood rapidly filled the cavity between the abdominal wall and leg, and was drawn directly, without a needle, into a 2 ml. disposable syringe.

(ii) Preparation of haemolysate

The plasma and buffy coat were separated by centrifugation. The packed red blood cells were washed twice with cold normal saline. A known volume of the cells was then lysed by a ten-fold dilution with distilled water, and was allowed to stand for 10 min. at $2^{o}C$ to ensure complete haemolysis.

(iii) Incubation

The procedure of Scharff and Vestergaard-Bogind (1966) was followed. The incubation medium contained

1 ml haemolysate

2 ml 0.25 M Tris buffer, pH 7.5

0.5 ml 0.032 M $MgCl_2$

19.4 mg ATP (32 µmoles)

A sample (0.2 ml) was removed prior to incubation (t_0) and the reaction stopped by the addition of 0.2 ml 12.5% (w/v) T.C.A. The sample was incubated for 30 min. at 37°C, and a second sample (0.2 ml) was taken and treated identically (t_{30}). The two samples were centrifuged at 3,000 x g for 5 min. to remove precipitated protein. 0.1 ml of the clear supernatant was employed for Pi determination.

A control was set up in which ATP was omitted, but all the other components were present.

(iv) Determination of inorganic phosphate

Inorganic phosphate was determined by a modification of the method of Fiske and Subbarow (1925), with the following reagent mixture;-

0.10 ml Elon

0.25 ml acid molybdate

0.10 ml supernatant

0.60 ml distilled water

After mixing, the tubes were kept at room temperature for 45 min. to allow for full colour development, and the O.D. was then measured in a Zeiss P.M.Q. II spectrophotometer at 660 nm.

0.1 ml of standard solution (4 mM KH_2PO_4) was employed as a reference for calibration.

Conversion to µmoles Pi/hr/ml packed cells was according to the following expression:

$$2\,(EA(t\ 30)-EA(t\ 0)-(EC(t\ 30)-EC(t\ 0)\ \times\ \frac{4}{ESTD}\ \times\ 805\ (\text{dilution factor})$$

Where:

EA = optical density (660 nm) of ATP-containing test sample at t=30 and t=0 respectively. EC = Extinction of control. ESTD = Extinction of standard.

Any rise in the concentration of inorganic phosphate due to endogenous ATP hydrolysis must be subtracted from the final value (Δ EA) obtained for the test values. In these experiments, Pi formed in the control (no ATP) run concurrently for 30 min. with test samples, was negligible, and consequently no adjustment was necessary.

Results

(i) The effect of Cd^{II} on purified ATPase, at optimum Mg^{II} concentration.

Apyrase (purified from potatoes, and containing 10% ADPase activity) was the enzyme source. The optimum Mg^{II} concentration, 8 mM, was present in all assays. The work was conducted with concentrations of Cd^{II} in the range encountered *in vivo* in cadmium-poisoned chicks. The results obtained are shown below:-

TABLE 12 The influence of added Cd^{II} ions to the activity of pure Apyrase.

Sample	E 660 nm		ΔE660/hr.	µmoles Pi $hr.^{-1}ml.^{-1}$	% of Normal
	T 0	T 30			
Control	0.070	0.082	0.024		
Normal	0.065	1.380	2.630	526.0	100
2×10^{-5}M Cd	0.063	1.195	2.264	452.8	86
5×10^{-5}M Cd	0.067	1.430	2.726	545.2	104
2×10^{-4}M Cd	0.074	1.227	2.406	481.2	92
5×10^{-4}M Cd	0.071	1.393	2.644	528.8	101

When the activity of Apyrase was measured at optimal Mg^{II} concentration (8 mM), Cd^{II} concentrations in the range $0.2 - 5 \times 10^{-4}$M had no consistent effect on the enzyme.

(ii) <u>The effect on pure ATPase (Apyrase) of different Cd^{II} and Mg^{II} ionic concentrations</u>.

Mg^{II} is known to be essential for optimal ATPase activity, as it combines with ATP to form the activated substrate $ATP^{IV} - Mg^{II}$ (Racker, 1965). Schaub and Ermini (1969) have shown that cadmium may substitute as the metal-ion activator, with optimal concentrations between 0.2 and 0.5 mM, but only in the absence of Mg^{II}. When Mg^{II} is present, the two ions tend to act competitively.

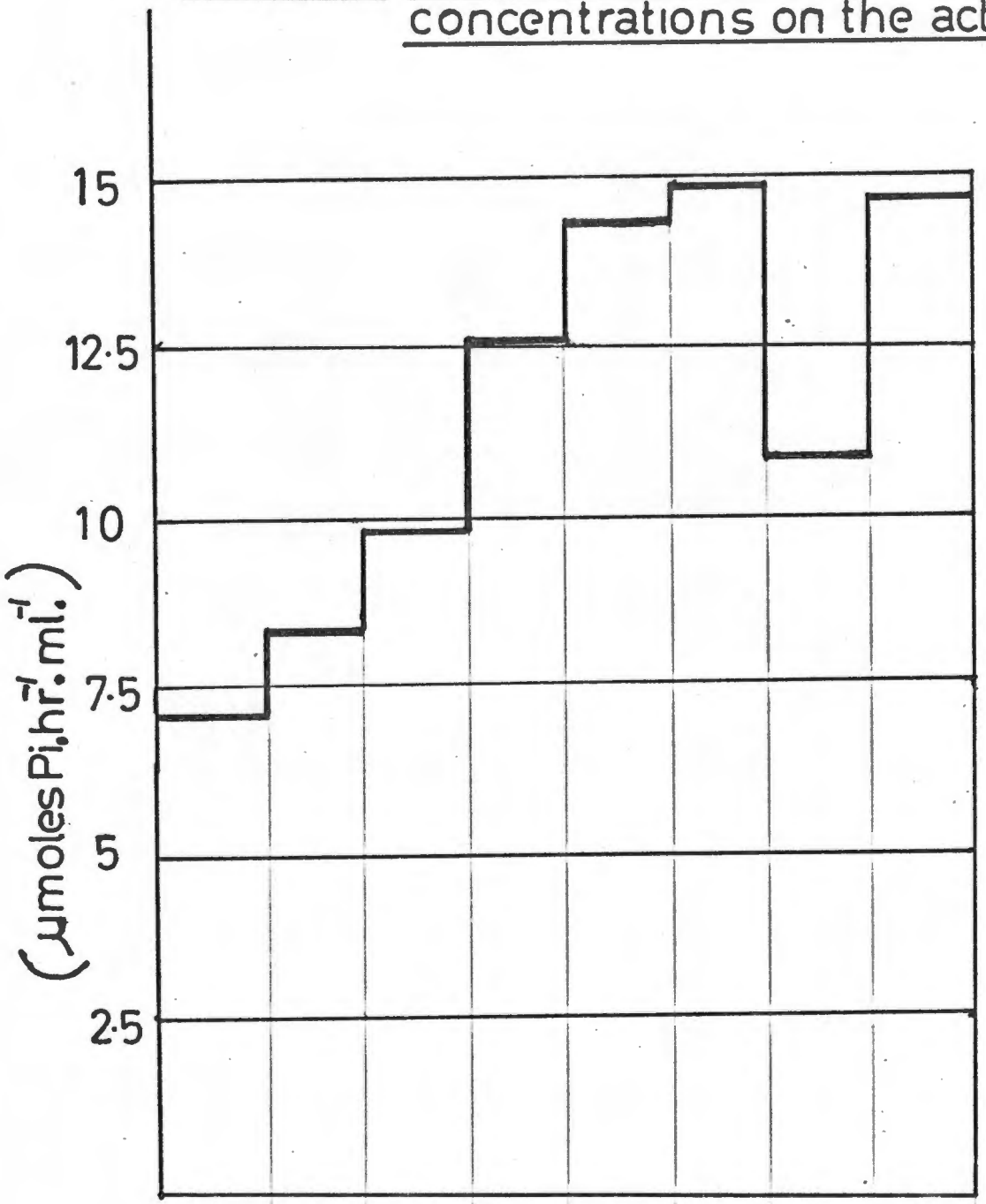
The results obtained in the present study are tabulated below, and shown in Fig. 14.

TABLE 13 Activation of purified ATPase by different concentrations of Mg^{II} and Cd^{II}.

Sample	Mg^{II} (mM)	Cd^{II} (M)	µmoles Pi. $hr.^{-1}ml.^{-1}$
A	Nil	Nil	7.2
B	Nil	2×10^{-5}M	8.4
C	Nil	2.5×10^{-4}M	9.8
D	Nil	5×10^{-4}M	12.6
E	4	5×10^{-4}M	14.4
F	8	5×10^{-4}M	14.9
G	4	Nil	10.9
H	8	Nil	14.8

FIG. 14.

ATPase: The effect of varying cation concentrations on the activity.



The activity of the enzyme in these experiments followed the Schaub and Ermini (1969) results to some extent. Mg^{II} and Cd^{II} activated the enzyme individually, but when the two metal ions were present together in the concentrations shown above, Cd^{II} was observed to have little influence on enzymic activity when Mg^{II} was present at optimal concentration (8 mM). Cd^{II} ions did, however, have an additive effect on Mg^{II} activation when the latter was present in less than optimal concentration (e.g. Samples E and G in Table 13). No evidence was forthcoming that Cd^{II} had a disruptive action on the ATP^{IV} - Mg^{II} complex.

(iii) <u>The in vitro effect of Cd^{II} on chick erythrocyte ATPase</u>.

Prior to assaying ATPase activity, each sample of haemolysate was preincubated for 10 min. with the respective concentration of Cd^{II} shown in Table 14. In order to obtain a measure of the production of inorganic phosphate, as a result of endogenous ATP hydrolysis, a control was included in which all components except ATP were present.

When Cd^{II} was added to haemolysates, there was a definite depression of ATPase activity (Table 14), although the concentration of Mg^{II} was maintained at optimal value as judged from the behaviour of Apyrase (Table 13).

TABLE 14 In vitro addition of Cd^{II} to haemolysates and subsequent assay for ATPase activity.

Sample	E 660 nm		$\Delta E.hr.^{-1}$	% of Normal
	T 0	T 30		
Control	0.044	0.035	-	-
Normal (no Cd)	0.134	0.700	1.132	100
2.5×10^{-6}M Cd	0.087	0.565	0.956	85
8×10^{-6}M Cd	0.093	0.521	0.859	76
2×10^{-5}M Cd	0.108	0.546	0.876	77
1×10^{-4}M Cd	0.104	0.498	0.788	70
5×10^{-4}M Cd	0.100	0.364	0.528	47

The enzyme activity expressed as µmoles $Pi.hr.^{-1}ml.^{-1}$ (RBC) was not calculated, as the relative effects of different concentrations of Cd^{II} on ATPase in the haemolysate could be observed from the values given by $\Delta E.hr.^{-1}$.

It is apparent that erythrocyte ATPase is much more prone to inhibition by cadmium, even at very low concentrations (2.5 and 8×10^{-6}M) of the metal. The reason for the increased sensitivity in this enzyme when compared with the purified potato enzyme is not discernible from this series of experiments. It is very possible that the Cd^{II} in the haemolysate inhibits an associated reaction,

thereby having an indirect effect. It is unlikely, however, that this would be sufficient to produce an inhibition of the magnitude indicated by the above results.

(iv) The induction of chick ATPase on hatching.

Several enzymes are known in which activity is considerably augmented once the hatching process begins. Evidence for post-natal stimulation of ATPase has been presented by Ermini and Schaub (1968) who reported a 4-fold increase in rat muscle ATPase within three weeks after birth over the neonatal level. From the present study it appears that avian erythrocyte ATPase belongs to this category (Table 15).

TABLE 15 The ATPase activity in erythrocytes of chick embryos at different ages.

Age (days)	Comment	ATPase activity (µmoles Pi./ml. packed cells/hr.)
14	2 chicks	12.9
17	2 chicks	16.0
20	2 chicks	27.4
21	Hatched	56.0
21	Cadmium-poisoned; yolk sac unabsorbed	24.1

The failure of the cadmium-poisoned chick to absorb its yolk-sac was accompanied by marked depression of ATP-ase activity, to less than 50% of normal values. The yolk sac is usually absorbed on the 19th - 20th day of incubation, hence it appears that some major mechanism in general embryological development had become unbalanced. This might account for the poor activity of ATPase if stimulation of activity does depend on a normal hatching process for full efficiency.

(v) Comparison of in vivo ATPase activity in normal and cadmium-poisoned chicks.

The variation of ATPase concentration amongst individual chicks was found to be great, but from the number of assays performed it was possible to obtain some indication of the effect exerted by cadmium on the erythrocytic enzyme in vivo.

TABLE 16 ATPase activity in normal and cadmium-poisoned chick erythrocytes.

Normal chicks μmoles Pi.hr.$^{-1}$ml.$^{-1}$	Cd-poisoned chicks μmoles Pi.hr.$^{-1}$ml.$^{-1}$
49.7	77.2
52.1	59.2
58.1	51.9
54.8	82.4
31.1	64.5
78.9	60.7
64.8	61.7
50.9	
63.1	
56.4	
Mean 56.0 $\pm$ 10.8	Mean 65.5 $\pm$ 12.1

Discussion

One of the more striking points that emerges from these investigations is the marked difference in susceptibility to Cd^{II} of the enzyme when in pure form as opposed to the red cell haemolysate. The Apyrase is

spectacularly resistant to inhibition when compared with most of the enzymes in this series. The *in vitro* studies on the haemolysate indicate a sensitivity of ATPase to Cd^{II} far exceeding that of the Apyrase.

ATPase is the enzyme primarily responsible for ionic transport across cellular and intracellular membranes. As the developing biological systems of the chick embryo are not subject to a wide variety of external factors, this transport mechanism may not be of great importance prior to hatching.

Erythrocytic ATPase activity is confined entirely to the membranes. The cytosol has no measurable activity *per se* (Scharff and Vestergaard-Bogind, 1966). The fact that nucleated red blood cells of birds have significantly greater ATPase activity than human erythrocytes may be ascribed largely to the presence of a greater quantity of membranous material (Scharff and Vestergaard-Bogind, 1966). Haemoglobin and catalase concentrations are much lower in avian erythrocytes than in those of man. The function of the nuclei in avian erythrocytes is, however, poorly understood at the present time.

In the presence of Mg^{II} the response to added Cd^{II} apparently depends on other factors in the system under scrutiny. Thus, Apyrase alone was stimulated by Cd^{II}

and not inhibited by this ion if optimal Mg^{II} was also present. In *in vivo* Cd^{II}-poisoned erythrocytes ATPase activity was enhanced, nevertheless when Cd^{II} was added to haemolysate from normal chicks the enzyme was depressed by almost one-half of its activity. We are at a loss to reconcile these conflicting observations. One possibility which should be considered is the concentration of enzyme. Certain effects, such as substrate inhibition, important *in vitro*,-witness the inhibition of certain isoenzymes of lactate dehydrogenase by pyruvate (Wilkinson, 1962) - do not take place *in vivo* where the intracellular concentration of enzyme is much greater.

In vivo, the cadmium-poisoned chick experienced an activated ATPase, and although an attempt was made to simulate the conditions *in vitro* by addition of a measured amount of Cd^{II} (0.5 - 1.0 ppm) to the erythrocytes, the resultant inhibition rather than the expected slight (15%) activation found in intoxicated chicks could not be explained on the basis of these results alone.

III. 2. CATALASE

(H_2O_2:H_2O_2 oxidoreductase)

(E.C. 1.11.1.6)

Catalase is the enzyme responsible for the biochemical degradation of hydrogen peroxide (H_2O_2) formed by all aerobic cells during normal metabolism.

The general reaction for peroxidases is

$$RO - OH + H_2 \longrightarrow R - OH + H_2O + X$$

With catalase R = H and X = O_2; giving an overall reaction:

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

Catalase has a molecular weight of 235,000, and possesses 4 ferriprotoporphyrin (haematin) prosthetic groups which are directly involved in activating the substrate. Its very large turnover number indicates an extremely rapid rate of catalysis (Dixon and Webb, 1965a).

Bonnichsen (1947) reported that liver catalase contains 3 haems and 1 "verdohaemochromogen" as opposed to catalase from horse erythrocytes which has 4 ferrihaem groups. Verdohaemochromogen is inactive and readily degraded to biliverdin. This fact probably accounts for hepatic catalase being much more labile than the erythrocytic enzyme.

The *in vivo* and *in vitro* action of cadmium on catalase activity in the chick was measured in both erythrocytes and in the liver. Since it was considered possible that cadmium could exert an effect on haem synthesis, as cadmium-poisoned chicks had markedly lowered haemoglobin and PCV levels, any influence the metal had on the availability of ferriprotoporphyrin for catalase should emerge from these investigations.

Materials and Methods

Activity of catalase was determined by the sodium perborate ($NaBO_3$) method of Feinstein (1949).

A 0.1 M sodium perborate substrate, adjusted to pH 7.0 with concentrated HCl, was prepared daily. The buffer in the assay medium was 0.1 M Na/K phosphate buffer, pH 7.0. Potassium permanganate solution (0.1 N) was standardised according to the method of Clowes and Coleman (1931).

Preparation of haemolysate

Whole blood was collected as described earlier (ATPase; methods). After centrifugation at 1,500 x g to remove plasma and buffy coat layers, the red cells were washed twice in cold saline and lysed with 4 volumes ice-cold distilled water. Repeated freezing (in dry-ice/acetone)

and thawing ensured complete lysis. After 10 min. at 2^{0}, the haemolysate was centrifuged at 12,000 x g for 15 min. to pack the stroma.

An aliquot of the clear supernatant was diluted 100 times for haemoglobin determination. The remaining haemolysate was subsequently diluted to a concentration of 0.15 gm. Hb/ml.

Preparation of liver homogenate

A standard procedure for isolating the high-speed supernatant fraction was adopted.

Fresh, weighed liver was homogenised in 10 volumes of 0.1 M phosphate buffer, pH 7.0, and centrifuged at 10,000 x g for 15 min. This sedimented all cell particles larger than ribosomes. The fatty layer at the top of the tube was removed, and the residual supernatant carefully aspirated for assay. 1 ml. aliquots were used for assay.

Determination of haemoglobin concentration of haemolysate

The method of Anderson, Kalckar, Kurahashi and Isselbacher (1957) was used with the following modifications:-

50 µl. particulate-free, diluted homogenate or haemolysate were blown into 5 ml. 0.4% (v/v) ammonia solution

and mixed well in air. The O.D. was read at 540 nm in a Zeiss PMQII spectrophotometer.

Globin will combine with only one haem for an equivalent molecular weight of 16,700 (Lemberg and Legge, 1949). Calculated on this basis, the millimolar coefficient for haemoglobin (EmM Hb) is 14.80.

The haemoglobin concentration was calculated as follows:

$$E\ 540 \times \frac{16,700}{14,800} \times 500\ \text{(diln. factor)} \times \frac{1}{1,000}$$

= E 540 X 0.564 gm. Hb/ml. undiluted haemolysate (Anderson et al. 1957).

The bulk of the haemolysate was then diluted to give a haemoglobin concentration of 0.15 gm. Hb/100 ml.

Assay Procedure (Feinstein, 1949)

1.0 ml. diluted haemolysate or homogenate was added by means of a blow-out pipette to 7.5 ml. perborate solution and 5 ml. phosphate buffer, previously equilibrated at 37°C for 5 min. After incubation, for exactly 5 min., the reaction was stopped by the rapid addition of 3.0 ml. 8 N.H_2SO_4. The reaction mixture was then titrated against the standardised $KMnO_4$.

A blank containing 1.0 ml. buffer in place of the haemolysate was run concurrently. The blank sample usually

required 1.0 to 2.5 ml. more $KMnO_4$ for neutralization than did the corresponding test.

Catalatic activity was calculated from the difference in volumes of $KMnO_4$ required to neutralise the $NaBO_3$ mixture in a blank as opposed to a test solution.

The difference (i.e. Blank - Test) increased with increasing activity. "Perborate units" were calculated by $(B-T).10^{-1}$. The incubation time was 5 min.

Activity of Preparations

(a) Activity of catalase in the haemolysate preparations was expressed as perborate units/0.15 gm. Hb.

(b) Activity of catalase in the liver homogenate of the chicks was expressed as perborate units/gm. liver (wet wt.).

Results (i) In vitro

The haemolysate of homogenate was preincubated with different concentrations of Cd^{II} for 10 min. prior to removal of 1.0 ml. haemolysate, which was immediately assayed. The control sample had no Cd^{II} ions added to

it, but was identically diluted.

The mean of three assays was calculated for each different Cd^{II} concentration.

TABLE 17 (a) Erythrocytic Catalase

Blank reading: 14.60 ml. $KMnO_4$

Test	Volume $KMnO_4$	Blank -Test	% Inhibition
Control	13.45	1.15	-
1×10^{-5}M Cd	13.50	1.10	5
1×10^{-4}M Cd	13.45	1.15	0
1×10^{-3}M Cd	13.60	1.00	13

TABLE 17 (b) Hepatic Catalase

Blank reading: 13.60 ml. $KMnO_4$

Test	Volume $KMnO_4$	Blank -Test	% Inhibition
Control	11.40	2.20	-
2×10^{-5}M Cd	11.40	2.20	0
8×10^{-4}M Cd	11.65	1.95	11

Results (ii) in vivo

The results of the normal and cadmium-poisoned chicks that were assayed are tabulated below.

The figures are "perborate units".

TABLE 18. Hepatic and erythrocytic catalase activities of normal and cadmium-poisoned chicks.

Normal chicks		Cadmium-poisoned chicks	
Liver	Erythrocytes	Liver	Erythrocytes
97.7 ± 13.1(14)	0.154 ± 0.04(20)	75.1 ± 13.8(13)	0.108 ± 0.024(18)

The figures represent the mean, standard deviation and number of chicks assayed in each case.

Both the hepatic and erythrocytic catalase were significantly ($p < 0.05$) lower in cadmium-poisoned chicks.

Figures 15 and 16 illustrate the differences in catalase activity between normal and cadmium-poisoned chicks.

FIG. 15.
ERYTHROCYTE CATALASE in vivo IN CHICKS.

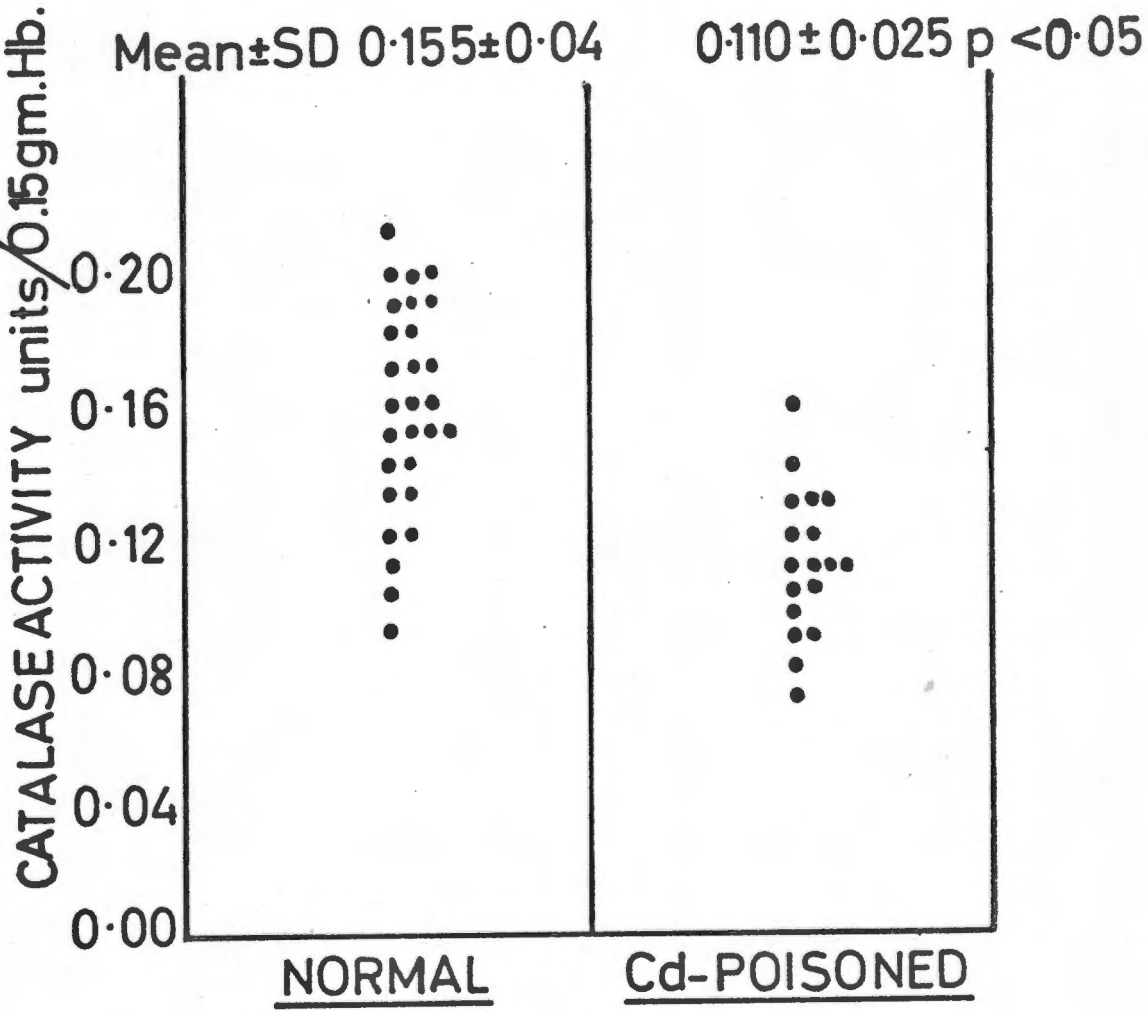
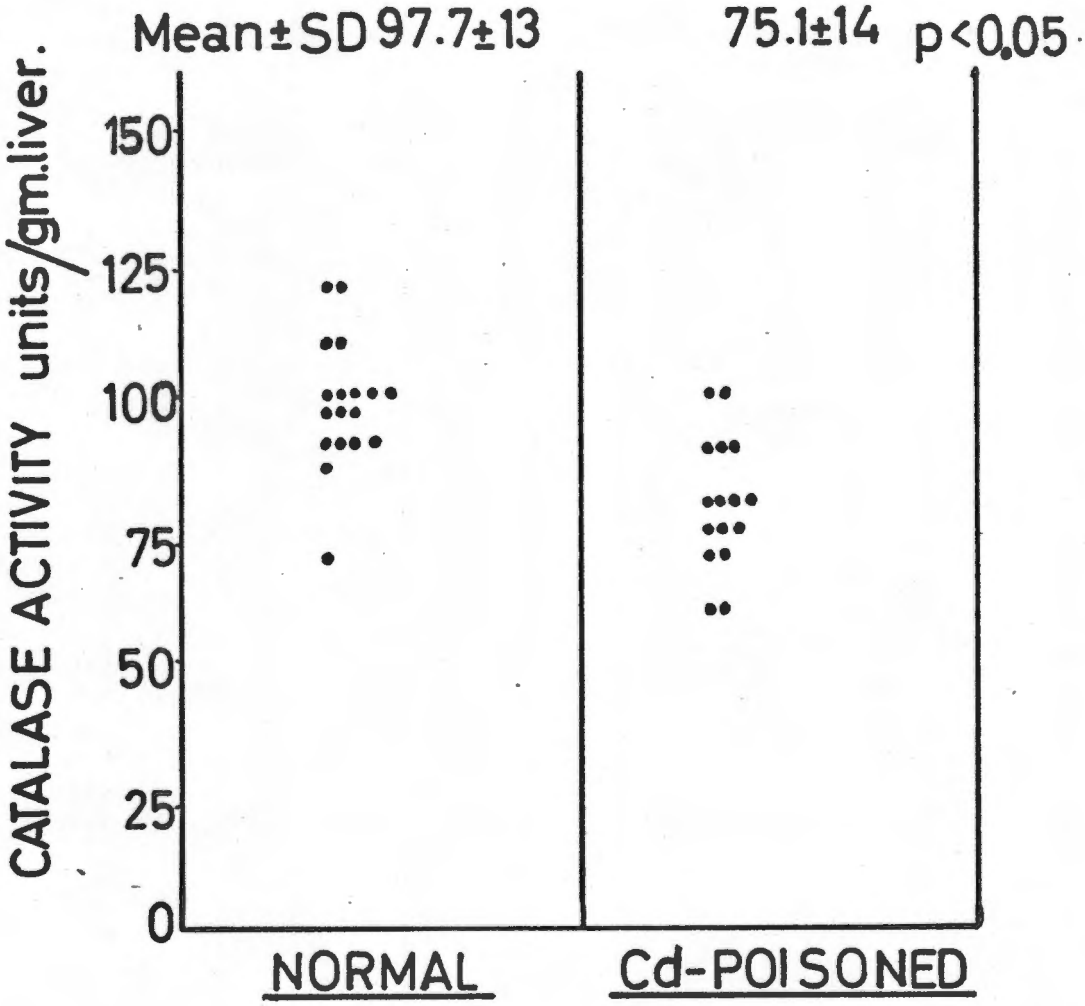


FIG. 16.

HEPATIC CATALASE in vivo IN CHICKS.



Discussion

The spectacular resistance of catalase to cadmium *in vitro* was unexpected. In the presence of a final Cd^{II} concentration as high as 2 mM, loss of activity was insignificant when compared with a normal control. The liver isozyme of catalase was almost as resistant to the metal, in spite of the fact that it is acknowledged to be physiologically more unstable than the red cell enzyme(Bonnichsen, 1947).

In vivo experiments showed that the mean values for catalase in cadmium-poisoned chicks were lower than those of normal chicks. The red cell isoenzyme was depressed by 30%, while the liver enzyme was decreased by 23%. If they are to be assessed critically, these figures should be related to depression of haemoglobin concentration (approximately 25%) and to the behaviours of the two haem-biosynthetic enzymes, ALA-synthetase and ALA-dehydrase, which were also investigated in the course of the present study.

III. 3. CYTOCHROME OXIDASE

(Cytochrome C: O_2 oxidoreductase)

(E.C. 1.9.3.1.)

The haemoprotein nature of cytochrome oxidase, the terminal enzyme of cellular respiration, then known as the "Atmung's ferment", was originally reported by Warburg (1926).

It was first identified with cytochrome a_3 by Keilin and Hartree in 1938.

The enzyme catalyses the following reaction:

$$\text{Ferrocytochrome C} + 2H + \tfrac{1}{2}O_2 \longrightarrow \text{Ferricytochrome C} + H_2O$$

Cytochrome oxidase is induced at an early age (5 days) in developing chick embryos, and its specific activity increases rapidly with embryo age (Albaum, Novikoff and Ogur, 1946).

The intention of the experiments on cytochrome oxidase now described, was to evaluate the effect of cadmium, both *in vivo* and *in vitro*, on the electron transport properties of chick liver mitochondria.

The enzyme mechanism has been well-documented, it being known to contain 1 atom of firmly bound Cu^{II} per haem, which undergoes oxidation and reduction with the haem. It is very strongly inhibited by cyanide, azide, sulphides, and CO (reversible by light). (Griffiths and Wharton, 1961).

Materials and Methods

The enzyme, cytochrome oxidase, and the substrate, cytochrome C, were obtained in pure form from Sigma, Ltd., California, U.S.A.

The cytochrome C was reduced to ferrocytochrome C by addition of a minimal quantity of ascorbate. Excess ascorbate was dialysed out of the solution for 24 hr., against three changes of 0.05 M phosphate buffer, pH 7.0, at $2^{o}C$.

The dialysed protein was scanned to ascertain whether or not there was any ascorbate remaining. A strong absorption maximum at 260 nm would have indicated ascorbate. Elimination of reductant is obligatory, since any residual ascorbate would certainly have impeded or even nullified oxidation of ferrocytochrome C by cytochrome oxidase. The difference spectrum of Fe^{II} cyt. C vs. Fe^{III} cyt. C is shown in Figure 18.

Ferricytochrome C, used in the blank, was prepared by complete oxidation of cytochrome C by potassium ferricyanide (Yonetani, 1965).

$$E^{mM}_{553\ nm} = 19.6 \text{ for } Fe^{II} \text{ cyt. C}$$

0.05 M potassium phosphate buffer, pH 7.0 was used for the assay.

The M.S.E. medium contained 0.225 M mannitol, 0.075 M sucrose, and 0.05 mM E.D.T.A. at pH 7.0.

Preparation of Mitochondria

The procedure followed is described by Johnson and Lardy (1967).

The tissues to be investigated, heart and liver, were removed rapidly from the stunned birds and placed in ice-cold M.S.E. medium. The heart was cut into small pieces with a pair of scissors prior to homogenisation. A smooth glass vessel with a motor-driven loose-fitting Teflon plunger was employed for homogenising all the tissues. The homogenization was limited to 5 downward passes of the plunger to avoid excessive damage to the mitochondria. The required mitochondrial fraction was then collected by differential centrifugation (Schneider, 1948).

A preliminary centrifugation at 1,500 x g (10 min.) sedimented all connective tissue and nuclei. Most of the fat accumulated as a thin pad at the top of the liver homogenate. After discarding the pellet, the supernatant was centrifuged at 10,000 x g (15 min.). The resulting mitochondrial pellet was harvested, and could be stored at $2^{o}C$ for 2 - 3 days without significant loss of enzymic activity.

For the assay, the mitochondria were resuspended in MSE/phosphate buffer 1:1 (v/v), and dissolved with a final Triton - X-100 concentration of 0.5% (v/v).

Assay Procedure

The most suitable method was that described by Smith (1955).

The rate of oxidation of ferrocytochrome C was continuously monitored at 550 nm in a Beckman D.B. recording spectrophotometer against a ferricytochrome C blank (Fe^{III} cyt. C has no absorption maximum at 550 nm.)

Each microcuvette contained:

0.4 ml. 0.05 M potassium phosphate buffer, pH 7.0

0.1 ml. Ferrocytochrome C (1.0 µmoles)

0.5 ml. distilled water

0.02 ml. diluted pure enzyme, or mitochondrial extract.

In vitro additions of cadmium directly to the enzyme required a preincubation period of 10 min., to enable Cd^{II} to become firmly attached to the enzyme, prior to mixing the enzyme with the other constituents of the assay mixture.

The pH was maintained at 7.0, as this was compatible with the oxidation reaction.

Ferrocytochrome C was checked (absorption spectrum) prior to use in the assay to ensure that no autoxidation to ferricytochrome C had occurred (Fig. 18).

The protein concentration of the extracts was determined by the microbiuret method (Lane and Mavrides, 1969).

The specific activity of the enzyme was then expressed

as:-

Spec. Act. = $\triangle$ E (550 nm) $min.^{-1}$ (mg. protein).$^{-1}$

Results

Assays on normal chicks were performed on mitochondria. These were prepared from liver and heart of 4 to 6 chicks pooled to provide an average value, thereby minimising the effect of individual fluctuation.

Cd^{II} was added *in vitro* to the isolated mitochondria, and the preparation incubated for 10 min. prior to assay. The *in vitro* tests were executed on both intact mitochondria, and on a fraction to which Triton - X-100 had been added to disrupt the mitochondrial membranes.

Preincubation of Cd^{II} with the enzyme had a marked effect on the maximal velocity, in spite of the subsequent high dilution on addition of enzyme to assay cuvette.

The Cd^{II} was maximally effective after 10 - 15 min. pre-incubation.

1. *In vitro*

(i) The effect of cadmium on the enzyme.

The effect of increasing Cd^{II} concentration on the

activity of heart mitochondrial cytochrome oxidase is shown in Table 19, which shows a decrease in activity associated with increasing concentration of Cd^{II}.

TABLE 19 The influence of Cd^{II} *in vitro* on heart mitochondrial cytochrome oxidase.

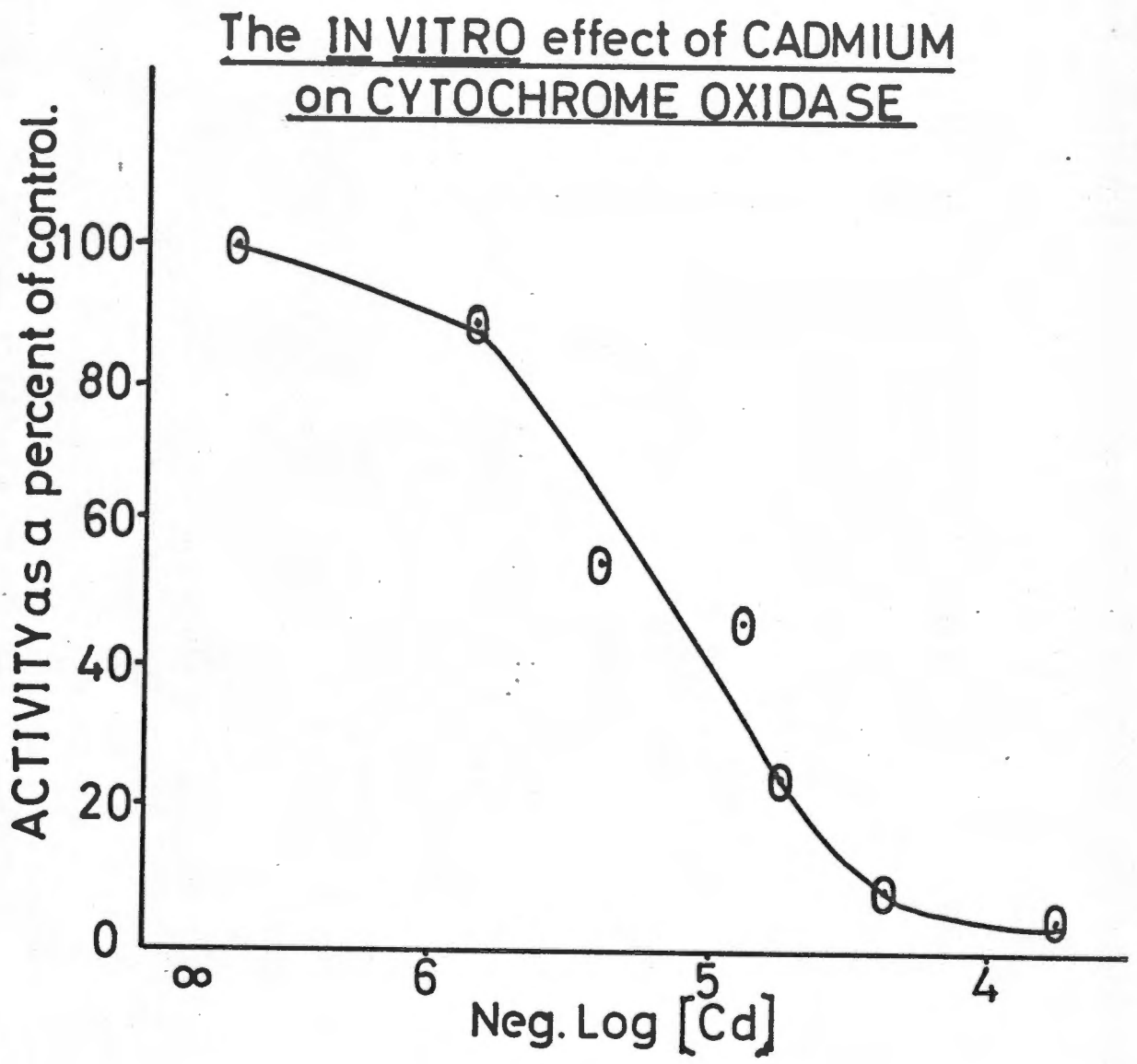
Cd^{II} concentration	Specific activity *	% inhibition
Nil (control)	0.281	0
2×10^{-6}M	0.247	12
5×10^{-6}M	0.180	36
1×10^{-5}M	0.141	50
2×10^{-5}M	0.053	81
5×10^{-5}M	0.022	92
2×10^{-4}M	0.011	96

* Activity is expressed as µmoles cyt. C oxid./min./mg. Protein.

The results are also shown in Fig. 17.

The incubation of relatively high cadmium concentrations (1×10^{-4}M), sufficient to cause 90% inactivation of most sensitive enzymes, with the substrate in the assay medium prior to addition of the enzyme did not influence the initial velocity of the reaction to a significant degree.

FIG. 17.


The IN VITRO effect of CADMIUM on CYTOCHROME OXIDASE
ACTIVITY as a percent of control.
100
80
60
40
20
0
8
6
5
4
Neg. Log [Cd]

The presence of the metal began to retard the rate of reaction after 10 - 20 seconds, as it became progressively more bound to the enzyme.

(ii) The effect of cadmium on the substrate.

The difference spectrum of oxidised and reduced cytochrome C was unchanged by the presence of Cd^{II}. This indicated that a Cd^{II} environment did not change the redox state or haem configuration of the substrate protein (Fig. 19).

2. In vivo

Cytochrome oxidase activity in 6 samples of pooled mitochondria from normal chicks was compared with that in the mitochrondria isolated from individual cadmium-poisoned chicks. The enzyme from both heart and liver preparations was assayed.

FIG.18

DIFFERENCE SPECTRUM: REDUCED VS. OXIDISED CYTOCHROME C.

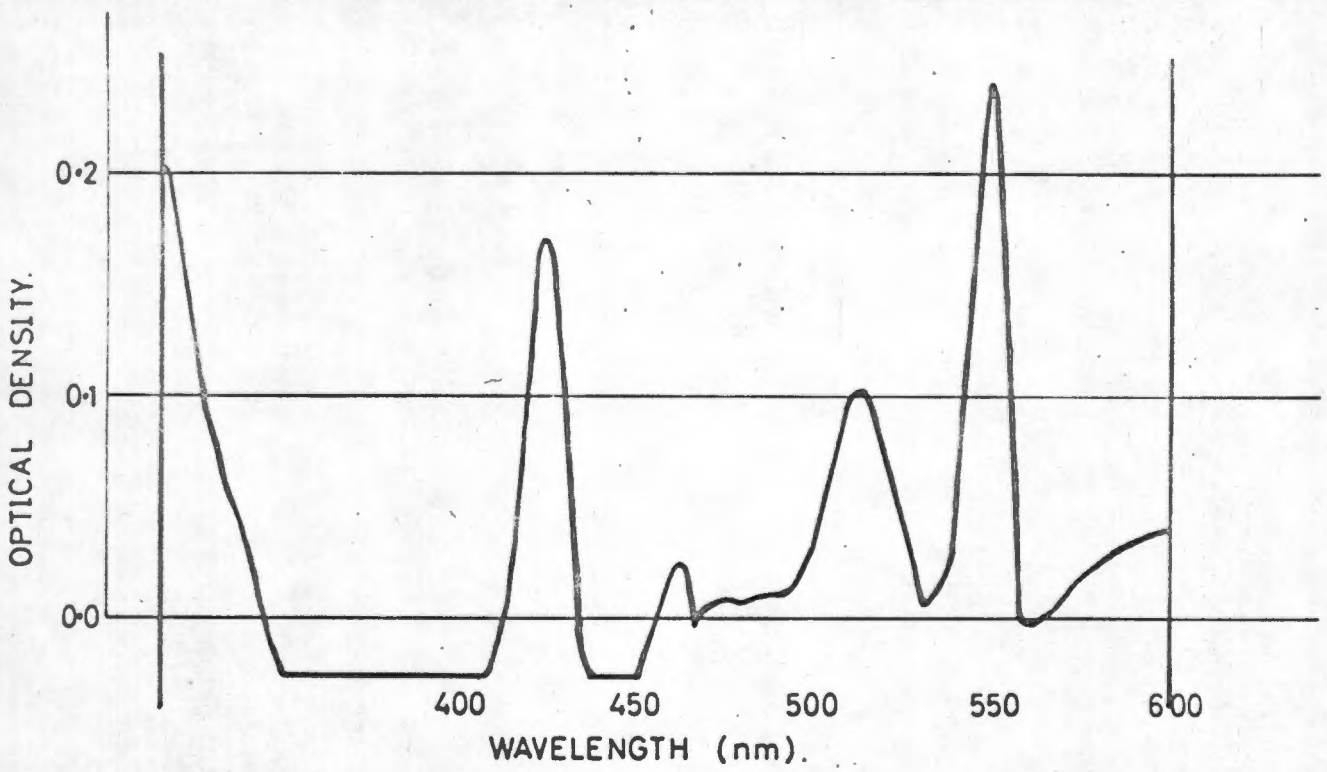


FIG.19

THE EFFECT OF ADDED CADMIUM ON FERROCYTOCHROME C.

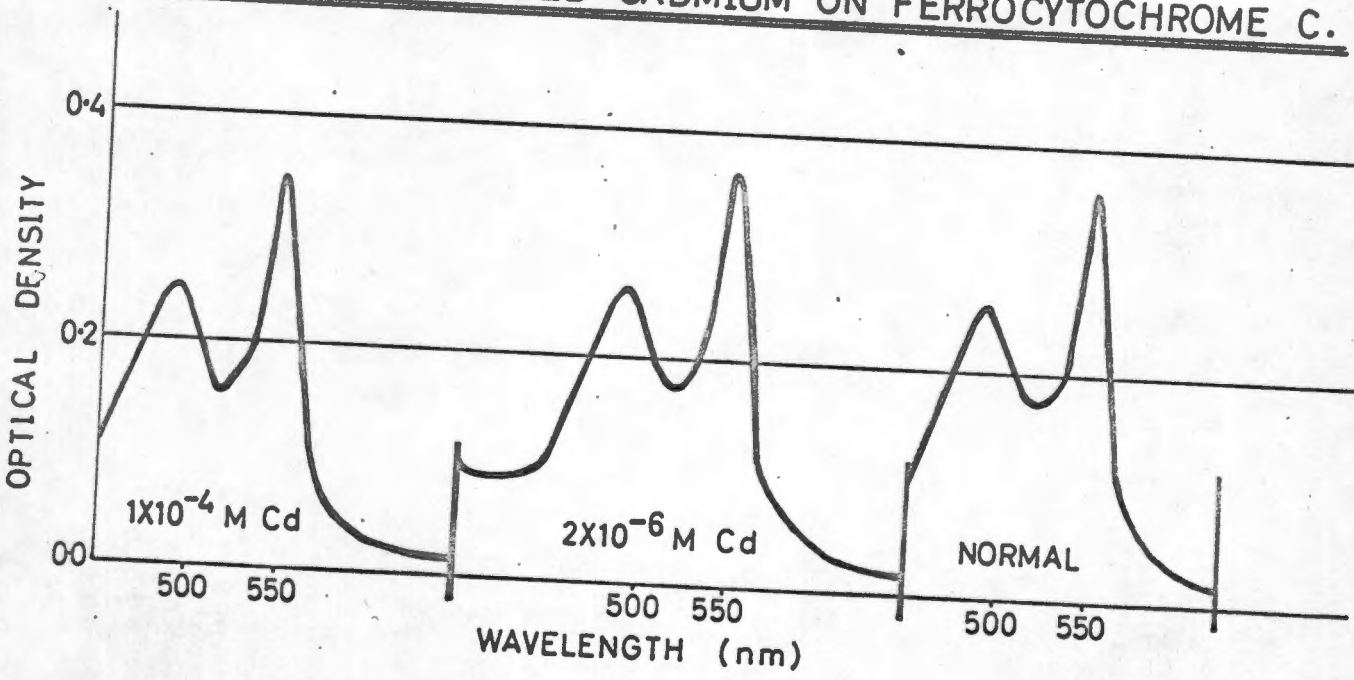


TABLE 20 The specific activity of cytochrome oxidase in normal and cadmium-poisoned chicks

	Normal Chicks (16)*		Cd-poisoned Chicks (10)*	
	Heart	Liver	Heart	Liver
Specific activity	0.377 ± 0.104	0.084 ± 0.020	0.429 ± 0.168	0.098 ± 0.037

* The number of individual chicks sampled

The cadmium-poisoned heart tissue had a 14% higher activity, and the liver a 17% higher activity than normal. The percentage increase in activity of the cadmium-poisoned mitochondrial preparations relative to controls was of the same order of magnitude as the degree of activation of succinic dehydrogenase. There was, however, a wide variation in the activity of the enzyme in different preparations and a 15 - 20% rise in the activity of cytochrome oxidase was not statistically significant in this series of experiments.

Hill, Matrone, Payne and Barker (1963) did not detect any depression of cytochrome oxidase activity in chicks fed cadmium chloride. It appears, therefore, that Cd^{II} ions are not capable of binding to the intramitochondrial enzyme when administered either orally or intravenously.

The nature of binding of cadmium to cytochrome oxidase

A pooled mitochondrial preparation was divided into two separate portions, and a constant Cd^{II} concentration (5×10^{-5}M) was added to one of them. The crude enzyme was assayed with different concentrations of cytochrome C as substrate to ascertain the type of inhibition the cadmium exerts on the enzyme. A reciprocal plot ($\frac{1}{s}$ vs. $\frac{1}{v}$) was drawn (Lineweaver and Burk, 1934). The results are shown in Table 21 and Fig. 20.

TABLE 21 The effect of varying substrate concentration on the rate of cytochrome C oxidation in heart mitochondria from normal and cadmium-poisoned chicks.

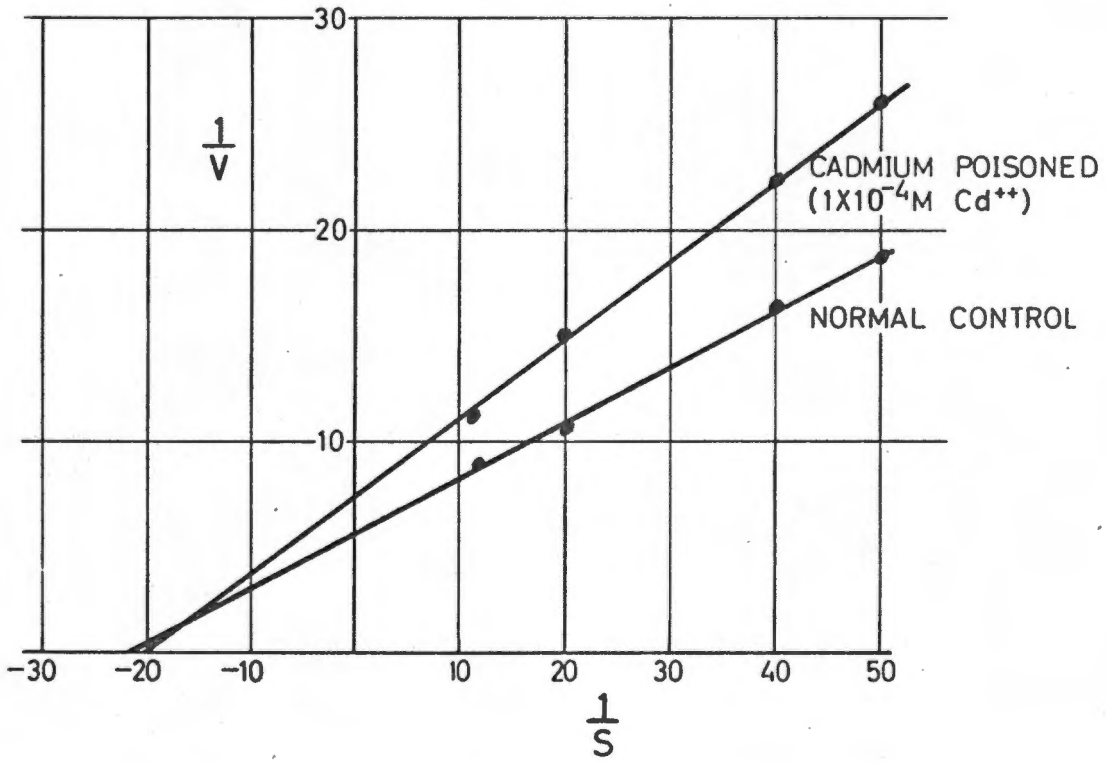
Sample	S (ml. Cyt. C)	V	$\frac{1}{s}$	$\frac{1}{v}$
A	0.020	0.054	50.0	18.5
A	0.025	0.062	40.0	16.0
A	0.050	0.094	20.0	10.6
A	0.075	0.105	13.3	9.5
B	0.020	0.030	50.0	33.3
B	0.025	0.038	40.0	26.3
B	0.050	0.067	20.0	14.8
B	0.075	0.089	13.3	12.6

A = Normal chick heart
B = Cadmium-poisoned heart extract $\left[Cd^{II}\right] = 5 \times 10^{-5}$M

FIG. 20.

INHIBITION STUDIES OF CADMIUM ON CYTOCHROME OXIDASE.

LINEWEAVER-BURK RECIPROCAL PLOT.



Whereas the Michaelis constants (Km) for the two rate processes were almost the same, there was a definite depression of the maximum velocity of the catalysed reaction in the presence of cadmium. The inhibition was, therefore, primarily of a non-competitive type, arising through some conformational change in structure of the enzyme brought about by cadmium ions. The change in quarternary structure was not directly in the configuration of the active site, but such as to impair the maximal rate of turnover of substrate molecules and products at the active centre, i.e. inhibition was of an allosteric kind. Since, however, there was a slight but perceptible alteration of Km, some change in orientation of the groups comprising the active centre must have taken place. This was persistent and depressed the rate of formation of enzyme-substrate complex. Other plots showed variations about the relative intercepts on $\frac{1}{v}$ and $\frac{1}{s}$ axes, and the Km for each assay varied according to the purity and activity of each preparation.

Once the metal concentration reached millimolar levels, the quarternary structure of cytochrome oxidase was so severely distorted by the Cd^{II} that the enzyme was irreversibly inactivated.

At such concentrations of cadmium, the inhibitor was very tightly bound to each of the three sensitive mitochondrial enzymes, succinate dehydrogenase, lipoamide dehydrogenase

and cytochrome oxidase, (K_1 was small), and restricted the number of molecules of enzyme available to combine with S to form ES. No elevation of the concentration of substrate (S) could augment the reaction rate, consequently inhibition was primarily of a non-competitive type.

Unfortunately it was impossible to evaluate the contribution of the various modes of inhibition with the simple kinetic analysis employed here. Metal ions seldom exert an "ideal" single effect on the E-S complex they inhibit. Inhibition is usually a manifestation of several types of metal-enzyme-substrate interaction. Furthermore, non-competitive inhibition due to irreversible binding of inhibitor to enzyme or enzyme-substrate complex, as evidenced by the above kinetic data, is rare. This is not generally appreciated, since non-competitive inhibition exhibits identical kinetics, regardless of whether reversible or irreversible interactions are involved (Webb, 1963).

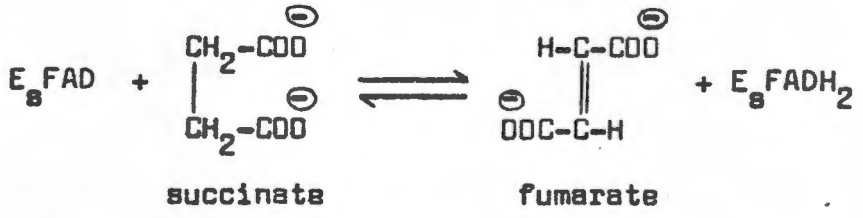
III. 4. SUCCINIC DEHYDROGENASE

(Succinate: (acceptor) oxidoreductase)

(E.C. 1.3.99.1)

Succinic dehydrogenase, a key enzyme in the citric acid cycle, is firmly bound to the inner mitochondrial membranes of all aerobic cells, and has frequently been used as a marker enzyme for the particles (Brunner and Bücher, 1970).

The enzyme catalyses the conversion of succinate to fumarate as follows:-



E_s = Apoprotein. The prosthetic group FAD(H_2) is tightly bound to the enzyme.

Several important features of the native physiological enzyme are not yet fully known, owing to the very close association of succinic dehydrogenase with the structural components of the mitochondria. This makes it very difficult to dissolve the enzyme and purify it.

The roles of the 4 gram-atoms of non-haem iron (N.H.I.) and the electron acceptor for $FADH_2$ are presently under intensive investigation (Koike, Reed and Carroll, 1963).

The isolated enzyme can react with the "cytochrome particle" to form succinate oxidase. It is very unstable. After 6 hours of storage in air at $0^{o}C$, only 1% of activity remains (King, 1966).

Succinate oxidation may be catalysed by a combination with the enzyme of several artificial electron acceptors, particularly ferricyanide, Würsters blue, methylene blue, Coenzyme Q, and in this series of experiments, 2,6-dichlorophenolindophenol (D.C.I.P.) (King, 1963).

The overall reaction can, therefore, be represented as follows:-

$$E_sFAD + \text{Succinate} \rightleftharpoons E_sFADH_2 + \text{Fumarate}$$

$$E_sFADH_2 + \text{dye} \rightleftharpoons E_sFAD + \text{dye } H_2$$

$$\text{Succinate} + \text{dye} \rightleftharpoons \text{Fumarate} + \text{dye } H_2$$

D.C.I.P., a royal blue in its oxidised form, is decolorised as it accepts 2 H atoms from succinate, and this provides a measure of the overall conversion (King, 1963).

Materials and Methods

Mannitol, 0.225 M, Sucrose, 0.075 M, and EDTA, 0.05 mM, pH 7.4 (M.S.E.) was the medium employed in the homogenisation and was diluted 50% (v/v) with 0.1 M Tris-glycine buffer, pH 7.0, for the mitochondrial resuspension. 2,6-Dichlorophenol-indophenol (D.C.I.P.) (B.D.H.) was obtained in pellet form. One pellet was dissolved in 5.0 ml. distilled water, and filtered to give a clear blue solution. A fresh solution was prepared every 3 - 4 days. Triton - X-100 detergent was employed to dissolve the resuspended mitochondria. A final concentration of 0.5% (v/v) was used. This involved a preliminary 50-fold dilution of the stock solution and gentle stirring until homogeneity was achieved. This solution was prepared anew every 4 days.

Preparation of homogenate

Succinic dehydrogenase from both cardiac and skeletal muscle was assayed in normal and cadmium-poisoned chicks. After stunning the chicks, the heart and thigh muscles were rapidly removed and cleaned free of non-muscle tissue. The tissue was then placed in ice-cold M.S.E., finely chopped with scissors, and homogenised in a glass homogeniser with a power-driven rotating Teflon plunger.

Differential centrifugation, as previously described, first at 600 x g (10 min.) and subsequently at 10,000 x g for 15 min., sedimented the mitochondrial pellet. The pellet was resuspended in 50% phosphate buffer - M.S.E., treated with a final concentration of 0.5% Triton-X, and assayed immediately for succinate dehydrogenase activity. The protein concentration of the solution was determined by the microbiuret method (Lane and Mavrides, 1969).

Assay Procedure.

The assay used was a modification of that described by Zeigler and Rieske (1967).
The reaction mixture contained

Potassium phosphate buffer 0.1 M pH 7.0	1.5 ml.
Sodium succinate 1.0 M	0.2 ml.
E.D.T.A. 1mM	0.1 ml.
D.C.I.P.	0.2 ml.
Muscle/heart mitochondrial suspension	0.5 ml.

When Cd^{II} was added for *in vitro* investigations, the E.D.T.A. was omitted. No coenzyme Q was added, as there was sufficient endogenous component for the overall assay.

The system was allowed to equilibrate at 37°C prior to the addition of the enzyme. The decrease in optical density at 600 nm due to decolorisation of D.C.I.P. was monitored on a Beckman DB recording spectrophotometer.

The activity of the enzyme preparation was expressed as the rate of change in optical density, i.e. ΔE600/min.

Specific activity is related to the protein concentration of the suspension, and was expressed here as

$$\Delta E600.min^{-1}.\ (mg.\ Protein).^{-1}\ \frac{1}{21*}$$

* D.C.I.P.:- $E^{mM}_{600\ nm} = 21$

Results

(1) in vitro

Cd^{II} was added to the mitochondrial preparation, which was then incubated for 10 min. before assay. This method of intoxication had a much greater influence on enzymatic activity than when Cd^{II} was added directly to the assay mixture as a whole, even although final concentrations of the metal were identical.

TABLE 22 The *in vitro* effect of Cd^{II} ions on the activity of succinic dehydrogenase

Concentration of Cd^{II}	Activity (Milliunits)	Activity as a Percent of control
0 (control preparation)	9.2	100
5×10^{-6}M	8.0	87
1×10^{-5}M	7.3	79
3×10^{-5}M	6.3	68
1×10^{-4}M	3.4	37
2.5×10^{-4}M	0.8	9

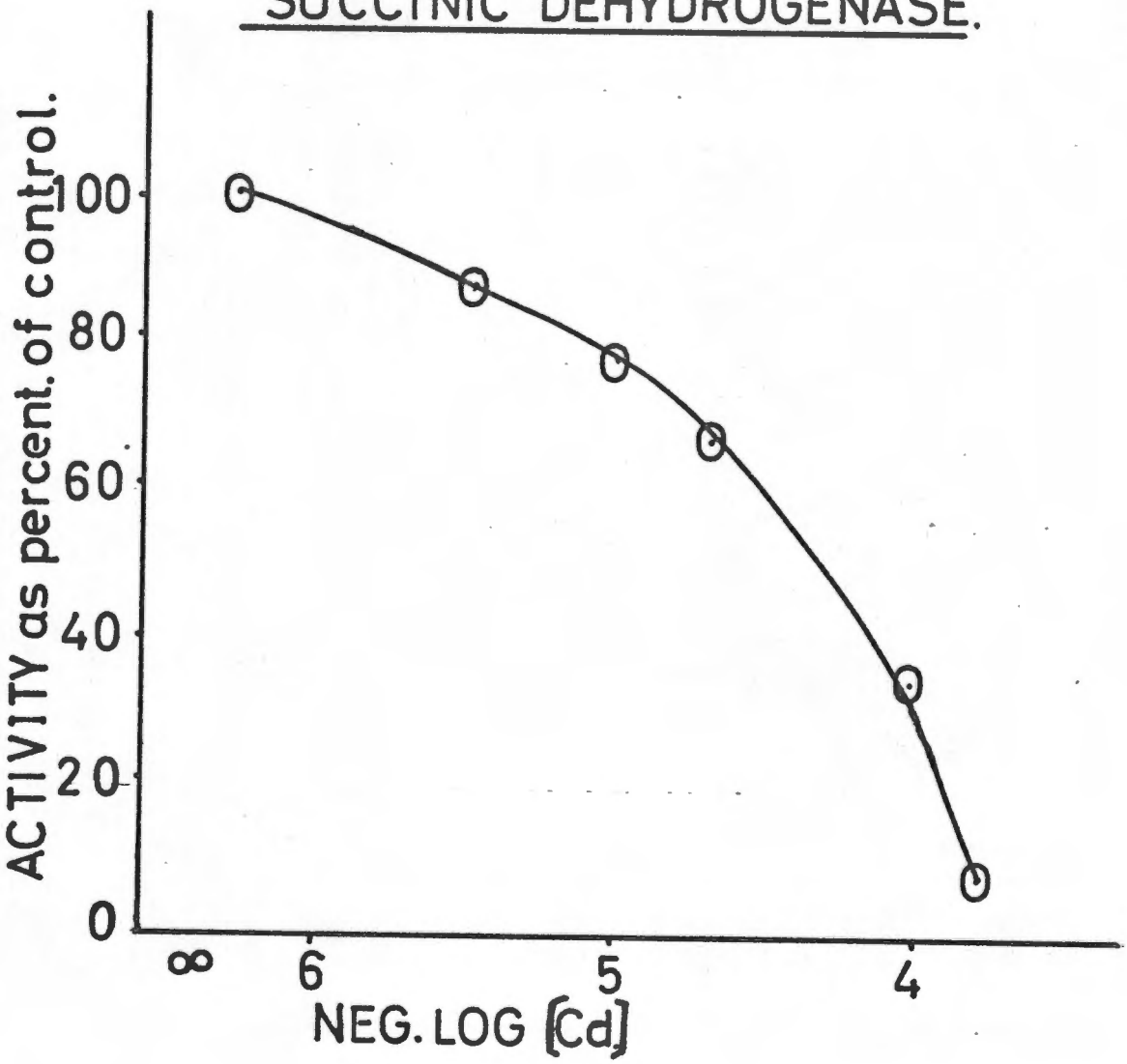
The results in Table 22 and Fig. 21 show that succinic dehydrogenase was progressively inhibited by elevation of the Cd^{II} concentration.

(2) *in vivo*

Both heart and skeletal muscle from normal and cadmium-poisoned chicks were assayed to evaluate the biochemical effect of cadmium on succinic dehydrogenase in the living animal. The aim of these experiments was to ascertain whether the magnitude of the *in vivo* change of activity

FIG. 21.

The IN VITRO effect of CADMIUM on SUCCINIC DEHYDROGENASE.



was comparable (or otherwise) with that observed when Cd^{II} was added *in vitro* to the mitochondrial preparations. Furthermore, the two individual tissues could be compared in this regard.

TABLE 23 *In vivo* effect of Cd^{II} on chick heart and skeletal muscle succinic dehydrogenase activity

	Normal Chicks		Cadmium-poisoned Chicks	
	Heart	Skeletal Muscle	Heart	Skeletal Muscle
Activity (in milliunits) of mitochondrial preparations.	10.8 ± 2.1(10)	8.6 ± 1.8(10)	12.7 ± 1.3(8)	9.10 ± 0.9(8)

Discussion

The magnitude of the *in vitro* inhibition of the enzyme by cadmium may be susceptible to several other factors, including ionic strength, pH, and type of buffer used in the assay. These variables may well influence the sensitivity of the enzyme enabling more or less Cd^{II} to bind to the active site in unit time.

It was crucial to standardise the preincubation time of Cd^{II} with enzyme for comparative studies to be made. Inhibition increased markedly with preincubation time. (See "Lipoamide Dehydrogenase, Results").

One aim of the present study was to assess the importance of cellular factors in the action of cadmium on the "poisoned" chick enzyme, and to correlate *in vivo* change, if possible, with alteration in the enzyme *in vitro*. To this end, the assay variables mentioned above were maintained as strictly comparable as possible. However, because the tests were not all executed simultaneously, but over a number of days, using fresh dye and co-factor solutions, the values presented in Tables 22 and 23 must be regarded as comparative activities of the heart or muscle preparations.

The activity of an unstable enzyme such as succinic dehydrogenase may be markedly influenced by slightly different conditions of assay, or, more likely, by slight variations in the isolation and preparation of the mitochondria.

The apparent activation of cardiac succinic dehyrogenase was not considered of such statistical significance as to justify any firm conclusion, since the specific activities of certain of the normal chick preparations were well below the mean. The probable reason for this

was delay in the isolation and dissolution of the mitochondria, which was responsible for a significant fall in activity of the labile enzyme.

The protection of succinic dehydrogenase afforded by dithiols and chelating agents against metal inactivation is well documented, and was first described by Kreke, Kroger and Cooke (1949), who examined Hg^{II} in the role of intoxicant in their investigation. The effect of E.D.T.A. on Cd^{II} sensitive mitochondrial dehydrogenases is reported in detail under the heading "Lipoamide Dehydrogenase". These findings indicated the concentration of Cd^{II} available for binding to the enzyme was markedly diminished in the presence of E.D.T.A. Consequently the mitochondrial enzyme was definitely rendered less susceptible to inhibition by cadmium through the presence of chelating agents. In vitro experiments were therefore conducted in the absence of E.D.T.A. (or other chelating agent) in the assay mixture.

The permeability barrier to succinate and cofactor (NAD^+) necessitates the complete solution of the lipoprotein membranes protecting the enzyme in order to secure maximal in vitro enzymatic activity.

When dissolved, the assay system still requires the presence of an artifical electron acceptor. D.C.I.P. is employed routinely in the diagnostic test for ascorbate, where it functions as a secondary standard.

The succinoxidase pathway, which incorporates succinic dehydrogenase linked through an FAD-bound enzyme to cytochrome oxidase, utilises O_2 as an electron acceptor. Succinic dehydrogenase, buried deeply in the inner mitochondrial membrane walls (Brunner and Bücher, 1970) is possibly located in an oxygen-depleted region, and functions as an anaerobic dehydrogenase.

The linkage of succinic dehydrogenase to a "cytochrome particle" does not prevent the transfer of electrons to a diaphorase. The dehydrogenase is, however, far more unstable than the succinoxidase system, losing 85% of its activity when stored at $4^{o}C$ in air for 24 hr. The assay should therefore be performed with minimum delay after the mitochondria have been isolated (King, 1963).

The incubation of oxidised glutathione with the enzyme abolished both succinoxidase and dehydrogenase activities, further evidence of the existence of a labile thiol group at the active centre. The thiol is shared by both the aerobic succinoxidase and anaerobic dehydrogenase (King, 1963).

The purified succinic dehydrogenase is free from lipid, haem, flavin or co-enzyme Q, but has an extremely labile sulphide, which chelates rapidly (and at least partially irreversibly) with divalent cations (King, 1966). The binding may be reversed to some extent by E.D.T.A. or dithiothreitol, but only about 40% of the metal could be displaced

once it had become firmly bound.

Why cadmium did not exert an effect on the *in vivo* activity of the enzyme when it was present in the mitochondrial preparation in a concentration of 10-20 µM was a crucial question, for which we have attempted to find a solution. The fact that the enzyme was so deeply embedded within the mitochondria may well account for the insensitivity of the enzyme to powerful *in vivo* inhibitors. Cadmium could either be prevented from crossing the mitochondrial membrane and consequently become bound to the outer membranes of the mitochondria; alternatively, if it did succeed in entering the inner folds, it was probably bound to thiol groups other than those in enzyme active sites.

The impression obtained from the series of experiments on succinic dehydrogenase and cytochrome oxidase was that cadmium was unable to exert its influence on intramitochondrial enzymes due to a very selective permeability barrier in the outer mitochondrial membranes. Any cadmium that was associated with mitochondria must be assumed to be attached to the outer structure.

Succinic dehydrogenase and cytochrome oxidase occupy the same intracellular site and behave similarly *in vivo* and *in vitro* in the presence of cadmium. The kinetics of cadmium inhibition of succinic dehydrogenase are therefore discussed under the heading "Cytochrome oxidase".

III. 5. LIPOAMIDE DEHYDROGENASE

(Reduced NAD: lipoamide oxidoreductase)

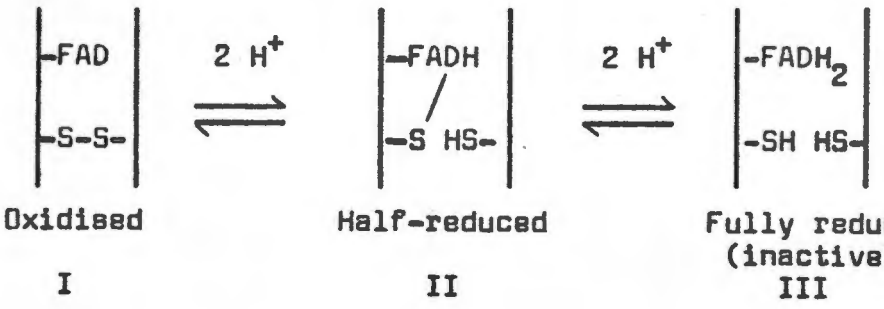
(E.C. 1.6.4.3.)

The function of this enzyme is to regenerate oxidised lipoamide, which plays a vital role in oxidative decarboxylation of α-keto acids, as for example, in the conversion of pyruvate to acetate and CO_2.

Lipoamide dehydrogenase is a mitochondrial-bound flavoprotein, with FAD as prosthetic group. It has a molecular weight of 100,000 (Massey, 1958). It was previously known as "Diaphorase" and was first isolated by Straub (1939).

The overall reaction process is shown in Fig. 22.

The reaction mechanism of lipoamide dehydrogenase has been investigated by Massey and Veeger (1961). They adduced evidence that, in the active enzyme, flavoprotein was only half reduced. Complete reduction of the enzyme by NAD-free $NADH_2$ rendered it catalytically inactive. Titration of the enzyme by $NADH_2$ revealed that an additional group was being reduced simultaneously. This they showed to be a disulphide group (Massey and Veeger, 1961). The three possible forms may be represented thus:-



Oxidised I

Half-reduced II

Fully reduced (inactive) III

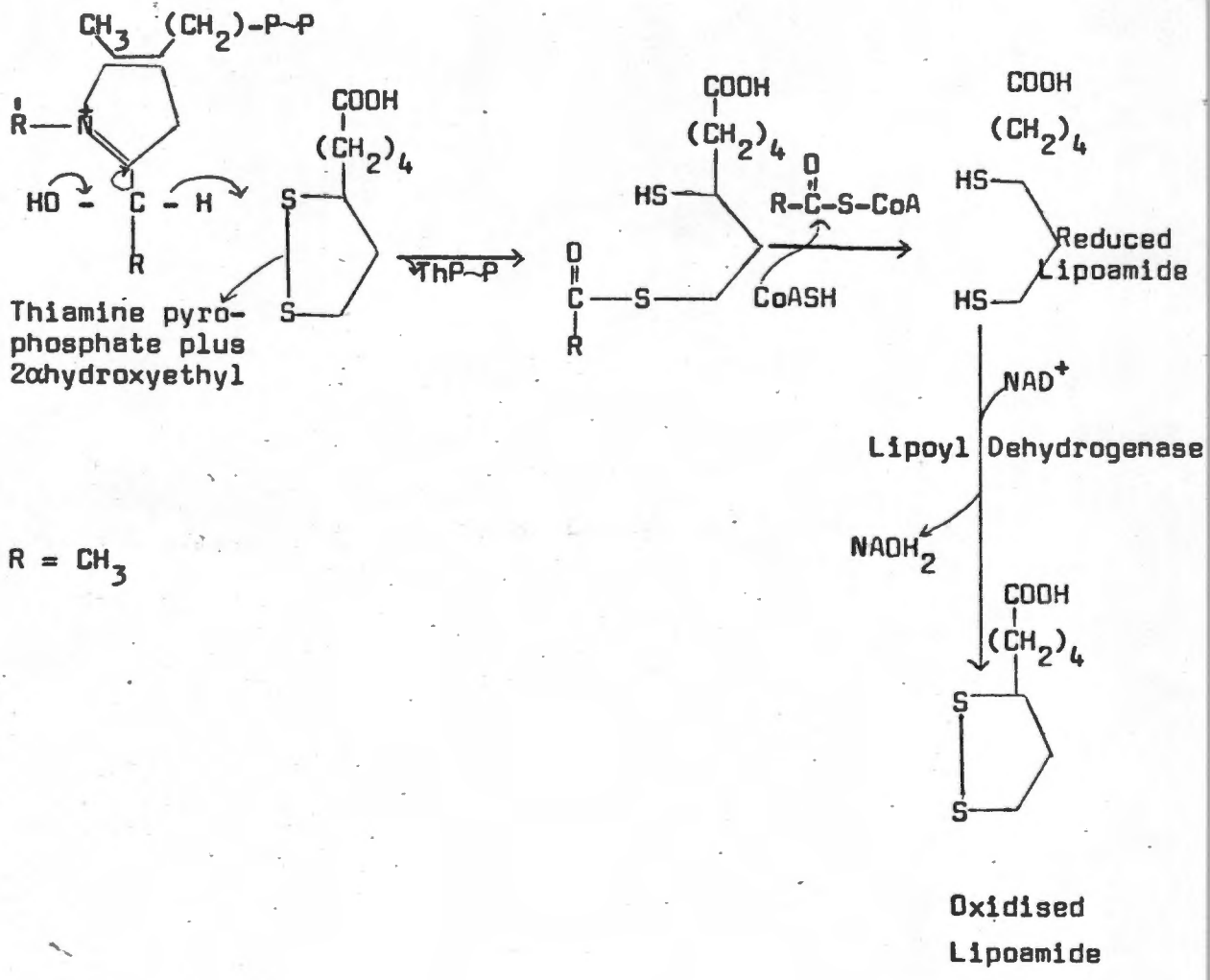
In view of this the importance of the equilibration period before the onset of the assay cannot be overstressed, as the enzyme must be in the fully oxidised form (State I) prior to $NADH_2$ addition. NAD^+ is essential for stability of the enzyme-substrate-cofactor complex, in order to prevent formation of the inactive State III on $NADH_2$ addition (Massey and Veeger, 1961).

Material and Methods

Both the purified enzyme and substrate were purchased from Sigma, Ltd. The enzyme was dissolved in water and diluted to a protein concentration of approximately 0.01 mg./ml. prior to use. NAD^+ and $NADH_2$ were obtained from Seravac, Ltd.

E.D.T.A. (10^{-4}M) was included in assay media routinely except when the direct effect of Cd^{II} on the enzyme was being investigated.

Fig 22: The role of lipoamide dehydrogenase in oxidative decarboxylation.



Bovine serum albumin (B.S.A.) 2% (w/v) was added to the assay as a stabiliser.

DL-lipoamide, the substrate, was found to be very insoluble in water, and was consequently dissolved in ethanol to give a stock concentration of 50 mM. (Flu-harty, Adelson and Gaber, 1969).

Preparation of homogenate.

The liver was excised from the chicks, homogenised in M.S.E. and differentially centrifuged to prepare the mitochondrial pellet exactly as described under the heading "Cytochrome Oxidase".

Immediately after preparation, the mitochondrial pellet was resuspended in the phosphate buffer (pH 7.4) employed in the assay. One aliquot was assayed as intact mitochondria; a second was dissolved in a final Triton-X-100 concentration of 0.5%, and assayed.

Assay Procedure

A modification of the method of Massey, Gibson and Veeger (1960) was followed.

In order to prevent precipitation of the albumin by the ethanol, the substrate/ethanol was added to the buffer

and the mixture stirred well prior to the addition of the 2% B.S.A.

The procedure was as follows:-

	Blank	Test
0.06M phosphate buffer pH 7.4	2.2 ml.	2.2 ml.
60 mM DL Lipoamide	0.05 ml.	0.05 ml.
Mix well to ensure dilution of ethanol		
20 mM NAD^+	0.05 ml.	0.05 ml.
2% B.S.A.	0.10 ml.	0.10 ml.
Pure enzyme or liver preparation.	0.02 ml.	0.02 ml.
Mix well, equilibrate 5 min. at 37^oC to allow formation of the Enzyme - Substrate - Cofactor complex.		
50 mM $NADH_2$		0.10 ml.

The decrease in O.D. at 340 nm was recorded on a Beckman DB recording spectrophotometer.

In separate *in vitro* experiments, Cd^{II} was added both to the whole assay system, and to the enzyme (or mitochondrial preparation) alone. The preincubation period in these experiments was a very important factor in determining the degree of inhibition of the enzyme by cadmium.

The enzyme was assayed by the method of Massey *et al.* (1960) and the specific activity expressed as follows:-

Spec.Act: Δ E 340 nm. min^{-1}. mg Protein^{-1} X $\frac{1}{6.22}$

$E^{mM}_{340\ nm}$ = 6.22 for DL-lipoamide

Results

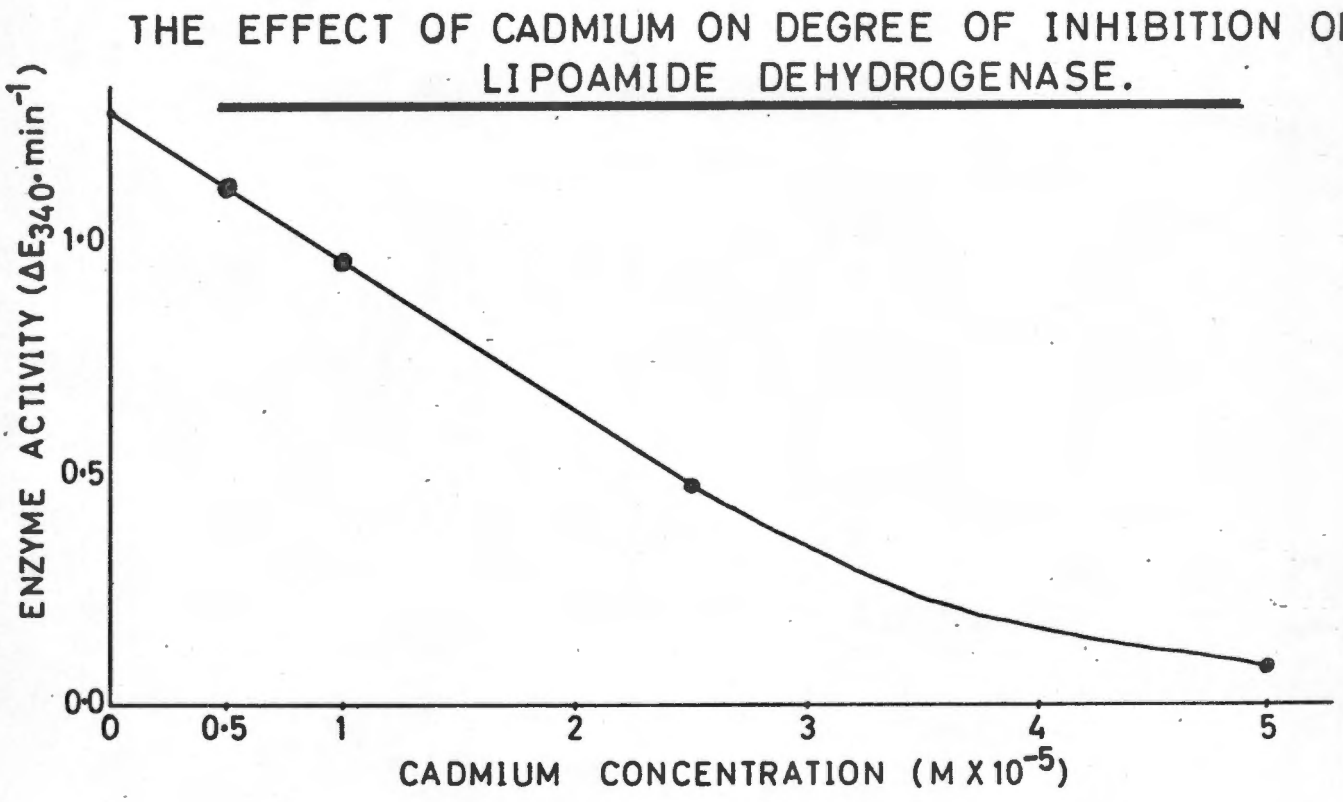
1. In vitro

The enzyme was studied in normal chick liver mitochondria and in mitochondria disintegrated by addition of the detergent Triton-X-100, which rapidly dispersed the lipoprotein membrane and liberated the enzymes. The results are shown in Table 24 and Fig. 23.

TABLE 24 In vitro studies of cadmium inhibition of lipoamide dehydrogenase in whole and in disintegrated mitochondria.

Sample	Specific Activity	% of Control
Whole Mitochondria		
Control	0.040	100
2.5×10^{-6} M Cd	0.036	90
1×10^{-5} M Cd	0.029	72
2×10^{-5} M Cd	0.018	46
Disintegrated Mitochondria		
Control	0.234	100
1×10^{-6} M Cd	0.134	57
2×10^{-6} M Cd	0.125	53
5×10^{-6} M Cd	0.098	42

FIG. 23.


THE EFFECT OF CADMIUM ON DEGREE OF INHIBITION OF LIPOAMIDE DEHYDROGENASE.
ENZYME ACTIVITY ($\Delta E_{340} \cdot min^{-1}$)
1·0
0·5
0·0
0
0·5
1
2
3
4
5
CADMIUM CONCENTRATION ($M \times 10^{-5}$)

The disintegrated mitochondrial preparation had nearly 6 times the specific activity of the intact mitochondria, and added cadmium exerted a more potent inhibitory effect on the "free" enzyme.

The effect of time on the preincubation of Cd^{II} with chick liver mitochondria, and the resultant change in lipoamide dehydrogenase activity.

The results are shown in Table 25. There was a steady increase in the percentage inhibition with time, although so far it has not been determined whether this phenomenon is related to attachment of more Cd^{II} ions to the protein. After 10 - 15 min. a steady state had been reached, after which there was little change in enzyme activities. (Fig. 24)

Comparative inhibitory studies of lipoamide dehydrogenase with cadmium at different concentrations were therefore strictly controlled as regards the period the metal was preincubated with the enzyme.

TABLE 25 / ....

TABLE 25 The effect of time on lipoamide dehydrogenase inhibition by cadmium.

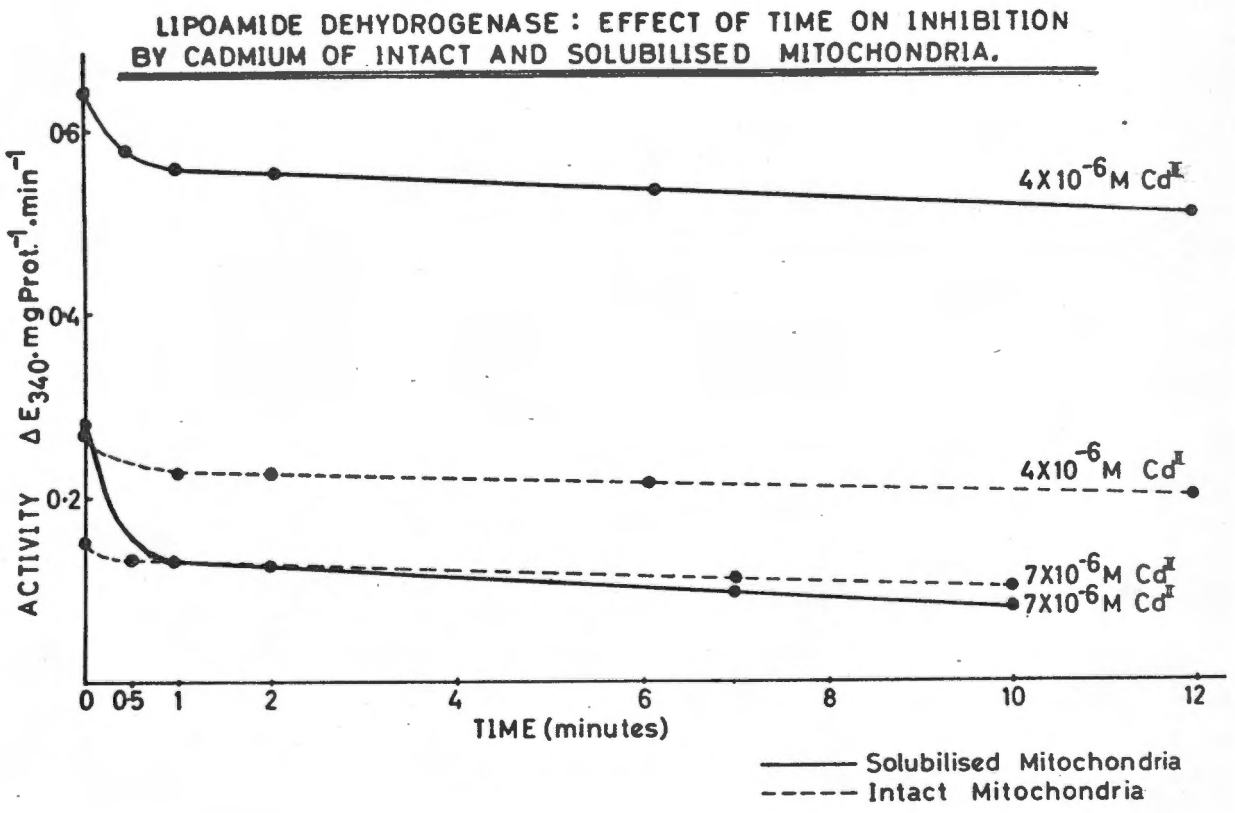
$[Cd^{II}]$ μM	Mitochondrial preparation	Percentage inhibition at 0 min.	5 min.	10 min.	20 min.
3	Intact	10	16	21	25
	Disintegrated	12	25	37	45
6	Intact	10	23	30	36
	Disintegrated	45	62	70	77
25	Intact	83	90	95	95
	Disintegrated	81	95	95	95

Optimum conditions for monitoring the effect of pre-incubation time on enzyme inhibition were achieved using 6 μM cadmium.

The protective effect of dithiols and chelating agents against cadmium inhibition of lipoamide dehydrogenase.

E.D.T.A. and dithioerythritol (D.T.E. - Cleland's reagent) were introduced into the assay system in order to assess the binding capacity of the enzyme for Cd^{II} in the presence of these protective agents. The results are shown in Tables 26 and 27.

FIG. 24.


LIPOAMIDE DEHYDROGENASE : EFFECT OF TIME ON INHIBITION BY CADMIUM OF INTACT AND SOLUBILISED MITOCHONDRIA.
ACTIVITY ΔE340·mgProt.-1.min-1
0·6
0·4
0·2
4X10-6M CdII
4X10-6M CdII
7X10-6M CdII
7X10-6M CdII
0 0·5 1 2 4 6 8 10 12
TIME (minutes)
Solubilised Mitochondria
Intact Mitochondria

(a) Dithioerythritol

TABLE 26 The effect of D.T.E. on the inhibition by cadmium (6×10^{-6}M) of lipoamide dehydrogenase in disintegrated mitochondria.

Sample	ΔE 340.nm	Percent of Control
Control (no Cd^{II})	0.28	100
0 min.	0.13	45
5 min.	0.11	39
10 min.	0.08	29
12 min.	0.12 *	43 *
17 min.	0.07 *	26 *

*1×10^{-4}M Dithioerythritol (D.T.E.) added after 10 min.

The activity (as a percent of control) rose 14% during the 2 min. following addition of D.T.E., where it could reasonably have been expected that, in the absence of the dithiol, there would have been a further 5% inhibition.

The progressive inhibition was not entirely prevented however, and 5 min. later the activity had fallen to 26% of the control level. By forming a stable non-ionised complex with Cd^{II}, D.T.E. would be expected to lower the concentration of ionic Cd^{II} free to attach itself to the enzyme. The fact that the enzyme activity increased after dithiol addition suggests that D.T.E. actually removed

some Cd^{II} which was attached to the enzyme. Whatever the mechanism of the stimulation, it was short-lived, and suggests that Cd^{II} still attached to the enzyme has induced conformational changes which lead to irreversible inactivation.

(b) E.D.T.A.

In a more definitive experiment, inactivation of the enzyme in a relatively concentrated cadmium environment (2.5×10^{-5}M) was very markedly ameliorated by addition of a 20-fold higher E.D.T.A. concentration (5×10^{-4}M). The assay was conducted on detergent-treated mitochondria.

TABLE 27 The effect of E.D.T.A. on the inhibition of lipoamide dehydrogenase by cadmium (2.5×10^{-5}M).

Sample	Activity	Percent of Control
Control (no Cd^{II})	0.280	100
20 sec.	0.053	19
2 min.	0.013	5
7 min.*	0.190*	67*
20 min.	0.012	4
30 min.*	0.055*	20*

*E.D.T.A. added at t = 3 min. of Cd^{II} preincubation

In the presence of E.D.T.A. (at 7 min.) a large percentage of enzyme activity had been restored, and at 30 min. there was still higher activity than that found in the absence of E.D.T.A. at 30 sec. In the E.D.T.A.-free assay, there remained only 4% of the original activity at 20 min., and at 30 min. the activity in that preparation was only a trace of the original, uninhibited value. The mechanism of metal ion inhibition is illustrated in Fig. 25.

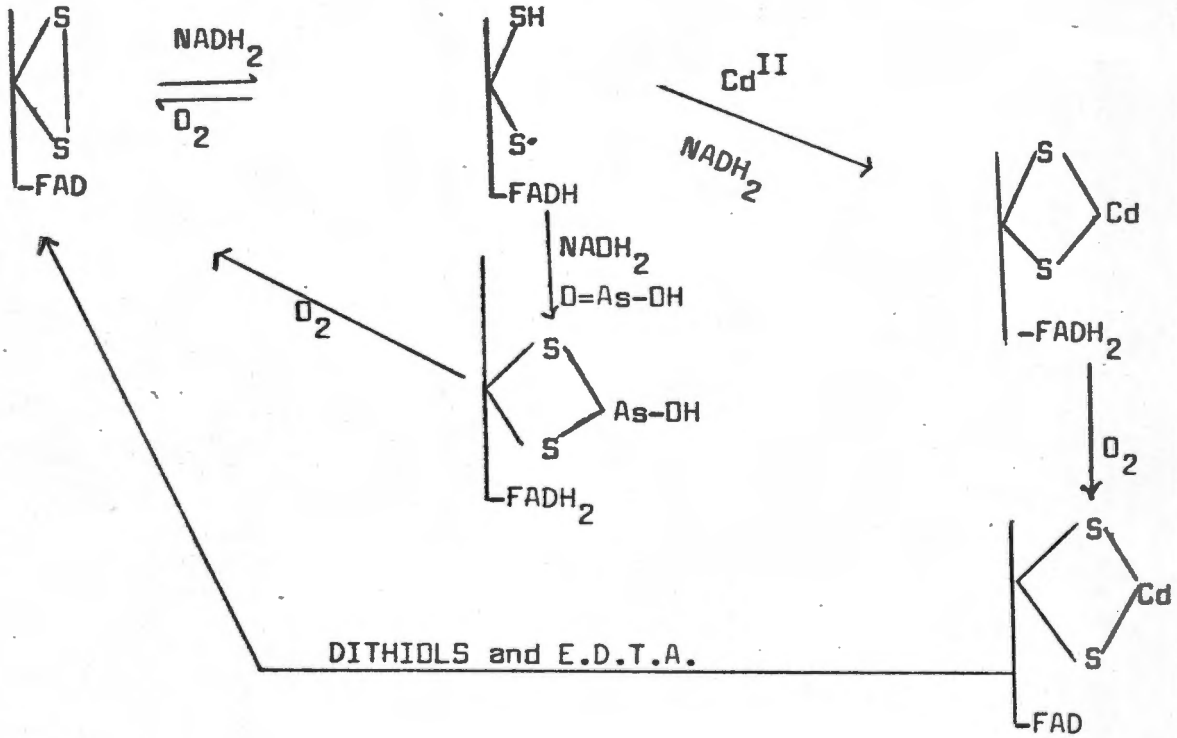
2. In vivo

Despite wide individual variation in the activity of lipoamide dehydrogenase in the liver of control and Cd^{II} poisoned chicks, there was a significant depression of 34% ($p < 0.05$) in the mean activity of lipoamide dehydrogenase in cadmium-poisoned chick liver mitochondria when compared with that of the normal chick (Table 28).

TABLE 28 The specific activity of lipoamide dehydrogenase in normal and cadmium-poisoned chicks

	Normal Chicks	Cd^{II}-poisoned Chicks
No. of samples	11	8
Sp. Activity	0.65 ± 0.18	0.43 ± 0.08

Fig 25: The mechanism of Cd^{II} binding to lipoamide dehydrogenase (Misaka and Nakanishi, 1966)



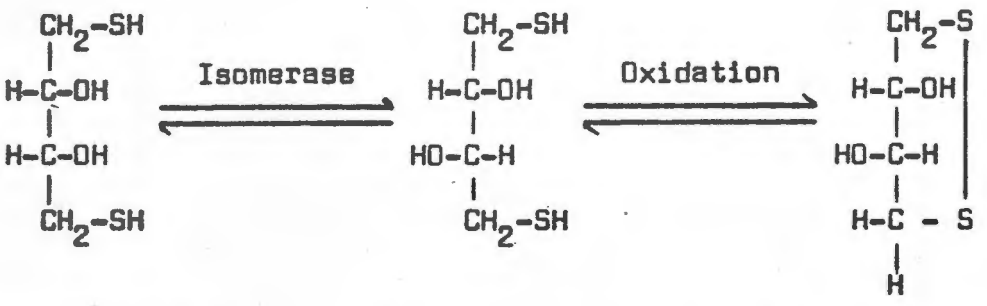
Arsenite, Fe^{II} and Co^{II} inhibition may be removed by oxygenation, but inhibition due to Cd^{II} must be ameliorated by E.D.T.A. or thiol groups.

The above observations were made on cadmium-poisoned chicks suffering severely from intoxication by the metal. The dose administered was 12 µg at day 15, and up to 65% of this Cd^{II} could be recovered from the liver of these chicks. Milder degrees of poisoning with lower doses of cadmium failed to elicit the same depression of lipoamide dehydrogenase activity.

Discussion

Addition of dithiol compounds could have minimised the effect of the Cd^{II} on lipoamide dehydrogenase by two mechanisms. The first, a mechanism common also to E.D.T.A., was by chelation of the Cd^{II} ions, thereby effectively reducing the number of ions available for combination with the enzyme. The second mechanism involved dynamic exchange of protons between the dithiol (D.T.E. in this case) and the inhibited active centre of the enzyme, in which a dithiol group is a vitally important feature. Dithiothreitol (trans form) could readily be converted to dithioerythritol (Cis) by an isomerase. Oxidation of the reagent causes cyclisation to Cis/trans 3,4 Dihydroxy - O - dithiane. (Cleland, 1964).

FIG. 26 Interconversion of Cleland's reagent



Dithioerythritol (Cis) | Dithiothreitol (Trans) | 3,4, dihydroxy-o-dithiane

Cleland (1964) noted that at low redox potential (-0.33 V at pH 7.0) cyclisation of this compound occurred more readily than did oxidation and cyclisation of lipoamide. Reduction of oxidised lipoamide by D.T.E. has an equilibrium constant of 31, indicating a very ready reaction, and the formation of a stable product.

It was advantageous to use Cleland's reagent in preference to other thiol-containing compounds such as cysteine, reduced glutathione or mercaptoethanol, because in addition to the above-mentioned factors, it was very soluble in aqueous media, had little odour, and was not autoxidised in air.

The enzymic process was initiated by addition of $NADH_2$ to the equilibrated enzyme-substrate-co-factor (NAD^+) complex. When intact mitochondria were the enzyme-source, a lag period preceded the linear fall in O.D. (340 nm) which was the manifestation of the conversion of $NADH_2$ to NAD^+.

A permeability barrier against $NADH_2$ exists at the mitochondrial membrane, and a shuttle must operate to transfer $NADH_2$ into the cell as NAD^+. Once inside the mitochondria, $NAD^+$was reduced, and could act as the electron donor for the lipoamide dehydrogenase. This shuttle was primarily motivated by malate dehydrogenase, as discussed elsewhere in this thesis.

The lack of constituent enzymes of the cytosol in the mitochondrial suspension may prevent immediate conversion of $NADH_2$ to NAD^+, and although malate dehydrogenase is located on the outer mitochondrial membranes, there may be insufficient shuttle operating to achieve maximum velocity for the lipoamide dehydrogenase immediately that $NADH_2$ was added to the assay medium. In other words, a rate-limiting step may be introduced through the inability of $NADH_2$ to combine rapidly with the intra-mitochondrial lipoamide dehydrogenase. This may partially explain the lag phase of the enzymic reaction and the greatly augmented reaction rate (8-10 times) of disrupted mitochondrial preparations.

If the mitochondria are ruptured, the malate-oxaloacetate shuttle is no longer needed to transport $NADH_2$ to the enzyme site, as the active centre of the enzyme would be openly exposed to both lipoamide and $NADH_2$.

From Table 24 it may be observed that the intact mitochondrial enzyme system was slightly, but consistently, more

resistant to inhibition by cadmium than was the enzyme from ruptured mitochondria. The postulated mechanism for this is that the mitochondrial membrane does not permit free passage of Cd^{II} ions into the mitochondria. Consequently those enzyme molecules buried in the mitochondria were protected from the powerful binding influence of cadmium to a greater extent than the enzyme liberated by detergent, notwithstanding the fact that there were many more lipoamide dehydrogenase (and other protein) molecules available as targets for the metal when the mitochondria had been disintegrated. The observed depression of lipoamide dehydrogenase contained within intact mitochondria must, therefore, be a maximal value for enzyme inhibition. Impairment of uptake of NADH, substrate and other factors would contribute towards the apparent inactivation of the enzyme. Furthermore, the difference observed before the inhibition of the enzyme from disintegrated mitochondria as opposed to the activity from the same intact organelles must be a minimal index of the degree of impermeability of the mitochondrial enzyme.

It might be argued that a similar difference in enzyme kinetics would emerge if Cd^{II} restricted the operation of the malate-NADH shuttle, as mitochondrial malate dehydrogenase remains intact in systems containing Cd^{II}. If preincubation of mitochondria caused the organelles to be less permeable to NADH or lipoamide, either of these compounds

could become rate-limiting if lipoamide dehydrogenase itself were not inhibited, i.e. the fall in apparent enzymic activity would be due to lack of NADH or lipoamide; not an inactive enzyme. Since the enzyme set free from mitochondria when they are detergent-disrupted is susceptible to inhibition by Cd^{II}, a decline in mitochondrial permeability to NADH or substrate would strengthen the evidence for a location of lipoamide dehydrogenase within the mitochondria impervious to Cd^{II}. One could not envisage that NADH or lipoamide could possibly become more available to the enzyme system than when the mitochondria have been thoroughly disintegrated. If, as a result of treatment with Cd^{II}, the mitochondria became freely permeable to NADH and lipoamide, differences between enzymic activity of intact and disrupted mitochondria could rest solely on inactivation of the enzyme, and be an indirect measure of the isolation of its mitochondrial site. To the extent that transport of NADH, lipoamide or any other essential component of the system was rate-restricting in Cd^{II}-treated mitochondria as compared with normal organelles, one would exaggerate the apparent inhibitive action of Cd^{II} on the enzyme.

The enzyme has a potent diaphorase activity, which was powerfully enhanced when catalytic activity towards lipoyl derivatives was eliminated by stoichiometric quantities of

divalent copper (Massey, 1963). This changeover was accompanied by oxidation of essential thiol groups. This was the first evidence that led to the elucidation of the structure of the active site of this enzyme. Divalent metal ions, particularly cadmium, have been instrumental in deciphering the mechanism of the reaction process at the active site (Searls, Peters and Sanadi, 1961; Misaka and Nakanishi, 1966).

CONCLUSION

In the liver of the developing chick embryo, significant inhibition by cadmium ions *in vivo* of the mitochondrial enzyme lipoamide dehydrogenase takes place only when the concentration of the metal is much greater (70 μM) than that (1 - 2 μM) adequate to effect a similar change in the enzyme *in vitro*.

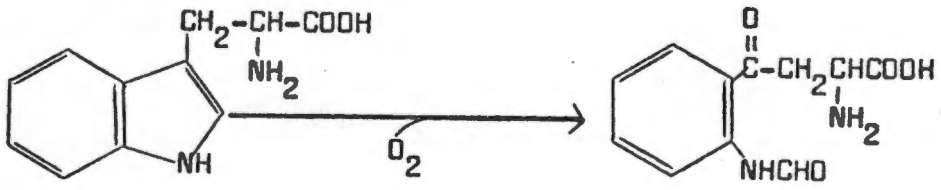
There appear to be specific binding sites, probably thiol in nature, in the cytoplasmic and mitochondrial membranes, which together constitute a metabolic barrier to the ingress of cadmium into the mitochondria. Cd^{II} access is largely excluded from the extremely sensitive dithiol group in the active centre of lipoamide dehydrogenase. Possible changes in permeability of membranes to other components of the enzyme system, such as substrate and co-enzymes,

may contributs to the overall derangement of the system, but changes in the enzyme itself are probably of paramount importance. The concentration of cadmium observed experimentally in the mitochondrial fraction of the liver (3×10^{-5}M) is not consistent with the degree of inhibition by cadmium *in vivo* of this and other mitochondrial enzymes, unless it is concluded that most of the Cd^{II} ions do not reach the intra-mitochondrial space, but are attached to and retained by the outer membranes, and thereby rendered metabolically inert insofar as the intra-mitochondrial enzymes are concerned.

III. 6. TRYPTOPHAN OXYGENASE

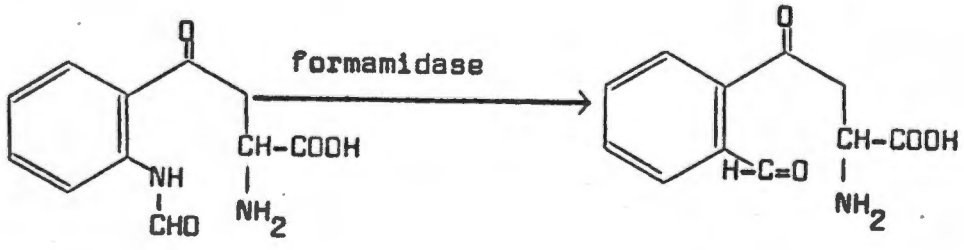
(E.C. 1.99.2.c)

Tryptophan oxygenase catalysss the conversion of L-tryptophan to N-formyl-kynurenine, as shown below.



L-tryptophan N-formylkynurenine

N-formyl kynurenine is converted to kynurenine by the enzyme formamidase. This reaction runs concurrently with the one above, and must be considered when assaying tryptophan oxygenase.



N-formylkynurenine Kynurenine

Tryptophan oxygenase normally exists in the inactive apoenzyme form (Greengard and Feigelson, 1962). Addition

of haem ferriprotoporphyrin IX, either as haematin, or as methaemoglobin, changes the apoenzyme to the active holoenzyme (Tanaka and Knox, 1959).

Tryptophan oxygenase incorporates 2 oxygen atoms into formyl kynurenine, deriving them from atmospheric oxygen (Hayaishi, Rothberg, Mehler and Sato, 1957). Thus, oxygen acts as a second substrate for which the Km of the enzyme decreases as it becomes saturated with L-tryptophan (Feigelson, 1969). The L-tryptophan acts as a stabiliser, and is added to the buffers used in homogenisation of tissue, and purification of tryptophan oxygenase.

Globin, an avid binder of protoporphyrin IX, markedly inhibits the enzyme (Feigelson and Greengard, 1961).

Subtle addition of a reducing agent protects the enzyme from oxidation (Tanaka and Knox, 1958). Ascorbate is usually used, but it has been found in this laboratory that ascorbate in slight excess will act as a potent oxidant of the indole ring of tryptophan. Consequently, the milder reducing agents dithiothreitol or cysteine have been employed (Feigelson and Maeno, 1966).

Greengard and Feigelson (1962) have shown that in the absence of exogenous reducing agent L-tryptophan is itself capable of reducing ferriprotoporphyrin IX to the ferrous state sufficiently for maximal activation of tryptophan oxygenase. This observation implies continuous cyclical

reduction and reoxidation of the enzyme's metalloporphyrin by tryptophan and oxygen respectively, during the course of the catalysis (Feigelson, Ishimura and Hayaishi, 1964).

Induction of tryptophan oxygenase by intra-peritoneal injection of L-tryptophan or hydrocortisone raised the activity several-fold four to six hours after administration (Knox and Mehler, 1950).

In the present investigation, tryptophan oxygenase was initially studied in relation to the development of normal chick embryos. Subsequently the effect of intravenously-administered cadmium on the activity of tryptophan oxygenase in the developing embryo was examined.

Materials

Analar reagents were used throughout the experiment unless otherwise stated.

Haematin was prepared from chick haemoglobin by the glacial acetic acid method (Rimington, 1942).

Methods

(i) Poisoning régime. In the first experiment, chicks were injected with 8 µgm. cadmium on day 10 of the 21-day incubation period. Representative numbers of poisoned chicks were sacrificed on subsequent days, i.e. from day 12 to day 21. The liver of each animal was assayed separately for enzymatic activity.

For the second experiment, the chicks were injected with 15 µgm. cadmium on day 14, and allowed to hatch.

(ii) Preparation of liver homogenate. The liver was removed, and immediately homogenised using 5 volumes of cold 0.1 M sodium phosphate buffer, pH 7.0, containing 2 mM L-tryptophan, in a glass-Teflon Potter-Elvjhem homogeniser. The homogenate was centrifuged at 12,000 x g for 15 min. in a Sorvall centrifuge. The supernatant was withdrawn carefully to avoid contamination from the thick fatty pad at the top of the tube.

(iii) Enzyme Assay. Determination of overall activity of tryptophan oxygenase in the supernatant necessitated activation of the stable apoenzyme by a preincubation period of 15 min. prior to the assay (Knox, Piras and Tokuyama, 1966).

1.0 ml. supernatant was incubated with 1.2 ml. of 0.15 M Na phosphate buffer pH 7.0, haematin (5 µmoles) and L-tryptophan (2 µmoles) at 37°C.

After 15 min., a further 15 µmoles L-tryptophan was added to initiate the assay. Incubation was continued for 40 min. at 37°C with continuous gentle agitation. The reaction was stopped by the addition of 0.5 ml. 20% (w/v) trichloroacetic acid (T.C.A.).

Controls in which T.C.A. was added prior to incubation were run concurrently. The precipitate was removed by centrifugation (1,500 x g) and the optical density of the supernatant read against the control at 321 nm and 360 nm in a Zeiss P.M.Q. spectrophotometer.

The total activity is calculated as follows:-

$$\Delta E\ 321 . \frac{1}{3.75} . (\text{mg Protein})^{-1} . hr.^{-1} + \Delta E\ 365 . \frac{1}{4.53} . (\text{mg Protein})^{-1} . hr.^{-1}$$

E^{mM}_{321} = 3.75 for formyl kynurenine

E^{mM}_{365} = 4.53 for kynurenine

The protein concentration of the liver supernatants was determined by the microbiuret method (Lane and Mavrides, 1969), using bovine serum albumin as a standard.

Results

(i) *In vitro*

Tryptophan oxygenase promotes the conversion of tryptophan to formyl kynurenine, and the next catabolic step, fission of the formyl group with production of kynurenine, is expedited by a separate enzyme, formamidase. It is possible that cadmium might exert a differential effect on the rates of these two enzymic processes *in vitro* or *in vivo*.

After incubation, the change in optical density at 321 nm due to formyl kynurenine exceeded that at 365 nm, the peak absorption of kynurenine (Table 29). These findings indicated that there was a greater increase in the formyl kynurenine (due to tryptophan oxygenase activity) than there was of kynurenine (due to formamidase activity).

TABLE 29 /

TABLE 29 The relative contributions of kynurenine (E 365) and formyl kynurenine (E 321) to the overall activity of tryptophan oxygenase

Sample	$\Delta E365.mg.^{-1} hr.^{-1}$	$\Delta E321.mg.^{-1} hr.^{-1}$	$\frac{\Delta E321}{\Delta E365}$	Total Activity
Control	0.017	0.037	2.17	0.0105
3×10^{-5}M Cd^{II}	0.023	0.042	1.83	0.0128
5×10^{-5}M Cd^{II}	0.022	0.039	1.77	0.0121
5×10^{-4}M Cd^{II}	0.019	0.033	1.74	0.0098
1×10^{-3}M Cd^{II}	0.011	0.020	1.82	0.0060

If the formamidase was less sensitive to cadmium, the relative proportion of kynurenine (E max = 365 nm) would rise, and the ratio ΔE 321 : $\Delta E365$ fall. The ratio in normal was in fact higher than that in cadmium-intoxicated samples. The overall activity, however, followed a course in relation to cadmium concentration closely similar to that already reported for tryptophan oxygenase in rat liver, viz. a stimulation of the enzyme at low concentrations of cadmium followed by inhibition at higher concentrations (Kench, Gubb and Sutherland, 1969). The results of these experiments are graphically illustrated in figure 27.

FIG. 27.

The IN VITRO effect of CADMIUM on TRYPTOPHAN OXYGENASE.

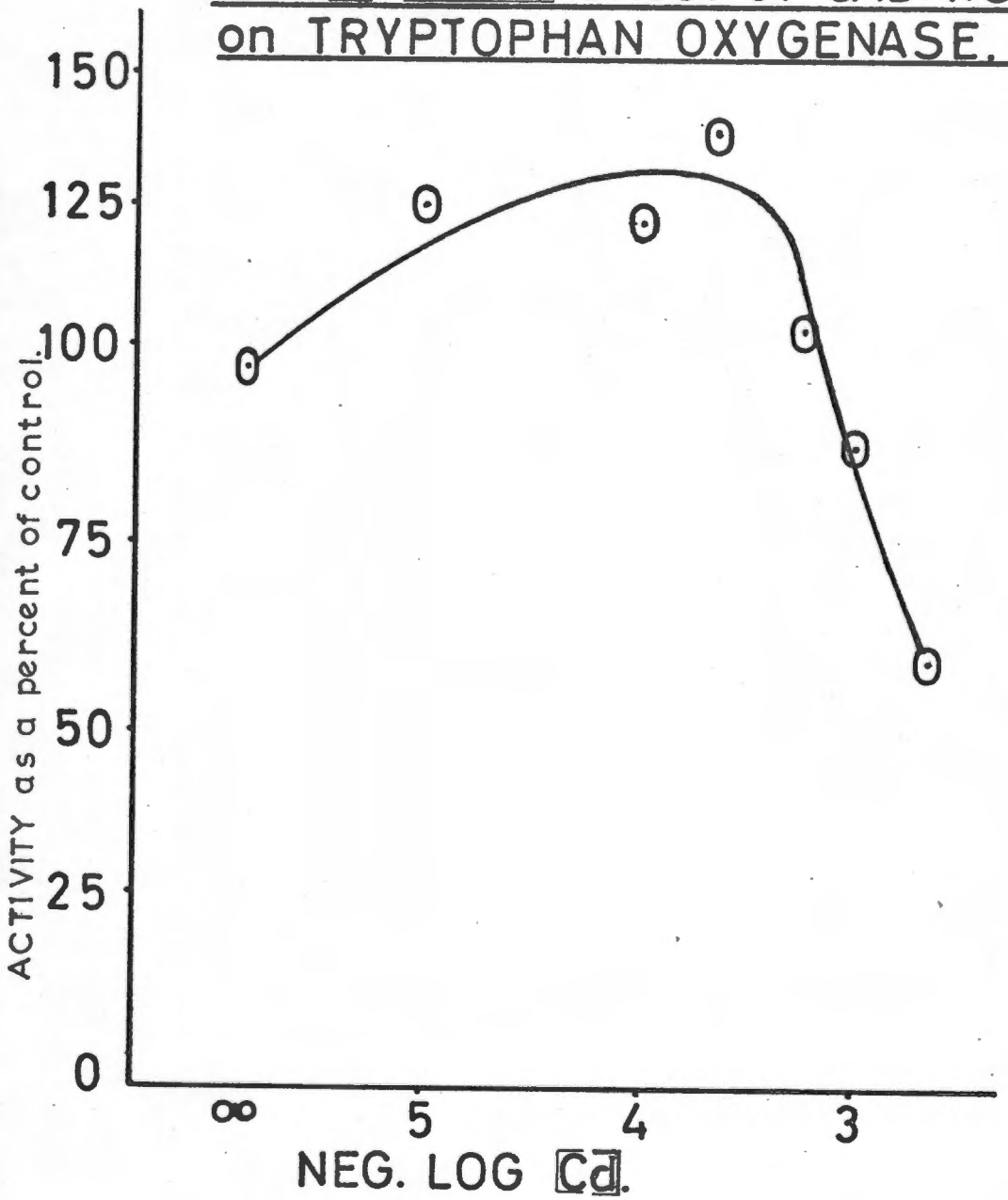


TABLE 30 The effect of different concentrations of Cd^{II} on the activity of tryptophan oxygenase

Experiment	Sample	Total Activity	Activity as % of normal
a.	Control (No Cd^{II})	0.0096	100
	2×10^{-4}M Cd^{II}	0.0136	141
	5×10^{-4}M Cd^{II}	0.0104	108
b.	Control (no Cd^{II})	0.0064	100
	6×10^{-4}M Cd^{II}	0.0056	89
	1×10^{-3}M Cd^{II}	0.0045	70
c.	Control (No Cd^{II})	0.0105	100
	5×10^{-5}M Cd^{II}	0.0128	123
	1×10^{-4}M Cd^{II}	0.0121	116
	5×10^{-4}M Cd^{II}	0.0108	103
	2×10^{-3}M Cd^{II}	0.0060	57

Activation of the enzyme was observed within the range of concentrations of Cd^{II} from 5×10^{-5}M to 5×10^{-4}M. The enzyme eventually became susceptible to cadmium inactivation when the *in vitro* concentration was raised to 1-2 mM. At this stage the activity fell away rapidly to 60 - 70% of the control value. There was, however, considerable difference in behaviour of the enzyme from individual chicks, presumably due to factors such as relative preponderance of other binding proteins. Nevertheless, all specimens examined

throughout a range of cadmium concentrations exhibited a peak of stimulation, followed by inhibition at higher concentrations of Cd^{II}. (Figure 27.)

(ii) In vivo

Tryptophan oxygenase was assayed in the liver cytosol of normal and of Cd^{II}-poisoned chicks. The chick-embryo liver was examined at different stages of development to discover any possible changes in the enzyme in normal as opposed to Cd^{II}-intoxicated chicks. The chick embryos were poisoned with 5 µg Cd^{II} injected into the allantoic sac on the 10th day.

The results of the enzyme activity at different incubation stages are shown in Table 31.

TABLE 31/

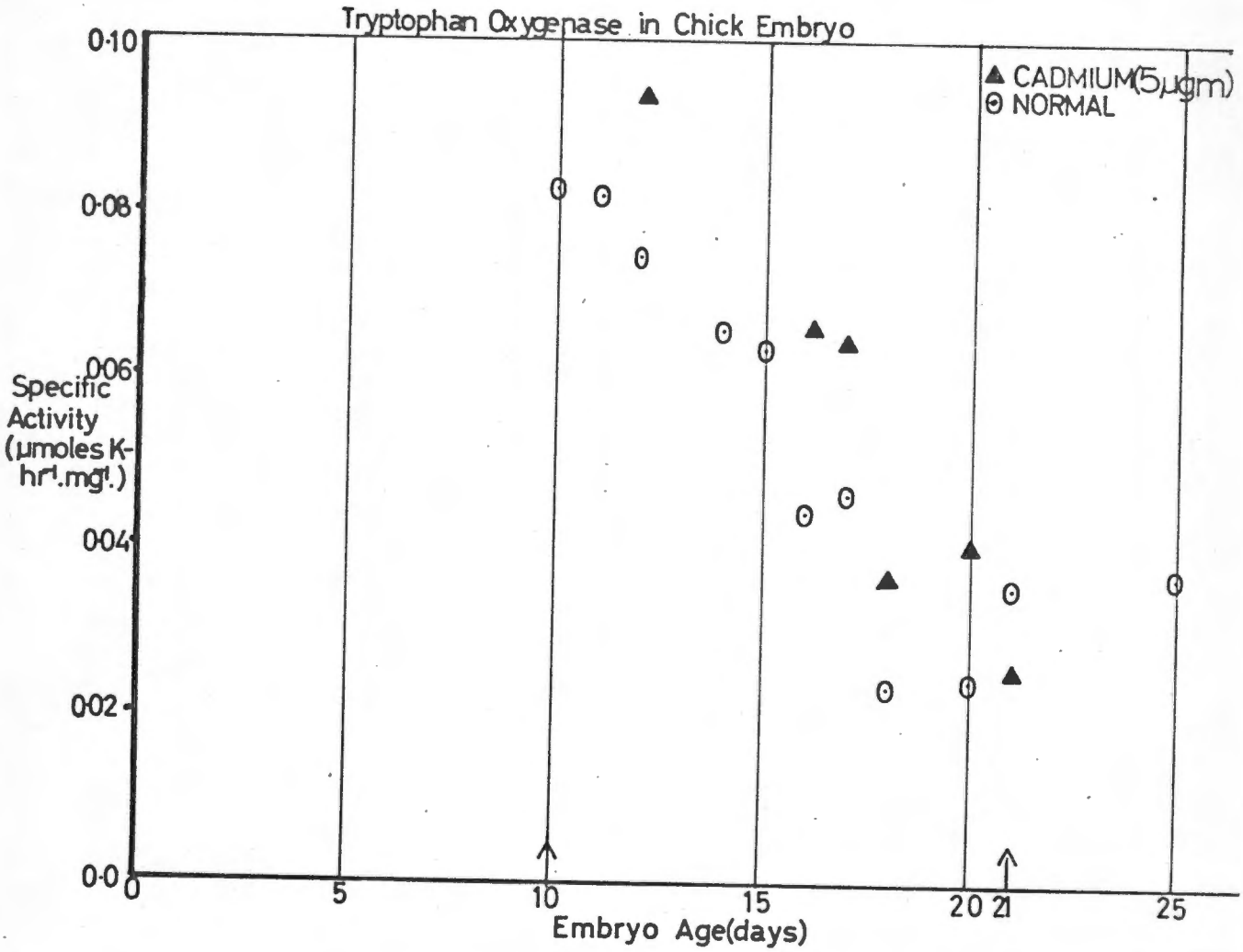
TABLE 31 The changes in specific activity of liver tryptophan oxygenase in normal and cadmium-poisoned chick embryos at different ages.

Age (days)	Normal/ Poisoned	Specific Activity	Mean wt. of whole liver (mg.)	Total tryptophan oxygenase activity per liver.
10	N	0.072	150	10.8
11	N	0.084	170	14.3
13	N	0.061	240	14.6
14	N	0.066	300	19.8
14	Cd^{II}	0.075		22.5
15	N	0.066	380	25.1
16	N	0.051	450	23.0
16	Cd^{II}	0.066		29.7
17	N	0.041	500	20.5
18	N	0.026	640	16.6
18	Cd^{II}	0.019		12.2
20	N	0.028	750	21.0
20	Cd^{II}	0.040		30.0
21	N	0.042	870	36.5
21	Cd^{II}	0.032		27.8

Table 31 illustrates that the specific activity of tryptophan oxygenase tends to fall steadily with age, but the total quantity of enzyme present in the liver rises as the embryo grows. Fig. 28 shows the decrease in specific activity with age of the embryo.

The difference between normal and cadmium-poisoned chicks was not significant, although generally the enzyme was a little more active in poisoned chicks.

FIG. 28.


Tryptophan Oxygenase in Chick Embryo
▲ CADMIUM(5µgm)
Θ NORMAL
0·10
0·08
0·06
0·04
0·02
0·0
Specific Activity (µmoles K-hr⁻¹.mg⁻¹.)
0
5
10
15
20
21
25
Embryo Age(days)

Chicks were also poisoned intravenously with 12 µg. cadmium, which represented a much increased level of toxicity. Tryptophan oxygenase was assayed on intravenously-poisoned chicks exactly as previously described (Table 32).

TABLE 32 The effect on liver tryptophan oxygenase of 12 µg. Cd^{II} injected intravenously into 14-day old chick embryos.

Sample and No. of chicks	Specific Activity µmoles kynurenine/ hr./mg. Protein	
Normal (4)	4.05	
Cd^{II} (3)	2.95	
Cd^{II} (3)	4.59	3.80 (mean)
Cd^{II} (5)	3.85	

Tryptophan oxygenase of cadmium-poisoned chicks was once again not significantly different from that of normal animals.

Discussion

A number of questions regarding the nature of the active centre of tryptophan oxygenase remain unsolved. Iron in haem, which is directly linked with enzyme-bound copper, may shuttle between ferrous and ferric states, as in the mitochondrial cytochromes (Maeno and Feigelson, 1965). Ascorbate stimulates maximal activity of the enzyme, perhaps by favouring the active ferro state rather than the more stable inactive ferri configuration (Feigelson and Maeno, 1966). On the other hand, autoxidation of ascorbic acid proceeds rapidly in the presence of Cu^{II} and could, thereby, provide hydrogen peroxide, and this could provide a potent source of the active oxygen required for the initial step in the catabolism of tryptophan. Consequently, ascorbate was not included in the assay in the experiments described here.

The role of Cd^{II} in this system is not understood; tryptophan oxygenase stands in marked contrast to catalase, the other haem enzyme investigated in this series, in which there was a significant depression of activity observed in chicks poisoned with cadmium. Either the haem pool was not sufficiently depressed to prevent full incorporation of the prosthetic group with the apoenzyme, or tryptophan oxygenase was preferential in acquiring the haem necessary for maximal activation.

In any event, the degree of intoxication induced in the young chick made insufficient impact on the synthesis of haem to significantly inhibit holoenzyme formation of tryptophan oxygenase. The role of the haem group in the binding of cadmium appears to be minimal, as both this enzyme and catalase were strongly resistant to cadmium *in vitro*.

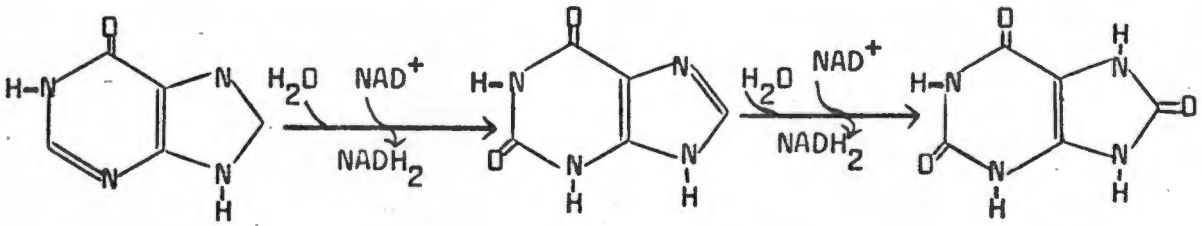
III. 7. XANTHINE DEHYDROGENASE

(Xanthine: NAD^+ oxidoreductase)

(E.C. 1.2.3.2.)

The enzyme, xanthine dehydrogenase, which is the terminal enzyme in avian purine catabolism, catalyses the following reaction sequence:

(hypo)xanthine + H_2O + NAD^+ = Urate + $NADH_2$



hypoxanthine xanthine Urate

The enzyme has a M.Wt. of 300,000, is soluble and found abundantly in liver cytosol. 2 FAD molecules constitute the flavin prosthetic group and are tightly bound to the apoenzyme, 2 molybdenum (Mo^{VI}) and 8 non-haem iron (NHI) atoms are additional obligatory cofactors.

Avian xanthine dehydrogenase is distinguished from milk and mammalian xanthine oxidases by the fact that NAD^+ is the physiological electron acceptor for the avian enzyme, as opposed to molecular oxygen for the oxidases. The avian enzyme was also believed to have twice the NHI and Mo^{VI} needs of the oxidases (Landon and Carter, 1960). However,

it has recently been reported that these cofactor differences were due to gross contamination, possibly by ferritin, in the earlier preparation of the avian enzyme (Rajagopalan and Handler, 1967).

The enzyme is relatively non-specific and has several possible substrates, including hypoxanthine, xanthine, pteridines, aldehydes and quinine. The components of the overall reaction, in sequential order, are:

$$DH_2 \longrightarrow Mo^{VI} \longrightarrow FAD \longrightarrow \left[(Fe^{3+}S) \equiv NHI\right] \longrightarrow NAD^+$$

Where DH_2 = the proton-donor, and

NHI = Non-haem iron

(Palmer, Bray and Beinert, 1964)

The active site of the enzyme has both a disulphide group and one Mo^{VI} atom attached in close proximity. The Mo^{VI} may be primarily involved in electron transport, and not directly concerned in the initial activation of the substrate (Rajagopalan and Handler, 1967; Mackler, Mahler and Green, 1954).

Materials and Methods

The substrate utilized in this series of experiments was hypoxanthine (Merck, Ltd.). The cofactor, NAD^+ was purchased from Seravac Laboratories, Ltd. All other reagents were standard analytical grade.

Chick liver homogenates were prepared in a Potter-Elvjhem homogenizer with a rotating Teflon plunger. The liver was chilled to 2^oC in 10 volumes 0.05 M phosphate buffer, pH 7.4, in which the tissue was then homogenized. All particulate matter was separated from the supernatant by centrifugation for 1 hr. at 105,000 x g in a Beckman Model L ultracentrifuge. The supernatant was carefully aspirated and could be stored at 4^oC for up to 24 hr. without noticeable loss of xanthine dehydrogenase activity.

A single liver was used for each assay for the cadmium-poisoned chicks, whereas the control values were obtained from a pooled preparation of the liver from 4 chicks.

Assay Procedure (Landon and Carter, 1960)

Enzyme activity was measured in terms of the increase in E 340 nm due to reduction of NAD^+ to $NADH_2$. The assay was conducted in a thermostatically controlled compartment

in a Beckman D.B. recording spectrophotometer.

The assay system contained, in a final volume of 1.25 ml.:

0.05 M phosphate buffer	0.80 ml.
10 mM hypoxanthine	0.20 ml.
10 mM NAD^+	0.10 ml.
1 mM E.D.T.A.	0.10 ml.
Enzyme solution	0.05 ml.

There was a short lag period during the assay, followed by a period of 10 - 15 min. during which there was a linear rise in E 340 nm.

The protein content of the supernatant was determined spectrophotometrically by the microbiuret method (Lane and Mavrides, 1969).

Results

The specific activity of a liver cytosol fraction was expressed as $\Delta 340$ min.$^{-1}$mg.$^{-1}$.

The protein content of the assay mixture usually varied from 4 - 7 mg.

In vitro tests were conducted as usual and some trials

with cadmium included 2×10^{-4} M E.D.T.A. to ascertain whether or not its chelating propensity could influence the overall enzyme activity.

(i) In vitro

The effect of cadmium on the enzyme was again far more marked when incubation preceded the assay. Preincubation of the cytosol with cadmium (1×10^{-6}M - 5×10^{-5}M) caused a definite decline in activity, the extent of which is shown in Table 33.

TABLE 33 In vitro effect of cadmium on chick liver xanthine dehydrogenase, and the influence of E.D.T.A.

Series	Sample (no. of chicks)	ΔE 340/ml. /min.	Specific Activity	Activity as % of control
A	Control (7)	0.80	0.067	100
	1×10^{-4}M Cd^{II} (2)	0.76	0.063	95
	2.5×10^{-4}M Cd^{II} (2)	0.60	0.050	75
	5×10^{-4}M Cd^{II} (2)	0.05	0.043	64
	5×10^{-4}M Cd^{II} + E.D.T.A. (2)	0.07	0.053	87
B	Control (4)	0.53	0.044	100
	1×10^{-5}M Cd^{II} (2)	0.42	0.035	80
	1×10^{-5}M Cd^{II} + E.D.T.A. (2)	0.49	0.041	93
	1×10^{-5}M Cd^{II} + 2.5×10^{-6}M Mo^{VI} (2)	0.42	0.035	80

2. in vivo

Cytosol preparations were diluted to a protein concentration of 4 - 8 mg./ml. The assays were duplicated for each chick, and are tabulated below. Some chicks were more obviously afflicted by the cadmium than others. The figures given in Table 34 are from chicks poisoned with 10 - 15 µg Cd^{II} between the 12th - 14th day of incubation.

TABLE 34 Specific activity of hepatic xanthine dehydrogenase in normal and in cadmium-poisoned chicks.

Type	No. of chicks investigated	Mean specific activity	S.D.	% of normal
Normal	10	0.037	±0.014	100
Cadmium-poisoned	10	0.021	±0.010	57

There was not always a significant loss of activity as described above; 5 - 7 µg cadmium injected intra-amniotically was not enough to cause hepatic necrosis, or to depress xanthine dehydrogenase activity.

TABLE 35 Specific activity of xanthine dehydrogenase in cadmium-poisoned chicks injected at 10 days intra-amniotically.

Type	No. of chicks investigated	Mean specific activity	% of normal
Normal	3	0.048	100
10 day Cd^{II}-poisoned	6	0.044	92

Discussion

The activity of hepatic xanthine dehydrogenase in newly-hatched chicks after small quantities of cadmium had been injected into them intra-amniotically at 10 or 12 days were not significantly different from normal values. At this age, and by this route, only a very narrow margin separates the lethal dose of cadmium from the quantity necessary to cause extensive hepatic cellular necrosis and diminished liver xanthine dehydrogenase. The precise dosage was seldom achieved, and many fatalities

resulted.

The effect of adding Mo^{VI} to the enzyme, and preincubation prior to assay was investigated, but free Mo^{VI} did not alter the ultimate activity of xanthine dehydrogenase.

The possibility that Cd^{II} displaced Mo^{VI} from the enzyme was considered, and a method for determining Mo^{VI} in micro-quantities (Ellis and Olsen, 1950) was employed to investigate Mo^{VI} concentrations in different chicks. The results, however, were inconclusive as no molybdenum was detected in any enzyme preparation.

It is now considered extremely unlikely that the Cd^{II} did in fact displace any Mo^{VI}, for it is tightly bound to the flavin (Bray, Petterson and Ehrenberg, 1961). The labile disulphide too (Rajagopalan and Handler, 1967), is well protected in the enzyme, and is not nearly so vulnerable as the similar grouping in mitochondrial succinic dehydrogenase. Succinic dehydrogenase was inhibited by 2×10^{-5}M Cd^{II} to the same degree as xanthine dehydrogenase by 5×10^{-4}M Cd^{II}. The cadmium concentration able to induce a significant fall in activity of xanthine dehydrogenase was 50 - 100 times that which sufficed to inhibit equally the other flavo-enzyme, lipoamide dehydrogenase.

In standard assay procedures E.D.T.A. was present in a protective capacity (Rajagopalan and Handler, 1967). When Cd^{II} was incubated with the enzyme, either in the

presence of E.D.T.A. or in its absence, there was a progressive binding of the metal to the enzyme in such a way as to inhibit the enzyme. E.D.T.A. protected the enzyme against inhibition by cadmium to a greater extent than it did when added to *in vitro* preparations of mitochondrial dehydrogenases. The metal did not have as powerful an avidity for xanthine dehydrogenase as it had for lipoamide or succinic dehydrogenase. The extent of the reactivation of enzyme activity was between 40% and 60%, depending on the relative concentrations of cadmium and E.D.T.A. present in the solution (Table 33).

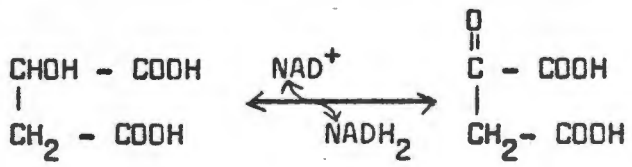
In conclusion, it appeared that xanthine dehydrogenase in chick liver cytosol was inhibited in extensive cadmium poisoning, particularly when there were obvious signs of liver damage. The enzyme was inactivated by the extent of 25 - 35% by cadmium *in vitro* when present in the same concentrations as might be expected in heavily poisoned chick liver cytosol ($4 - 7 \times 10^{-5}$M).

III. 8. MALATE DEHYDROGENASE

(Malate: NAD^+ oxidoreductase)

(E.C. 1.1.1.37)

Malate dehydrogenase (MDH) participates in the process of aerobic respiration in mitochondria and, in common with other enzymes present in these organelles, plays a vitally important role in the regulation of aerobic metabolism.



malate oxaloacetate

Malate dehydrogenase is present both in the mitochondria and the cytosol of hepatic cells. The enzyme has a molecular weight of 68,000, and the number of titratable SH groups varies from 8 or 9 in the cytoplasmic to 14 in the mitochondrial isoenzyme (Skilleter, Lee and Kun, 1970).

The enzyme brings the redox system formed by malate, oxaloacetate and the oxidized and reduced forms of nicotinamide adenine dinucleotide rapidly into equilibrium.

Boosted rate of synthesis of malate, NAD^+, or both, through the mediation of MDH, is offset by the formation of oxaloacetate.

Metabolic processes, such as aerobic oxidation of fatty acids, which contribute largely to the pool of $NADH_2$, promote "back-peddling" of the tricarboxylic acid cycle with the formation of malate and NAD^+. Although it appears that NAD^+ can to some extent be actively transported from the cytosol into mitochondria, the membranes of these organelles are impermeable to oxaloacetate and $NADH_2$, but freely permeable to malate. When the mitochondrial pool of malate is replenished, this acid diffuses into the cytosol down a concentration gradient (Krebs, 1968).

In the cytosol, through the agency of MDH, oxaloacetate is regenerated eventually via phosphoenolpyruvate kinase, and glucose is produced. The cue to the whole process of gluconeogenesis in the liver cell is the use of NADH within the mitochondria consequent to oxidative catabolism of fatty acids. Malate dehydrogenase and the malate shuttle are crucial to gluconeogenesis.

The activity of MDH was assayed in both cellular compartments of the liver of normal and cadmium-poisoned chicks to determine whether cadmium interfered in any way with the malate shunt mechanism. The impact of Cd^{II} *in vitro* on the liver fractions and on the pure enzyme was also investigated.

Preparation of cytosol and mitochondrial fractions

Homogenisation and centrifugation of the liver was executed exactly as for cytochrome oxidase, with the 10,000 x g supernatant retained as the cytosol fraction. The mitochondrial fraction was disintegrated with 0.5% (v/v) (final volume) Triton-X.

The protein concentration was measured in both fractions (Lane and Mavrides, 1969).

Materials and Methods.

Malate dehydrogenase (pure) was purchased from Seravec Ltd., Epping, Cape Town.

The assay reagents, as supplied by Boehringer (Mannheim) Ltd., were:-

1. 0.1 M phosphate buffer, pH 7.4 + 0.042 M aspartate.
2. 0.065 M α-oxoglutarate
3. 0.012 M NADH
4. 0.01 mg./ml. glutamate-oxaloacetate transaminase (G.O.T.).

The pure malate dehydrogenase was diluted 250 times to a final concentration of 0.04 mg./ml.

Assay Procedure

The volumes to be used in the kit were adjusted for microcuvettes, and were:-

1.2 ml. buffer

0.025 ml. α-oxoglutarate

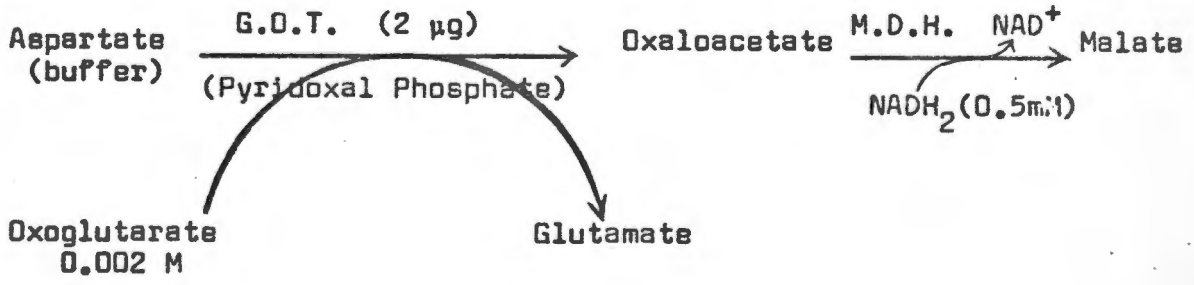
0.025 ml. $NADH_2$

0.025 ml. G.O.T.

The assay contents were well stirred, and preincubated in the thermostatically controlled Beckman DB recording spectrophotometer at 25°C. Pure enzyme or liver fraction was added (0.02 ml.), and the initial linear decline in O.D. (340 nm) was recorded against time.

For in vitro determinations, various concentrations of cadmium were preincubated with the preparations prior to assay.

FIG. 29 The reaction sequence followed for assay of malate dehydrogenase using the test-kit.



0.025 ml. 2-oxoglutarate, G.O.T. and $NADH_2$ were added to 1.0 ml. phosphate buffer (pH 7.4) which contained aspartate. After thorough equilibration, the sample to be assayed was added, and the decrease in O.D. at 340 nm was measured as $NADH_2$ was oxidised to NAD^+, and oxaloacetate simultaneously reduced to malate.

Activity of preparations.

Specific activity was calculated by dividing the activity by the protein concentration (expressed in mg./ml.).

Units of S.A. were

$$\Delta E_{340} \cdot mg^{-1} \cdot min.^{-1}$$

Results/

Results

1. In vitro

(a) Pure Malate Dehydrogenase

Malate dehydrogenase isolated and purified from pig heart mitochondria (Sigma) was assayed in a Cd^{II} environment at several different concentrations of the cation. A preincubation time of fifteen min. at 25°C was sufficient to ensure binding of Cd^{II} to the MDH.

The original enzyme suspension, in 70% $(NH_4)_2SO_4$, contained 10 mg. enzyme per ml. This preparation was diluted 250 times, and 0.02 ml. of diluted enzyme was assayed for activity in a final volume of 1.1 ml. The diluted preparation thus had a concentration of 40 µg/ml.; 0.8 µg MDH was used for each assay.

TABLE 36 The effect of Cd^{II} ions on the activity of pure malate dehydrogenase.

Sample	$\Delta E340.min.^{-1}$	Percent of Control
Control	0.848	100
1×10^{-4}M Cd^{II}	0.736	87
5×10^{-4}M Cd^{II}	0.608	71
2×10^{-3}M Cd^{II}	0.488	58

At 2 mM Cd^{II} there were 2.0 µg-atoms Cd^{II}/ml. present in the enzyme solution. This was responsible for the observed 42% inhibition of the 40 µg./ml. enzyme concentration.

The M.Wt. of MDH is 68,000, thus 40 µg./ml. represents $\frac{40}{68,000}$ µmoles/ml. or approximately 0.6×10^{-3} µmoles enzyme/ml. Thus the ratio of Cd^{II} to diluted MDH at 2 mM Cd^{II} concentrations is $\frac{2}{0.6 \times 10^{-3}}$:

or 3.3×10^{3} Cd^{II} atoms/MDH molecule.

Cadmium was unquestionably in great excess at millimolar concentrations, and appears to inhibit MDH via a completely different mechanism from that occurring when the metal acts on lipoamide dehydrogenase. The ratio of Cd^{II} to MDH suggests more a hydrolytic rather than a specific reactive-group binding type of inhibition.

(b) (i) Mitochondrial and cytoplasmic preparations of chick liver

The *in vitro* action of Cd^{II} on MDH in the cytosol and mitochondria of the livers of newly-hatched chicks was studied to evaluate the importance of the biological environment on enzyme heavy-metal system interactions.

Unfortunately, a pure enzyme from hepatic tissue was not available, and the comparison was made on data gathered from

malate dehydrogenase prepared from pig heart mitochondria.

TABLE 37 The effect of Cd^{II} on the activity of malate dehydrogenase from the cytosol and mitochondria of chick livers.

Cd^{II} concentrations	$\Delta E340.min.^{-1}$	% activity of control
(a) Cytosol		
Control (no Cd^{II})	0.57	100
4×10^{-5}M	0.59	103
1×10^{-4}M	0.57	100
2×10^{-4}M	0.58	102
5×10^{-4}M	0.56	98
8×10^{-4}M	0.51	89
2×10^{-3}M	0.42	74
(b) Mitochondria		
Control (no Cd^{II})	67	100
4×10^{-5}M	64	95
1×10^{-4}M	64	95
2×10^{-4}M	62	92
5×10^{-4}M	60	89
1×10^{-3}M	41	61

At millimolar concentrations, the mitochondrial isoenzyme was inhibited by Cd^{II} to a greater degree than was the cytoplasmic enzyme.

(b) (ii) The inhibitory effects of Cd^{II} in the presence of I_2, a known inhibitor of malate dehydrogenase.

Varrone, Consiglio and Covelli (1970) described the inhibition of pure MDH by I_2, thyroxine and iodine cyanide. Each enzyme molecule had thiol groups (Varrone et al. 1970) and activity was not depressed until more than 4 had been oxidised. All activity was dispelled when 10 thiol groups had become oxidised.

The possibility that Cd^{II} could protect the enzyme against inhibition by I_2, through the formation of relatively non-ionized CdI_2, was explored in a series of experiments.

Fresh solutions of tetra-iodothyroxine and I_2 were prepared daily in 10 mM concentrations. Thyroxine was not available as an analytical preparation, but was prepared from a pharmaceutical drug, Choloxin.

I_2 was dissolved in 0.05 N NaOH and the solution neutralised before use. MDH was diluted to 0.05 mg./ml. or 0.65 µM giving, in the experiment, a molecular ratio of I_2:enzyme of nearly 2000:1.

The oxidation of MDH by I_2 increased with time. The enzymic activity of preparations with I_2 or Cd^{II}, added singly and with both ions simultaneously, was determined, following mixing, at intervals during incubation at $25^{o}C$.

TABLE 38 Decrease in activity of malate dehydrogenase with time after addition of 2 mM I_2 alone, or when together with 2 mM Cd^{II}.

Sample	$[Cd^{II}]$	$[I_2]$	Time	$\Delta E340.min.^{-1}$	Activity as a % of control
Control	Nil	2 mM	1 min.	1.06	100
Test 1	2 mM	2 mM	1 min.	0.76	72
Test 2	2 mM	2 mM	2½ min.	0.53	50
Test 3	2 mM	2 mM	4 min.	0.25	23
Test 4	2 mM	2 mM	6 min.	0.11	11

After 1 min., Cd^{II} and I_2 together had inhibited the enzyme to the extent of 28% as compared with the enzyme subjected to I_2 alone. In assays designed to compare the potency of Cd^{II} and I_2 a less concentrated I_2 solution was employed.

Table 39 gives the changes in enzymatic activity of MDH treated with Cd^{II} or I_2 separately or together.

TABLE 39 /

TABLE 39 Activity of malate dehydrogenase treated with Cd^{II} (0.5 mM), I_2 (0.5 mM) or with Cd^{II} and I_2 together.

Sample	Time (min.)	$\Delta E340.min.^{-1}$	Activity as a % of control
Control (C)	0	0.55	100
	45	0.49	89
C + Cd^{II}	7	0.48	88
	25	0.40	73
C + I_2	5	0.33	60
	10	0.30	54
C + Cd^{II} + I_2	6	0.19	36
	8	0.19	36
	20	0.14	28

It is clear that the presence together of the two ions enhanced, rather than diminished, the inhibition of malate dehydrogenase.

Further evidence in support of this observation emerged when the two aliquots in Table 39, C + Cd^{II} and C + I_2 were mixed in equal proportions after incubation for 25 min. With this mixture, the expected ΔE 340 was approximately 0.33, but

after 4 min. the activity of the enzyme had declined, to give a value for ΔE 340 of only 0.24, being a further 27% inactivation of the enzyme.

2. *In vivo*

Malate dehydrogenase in the soluble and mitochondrial fractions of the liver of normal chicks was compared with the corresponding enzyme in cadmium-poisoned chicks (inoculated with 16 µg Cd^{II} on day 15 - 16). The two hepatic fractions were prepared by differential centrifugation as described in "Cytochrome oxidase" methods. The mitochondrial pellet was resuspended in 0.05 M potassium phosphate buffer, pH 7.4, and disrupted by addition of Triton-X-100 to a final concentration of 0.5%.

Each determination entailed pooled liver of 2 - 4 chicks, which was assayed for both cytoplasmic and mitochondrial isoenzymes.

Experimental data on the *in vivo* studies of the chicks are shown in Table 40. Figures in the Table are activities of the enzyme preparations expressed as $\Delta E340.min^{-1}.mg\ protein^{-1}$.

TABLE 40 The activity of malate dehydrogenase in liver cytosol and mitochondrial fractions of normal and cadmium-poisoned chicks.

Fraction	Normal Chicks	Cadmium-poisoned Chicks	p
Cytosol	0.91 ± 0.17	1.23 ± 0.36	<0.05
Mitochondria	0.85 ± 0.17	0.87 ± 0.33	NS

There was no significant difference between the mitochondrial preparations of normal and cadmium-poisoned chicks, but the cytoplasmic activity of MDH of cadmium-poisoned chicks was elevated above normal by 25 - 35%.

3. The kinetics of inhibition of malate dehydrogenase by cadmium.

The conventional assay of MDH using the Boehringer Test kit was performed on two samples of the same enzyme solution. One contained Cd^{II} present at 0.5 mM concentration. Both solutions were incubated for 20 min. before assay.

The assay was carried out with 5 different 2-oxoglutarate concentrations employed for each sample, although 2-oxoglutarate was not the true substrate, but was present to generate oxaloacetate (Fig. 29). A range of substrate concentrations was prepared by taking successive 3,6,13,25 and 50 µl. aliquots of the 0.065 M 2-oxoglutarate in a final volume of 1.1 ml. of assay mixture. The standard assay procedure employs 20 µl. of substrate under similar conditions.

The initial linear velocity for each sample at each concentration of 2-oxoglutarate was then calculated exactly as previously described.

Anomalies of reaction velocity were observed with 3, 25 and 50 µl. 2-oxoglutarate. The reason for these untoward findings was undoubtedly because the oxoglutarate was included only as an acceptor for the α-amino group of aspartate, present in the buffer (Fig. 29). Transfer of the amino group from aspartate to oxoglutarate catalysed by glutamate-oxaloacetate-transaminase provided a continuous supply of oxaloacetate, the substrate of MDH. The instability of oxaloacetate precludes its being supplied as such in the assay kit.

An essential requirement with regard to comparative studies of reaction kinetics of enzymes is that the substrate should saturate all the active centres of the enzyme molecules present, i.e. substrate concentration must never be rate-limiting, and kinetics must be zero order with respect to substrate.

If the coupled enzyme system incorporated in the kit for assay of MDH is considered, it is clear that the concentration of oxaloacetate at any time will depend on: (1) its rate of formation from aspartate through the agency of GOT, and (2) the rate of its conversion to malate promoted by MDH.

The overall production of oxaloacetate will, indeed, depend on the activity of MDH, which prevents the transamination process from coming to equilibrium (Wilkinson, 1962).

These conditions do not allow a fine control of the substrate concentration, which is obligatory in examining the kinetics of the enzyme. It seemed that inhibition would be similar in type to that operative on cytochrome oxidase, although the avidity of metal for enzyme was obviously a great deal less for malate dehydrogenase.

The action of Cd^{II} was not directed against GOT, for cadmium was preincubated with MDH only, a small quantity of which was then transferred to the reaction-mixture to initiate the assay.

Discussion

A dehydrogenase found to be noteworthy for its relative insensitivity to inhibition by Cd^{II}, malate dehydrogenase was extensively investigated in an attempt to ascertain whether or not Cd^{II} in the cellular components of chick liver would manifest its presence by change in the activity of one or both of the MDH isoenzymes.

Cd^{II} added *in vitro* did not inhibit the enzyme significantly until the concentration of the heavy metal reached 2×10^{-4}M, considerably higher than that to be found in poisoned chicks.

The *in vivo* results produced the first anomaly, for whereas the activity of the mitochondrial isoenzyme was not altered by Cd^{II}, that of cytosol MDH appeared to be elevated by approximately 30%. The concentration of Cd^{II} intramitochondrially was 10 - 20 µM, a concentration which had no measureable influence on malate dehydrogenase when assayed *in vitro* on the pure enzyme. The reason for this could not be established from the experiments conducted here. At a concentration of 5×10^{-5}M Cd^{II} (the order of concentration of Cd^{II} found in the cytoplasm), Cd^{II} could enhance the effect of Mg^{II} or Mn^{II} on the activation of the enzyme.

The pure enzyme available for the experiments had been prepared from pig cardiac muscle. One cannot exclude the

possibility that purified MDH from hepatic mitochondria of the newly-hatched chick may react differently with Cd^{II}. Another possibility to be borne in mind is that *in vivo* biosynthesis of the enzyme itself could be deranged by the presence of heavy metal ions; witness the abnormal minialbumin of cadmium-poisoned men and animals (Kench, Gain and Sutherland, 1965). Such a derangement of biosynthesis could change the conformation of the enzyme and of its active site and susceptibility to activation or inhibition by a variety of chemical compounds. This latter possibility once more poses the query, based on the activity of MDH in cadmium-poisoned animals, as to the accessibility of the enzyme within the mitochondrion to Cd^{II}. Some of these uncertainties should be resolved if the isoenzymes of MDH in the newly-hatched chick could be prepared in pure form, and scrutinized according to the protocols of the present experiment. It is hoped to do so in this laboratory.

As regards assay of the enzyme, the procedure described here proved adequate when used within certain limitations. The quantitative estimation of malate dehydrogenase activity in liver, as opposed to that of the pure enzyme, was a valid use of the test kit, provided all variable factors such as concentration, temperature and equilibration time were rigorously controlled.

A small variation in the concentration of, for example,

the GOT in the reaction mixture could give an exaggerated rise or fall in the oxaloacetate produced, resulting in a marked change in the apparent activity of malate dehydrogenase in the preparation.

In the current investigations however, where an uncomplicated parameter was to be determined, and where the difference in activity between any two mitochondrial or cytoplasmic fractions was not great, the test kit was a very convenient tool for the enzymic assay. This applied equally to inhibition studies of the pure enzyme or of malate dehydrogenase in a cellular preparation, wherein a constant quantity of enzyme was subjected to repeated identical assays, and the measured activity was directly proportional to the slope of the straight line traced by the recorder. This measurement was again valid within certain limits. Provided the linear fall in O.D. (340 nm) was of a similar order of magnitude for a given time interval, a number of assays of different enzyme preparations could reliably be compared.

Finally, the test kit proved useful in the study of the activity of malate dehydrogenase of the pure reference enzyme and of similar preparations of liver. The test kit could not be used for kinetic studies, since the absolute concentration of substrate in each assay could not be determined.

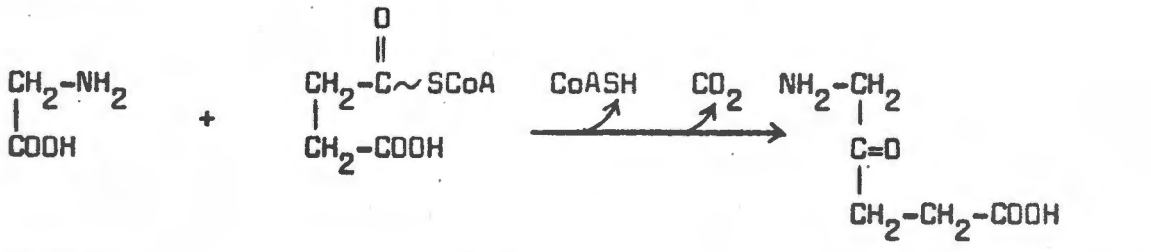
III. 9. A L A - S Y N T H E T A S E

(E.C. 2.3.1.13)

It is generally accepted that condensation of glycine with succinyl CoA is one of the principal controlling steps in both porphyrin and chlorophyll biosynthesis.

The enzyme catalysing this step is δ-aminolaevulinate synthetase, (ALA-synthetase), and is present in all cells with an aerobic metabolism.

The condensation reaction is as follows:-



Glycine Succinyl CoA δ-aminolaevulic acid

The enzyme requires pyridoxal phosphate as an essential co-factor. ALA synthetase was originally thought to be confined to mitochondria, but more recently it has been shown that, when the enzyme was increased several-fold by induction with certain compounds, it was also detectable in extramitochondrial cellular fractions (Hayaishi, Yoda and Kikuchi, 1969).

ALA synthetase activity in chicks poisoned with cadmium was examined in view of the severe anaemia induced by poisoning the chicks with the metal.

Materials and Methods

ATP, pyridoxal phosphate and coenzyme A were obtained from Sigma Chemical Co., Ltd.

Glycine and sodium succinate solutions were prepared in 0.05 M Tris/HCl buffer, the same as was used throughout the assay.

Sucrose and E.D.T.A. were standard analytical grade reagents.

Assay Procedure

The method of Hayaishi *et al.* (1969) was followed as published, except that the final volume for the incubation was increased to 2.5 ml.

The reagents for the assay were:-

Tris-HCl buffer pH 7.5	150 µmoles
Glycine	200 µmoles
Sodium succinate	20 µmoles
ATP	20 µmoles
Pyridoxal phosphate	0.5 µmoles
Coenzyme A	0.25 µmoles
$MgCl_2$	10 µmoles
Sucrose	100 µmoles
E.D.T.A.	5 µmoles

The above reagents were incubated for 5 min. at 37° prior to addition of 1 ml. (5 - 8 mg. protein) mitochondrial preparation (as prepared under "Cytochrome oxidase - Methods").

A blank, prepared by the addition of 0.5 ml. 20% (w/v) T.C.A. to the assay mixture, was run concurrently.

After an incubation period of 30 min., the reaction was stopped by the addition of 0.5 ml. T.C.A. The incubation mixture was then centrifuged at 2,000 x g (5 min.). 2.0 ml. of the supernatant was removed for determination of ALA. The method of Narisawa and Kikuchi (1966), as outlined below, was used.

Samples were adjusted to pH 4.6 with 2.5 N NaOH and acetate buffer, 1.0 M, pH 4.6. After the addition of 0.1 ml. acetylacetone, the samples were boiled for 10 min. in a water bath to yield a pyrrole complex by condensation of ALA with acetylacetone.

The solution was applied to a 1 x 3 cm. Dowex 1 - acetate column. Three fractions were eluted from the column, using:-

4 ml. 50% methanol/ammonia (0.1 N)

4 ml. 1 M acetic acid

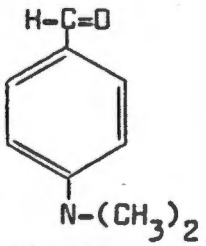
4 ml. glacial acetic acid

The ALA/acetylacetone condensation product was recovered quantitatively in the third fraction. To this was added an

equal volume of modified Ehrlich's aldehyde reagent (Urata and Granick, 1963).

FIG. 30 The structure of Ehrlich's reagent.

P-dimethylaminobenzaldehyde (p-DMAB)



The composition of Ehrlich's reagent was as follows:-

0.5 gm. p-dimethylaminobenzaldehyde
21 ml. glacial acetic acid
7 ml. 60% perchloric acid

The resultant colour was measured after 7 - 12 min. in a Zeiss spectrophotometer at 553 nm. Any turbidity occurring in the tubes was cleared by a brief centrifugation in a bench centrifuge at 2,000 x g prior to evaluating the optical censity of the samples.

Blank readings were subtracted from test results to obtain nett ALA-synthesis during the incubation time.

Calculation of enzymatic activity of ALA-synthetase in chick liver mitochondria.

E^{mM}_{553} for ALA = 53 (Narisawa and Kikuchi, 1966)

$$\text{Activity} = \Delta E\ 553 . \frac{1}{53} . 1.2 . \frac{1}{(\text{mg. protein})}$$

Factor - 2 to convert to "per hour"

Units are millimoles ALA.$hr.^{-1}$ mg. $protein^{-1}$.

Notes on the assay of ALA-synthetase

In the assay of ALA-synthetase, the quantity of ALA formed was measured by ring closure with acetyl acetone (Knorr reaction), following its conversion to a pyrrole derivative, which gave a purple-coloured product with Ehrlich's aldehyde reagent. Any free primary amines present could combine with acetylacetone to form aminoacetone during the strong heating at pH 4.6 necessary for ALA-acetylacetone complex formation. Several contaminating compounds were also formed during the procedure thereby necessitating isolation of ALA, which was achieved by column chromatography.

The elution of ALA from a Dowex 1 x 8-ion exchange column involved three steps. Aminoacetone, which is Ehrlich's aldehyde-sensitive, was first eluted with methanol-ammonia. The column was then washed with dilute acetic

acid prior to quantitative elution of the ALA by glacial acetic acid.

Addition of mercuric chloride to the 20% (w/v) T.C.A. caused a turbidity in the tubes once the Ehrlich's reagent was added. The glacial acetic acid in the colour reagent caused precipitation of the mercuric salt. A short centrifugation in a bench centrifuge just before reading the optical density of the samples ensured optically clear solutions. Addition of mercuric chloride to Ehrlich's reagent was a modification first introduced by Urata and Granick (1963) to increase the stability of the purple colour-complex. Addition of $HgCl_2$ to the T.C.A. obviated the making up of a fresh solution daily, as was necessary with the unmodified Ehrlich's reagent.

The range of variation of the results was much greater for this enzyme than for other enzymes investigated. Probably both the biochemical action of Cd^{II} on the enzyme-system and also the inaccuracy inherent in the assay method were responsible for the variations. The assay procedure was complex and had many more stages than other enzyme-assays. The column separation particularly, seemed prone to error.

Results

(i) Activity of intact and disintegrated mitochondria

A mitochondrial sample was disrupted, i.e. dispersed and dissolved, by the addition of 0.5% (v/v) Triton X-100 and the activity of this preparation compared with that of intact mitochondria.

TABLE 41 The activity of ALA-synthetase in intact and detergent-disrupted chick liver mitochondria.

Fraction	Time	ΔE 553	Protein (mg./ml.)	Activity
Intact mitochondria	12 min.	0.027	6.5	3.5
Intact mitochondria	30 min.	0.052	6.5	2.7
Disintegrated mitochondria	10 min.	0.049	6.5	7.5
Disintegrated mitochondria	20 min.	0.087	6.5	6.7
Disintegrated mitochondria	30 min.	0.142	6.5	7.3

Assay of the intact mitochondria did not result in a linear rate of formation of ALA throughout the full incubation period, nor did the rate reach more than approximately 60% of that of the Triton-X treated organelles. Consequently, this latter method of measurement of the intrinsic activity of the enzyme was employed for all subsequent assays.

(ii) In vitro studies

Mitochondria isolated from the liver of 4 newly-hatched normal chicks were pooled and subjected to the assay procedure.

Two such mitochondrial preparations were made and labelled N1 and N2.

Studies with cadmium were performed on mitochondria consisting of equal proportions of N1 and N2. This mixture was preincubated with two different cadmium concentrations, viz.

Cd^{II}_B was incubated with 3×10^{-4}M cadmium

Cd^{II}_A was incubated with 1×10^{-4}M cadmium

These cadmium concentrations were chosen as they were in the range expected in the liver of chronically and acutely poisoned chicks.

TABLE 42 The in vitro effect of two different Cd^{II} concentrations on the activity of chick liver ALA-synthetase.

Sample	ΔE 553/ 30 min.	Protein (mg./ml.$^{-1}$)	Activity ($\times 10^4$)	Activity as % of control
N1	0.035	6.5	2.04) 2.48	100
N2	0.048	6.2	2.92)	
Cd^{II}_A	0.029	6.3	1.78	71
Cd^{II}_B	0.022	6.3	1.34	54

Each value presented is the mean of duplicate assays. There was a large difference in ALA-synthetase activity of the two preparations of normal chick liver. The activity of the enzyme was depressed by incubation with Cd^{II}, particularly at a concentration of 3×10^{-4}M.

(iii) In vivo studies

Observations on ALA-synthetase activity in the liver of normal and cadmium-poisoned chicks were made over a period of a month, as the intoxicated chicks were not all available at one time. The liver of 2 - 4 chicks was used in each assay. The chicks were poisoned with 16 µg. Cd^{II} each on the 15th day of the 21 day incubation period.

After each programme of poisoning, ALA-synthetase activity of the pooled livers of cadmium poisoned chicks was compared with that of the pooled normal chick liver and was expressed as a percentage of the normal activity.

TABLE 43 /

TABLE 43 *In vivo* normal and Cd^{II}-poisoned chick ALA-synthetase.

Series	Sample (No. of chicks)	ΔE 553. $hr.^{-1}$	Protein ($mg.ml.^{-1}$)	ΔE 553 $mg.^{-1}hr.^{-1}$	Activity as % of normal
A	Normal (4)	0.071	5.2	0.014	100
	Cd^{II}-pois. (2)	0.022	5.8	0.004	28
	Cd^{II}-pois. (3)	0.052	6.1	0.009	62.5
B	Normal (4)	0.102	9.6	0.011	100
	Cd^{II}-pois. (2)	0.084	8.5	0.010	92
	Cd^{II}-pois. (2)	0.114	9.2	0.012	117
C	Normal (2)	0.136	4.5	0.030)	100
	Normal (2)	0.162	4.5	0.036)	
	Cd^{II}-pois. (2)	0.076	4.7	0.020	62
	Cd^{II}-pois. (2)	0.112	4.7	0.029	89

The ALA-synthetase activity appeared to be depressed in most of the cadmium-poisoned animals.

There is a wide variation in observed values, and a much larger series of chicks must be studied before firm views could be expressed on the biochemical changes which have taken place in the cadmium-poisoned chicks.

Recovery of ALA from the assay procedure

In view of the difficulties inherent in the determination of ALA, recovery of ALA was evaluated. Three different concentrations of ALA were processed exactly as was the protein-free T.C.A. supernatant in the enzyme assay.

Aliquots of a freshly prepared 0.4 mM stock solution (2.55 mg. ALA in 50 ml. distilled water) containing 0.08, 0.04 and 0.02 µmoles of ALA were added to 1.0 ml. of 1.0 M acetate buffer, pH 4.6, and boiled with 0.02 ml. acetyl acetone for 15 min. The column separation was exactly as already described, followed by addition of an equal volume of Ehrlich's reagent.

The O.D. (533 nm) was measured after 10 min., which allowed for full colour development. Samples of each concentration were in duplicate. The elution volume was 4 ml., and final volume for colour-development 8 ml. The sample which contained 0.08 µmoles ALA should therefore, have had a final concentration of 0.01 nM. Similarly the other two samples containing 0.04 and 0.02 µmoles should contain ALA concentrations of 0.005 and 0.0025 nM respectively.

The expected optical density, assuming 100% recovery, was compared with the experimentally measured value to obtain the percentage recovery of ALA (Urata and Granick, 1963).

$$E^{mM}_{553} = 53$$

The following data were obtained

TABLE 44 The percentage recovery of ALA from the assay procedure

Sample (Final ALA concentration (nm))	E 553	E 553 - Blank (ΔE)	ΔE 553 for 100% recovery	Percent recovery
Blank	0.180	-	-	-
0.01	0.495	0.315	0.53	59
0.01	0.432	0.252	0.53	48
0.005	0.367	0.187	0.265	71
0.005	0.307	0.127	0.265	48
0.0025	0.261	0.081	0.133	61
0.0025	0.276	0.096	0.133	72

The mean recovery was 60%. It is evident that the advocated procedure for determination of ALA gives low and variable recovery. It is obvious that the method would require a thorough and detailed modification to provide satisfactory information. In the meantime, values for activity of ALA-synthetase have been corrected on the basis of 60% recovery of ALA. Taking into account the magnitude of changes witnessed in the enzyme when in con-

tact with added Cd^{II}, it is believed that the conclusions reached are valid and meaningful.

Discussion

ALA-synthetase is the prime rate-controlling step in haem biosynthesis (Gibson, Laver and Neuberger, 1956) and the investigation of this enzyme was undertaken as Cd^{II}-poisoning was observed to have caused severe anaemia in newly-hatched chicks.

ALA-synthetase is unique in that it is the only mitochondrial enzyme known to be inducible (Hayaishi, Yoda and Kikuchi, 1969). The most potent inducing agent is alkyl isopropyl acetamide (A.I.A.), which can stimulate the mammalian enzyme to an 8-fold increase in activity in a few hours (Granick, 1966).

The enzyme functions best in an anaerobic environment, or one in which the pO_2 is low. Bubbling oxygen through the reaction-mixture during assay markedly inhibited the formation of ALA (Marriott, 1968).

The enzyme has a thiol in its active centre, a characteristic elucidated by inhibition studies with iodoacetamide, arsenite and cyanide (Laver, Neuberger and Udenfriend, 1956). An essential cofactor of ALA biosynthesis, coenzyme A, also

has a free thiol group.

Heavy metals, particularly lead, are known to inhibit this enzyme in mammalian liver and in avian erythrocytes in *in vitro* studies (Driesel and Falk, 1956; Goldberg, Ashenbrucker, Cartwright and Wintrobe, 1956). More recently the effects of ferrous and ferric iron intoxication on ALA synthetase have been compared with lead poisoning (Morrow, Urata and Goldberg, 1969). Fe^{II} and Fe^{III} were found to be considerably less toxic than lead except when present in concentrations approaching millimolar levels. The results did indicate a mild stimulation of enzyme activity with Fe^{II} at a concentration of 10^{-5}M, which is postulated as a possible valid concentration of ferrous iron *in vivo* (Morrow, *et al.* 1969).

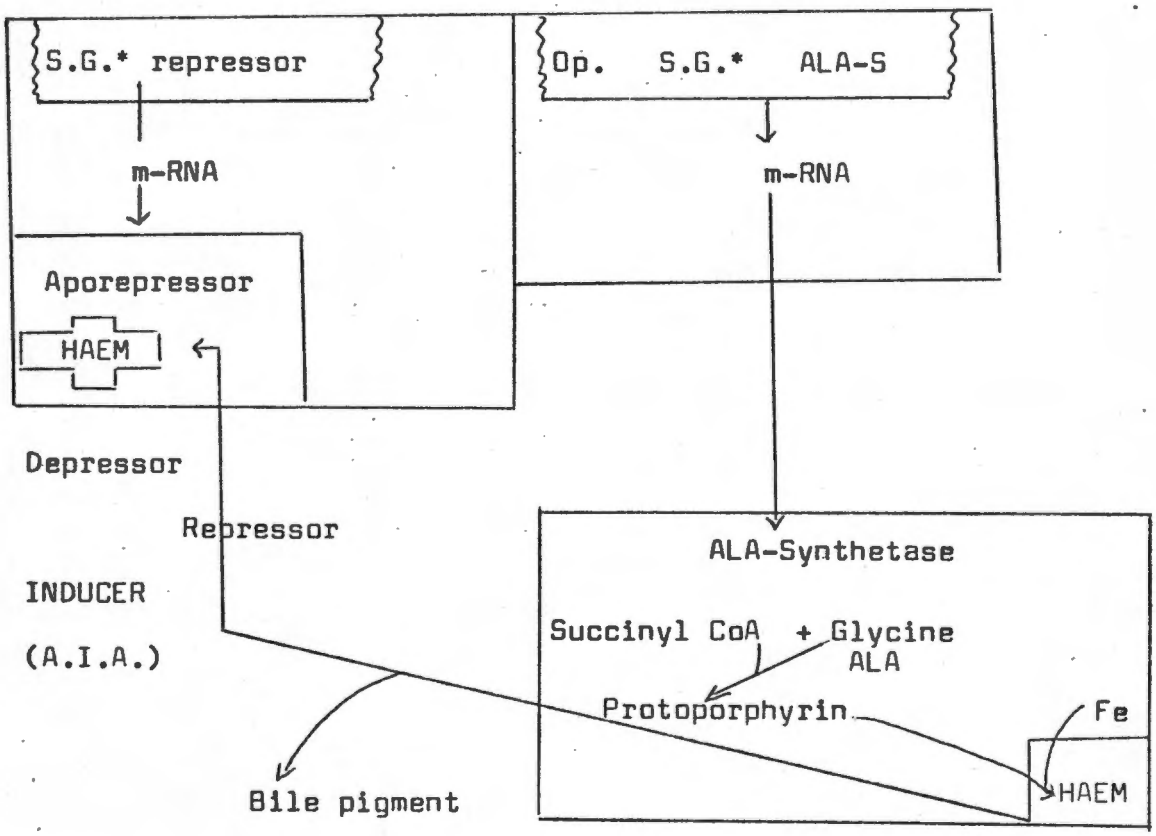
Millimolar concentrations of Fe^{II} or Fe^{III} caused marked ALA-synthetase inhibition. This suggested that some types of anaemia (siderochrestic) are caused by the inhibition of haem synthesis due to the accumulation of excessive quantities of iron in the mitochondria. Gibson and Goldberg (1970) failed to detect any difference in ALA-synthetase of normal and lead-poisoned rabbits *in vivo*. However, they noted that ALA-dehydrase was significantly depressed in the same animals. Tissues, particularly the liver, of rabbits with induced porphyria had markedly raised ALA-synthetase activity.

Regulation of the enzyme in the developing Cd^{II}-poisoned

chick liver cells appears to be through operation of a chemostat mechanism, based on the rate of synthesis of haem, the final product. There is a negative feed-back from haem itself to ALA-synthetase, formation of which is provoked whenever there is some impediment to haem synthesis, as, for example, in iron deficiency (Fig. 31). The enzyme is known to be markedly inhibited by haem (Marver, Schmid and Schutzel, 1968).

Several different control mechanisms whereby this enzyme regulates haem formation have been postulated. The two most likely to be the true operative mechanism appear to be those proposed by Burnham and Lascelles (1963) and Granick (1966). The original mechanism envisaged was a feedback inhibition, whereby the end product of the reaction sequence, i.e. haem, exerted an inhibitory influence on one of the initial steps in its biosynthesis. Such a negative feedback usually involves an allosteric binding site. The alternative theory of Marver et al (1966) - as expanded by Granick (1966) and Scholnick, Hammaker and Marver (1970) - was that, while ALA-synthetase is an allosteric enzyme and consequently contains a binding site for haem distinct from its active site, the haem functioned as a co-repressor in feedback repression. Regulation of the synthesis, therefore, could take place not only at the enzyme active site, but also through the behaviour of haem as a repressor of m-RNA transcription (Fig. 31).

Fig. 31: The postulated mechanism for feedback repression of ALA-synthetase. (Granick, 1966).



*, Structural-gene

Although the enzyme was found to be present in the cytosol of the chick livers, it was assumed that it appeared there as a contaminant from ruptured mitochondria during homogenisation or centrifugation. Nevertheless, it has been reported by Hayaishi et al. (1969) that the enzyme is present in the soluble fraction of A.I.A.-induced mammalian liver cells. These workers explained the presence of ALA-synthetase in the cytosol by proposing that it was "in transit" to the mitochondria.

Cadmium has been observed, in these experiments, to cause a severe anaemia in developing chicks. More information is needed before the origin of the anaemia can be understood.

Inhibition of ALA-synthetase by cadmium could certainly be a contributory factor, a consequence of the reaction of the metal ion with the sulphydryl of the active centre in addition, perhaps, to changes in other parts of the protein which are responsible for allosteric alterations in its catalytic function. Cadmium could also be expected to inactivate the thiol group of coenzyme A, although one would expect that chelation with thiols and other adjacent groups, such as carboxyl and imine would form a more stable, less ionized chelate and be much preferred (Vallee and Williams, 1968).

The inhibition of the active enzyme in the nucleated erythrocytes of cadmium-poisoned chicks *in vivo* was not investigated, and the questions regarding the anaemia present in chicks and the inhibition of ALA-synthetase cannot be answered confidently until this has been done. Sutherland (1967) did not find that an anaemia developed in monkeys chronically poisoned with Cd^{II}, but possibly the reason for this was the lack of active haemoglobin-synthesising enzyme in the mammalian erythrocyte.

ALA-dehydrase should also be investigated in avian erythrocytes, particularly since it was found to be more sensitive to cadmium in hepatic preparations than was ALA-synthetase.

III. 10. A . L . A . D E H Y D R A S E

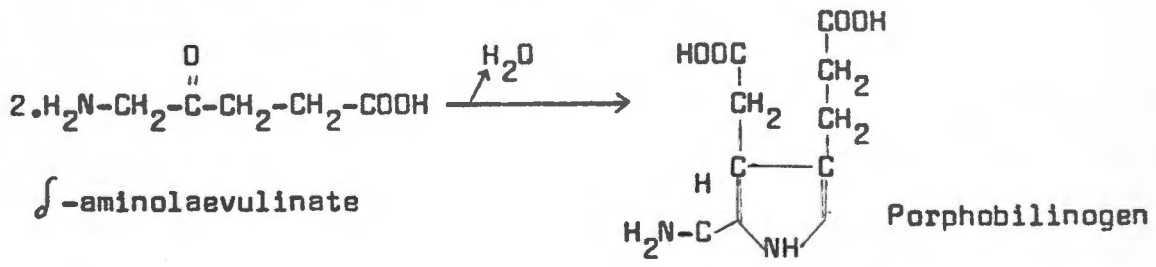
(5-amino laevulinate hydro-lyase: cyclising enzyme)

(E.C. 4.2.1.24)

In the biosynthesis of porphyrins, the second step is catalysed by an enzyme in the cytosol, as opposed to the first step, which is catalysed by the mitochondrial ALA-synthetase. The enzyme responsible for the cytosol reaction is δ-aminolaevulinate dehydrase (ALA-dehydrase), which is absent from mitochondria (Urata and Granick, 1963).

It catalyses the condensation of 2 molecules of ALA to 1 molecule of porphobilinogen (PBG), with a water molecule formed in the process.

The reaction is as follows:-



The enzyme reaction is not generally considered to be as important in controlling the rate of porphyrin biosynthesis as is ALA-synthetase, but it is well known that certain heavy metals, particularly lead, inhibit this enzyme, with a resultant decrease in haem formation. (Gibson and Goldberg, 1970).

The system does not require any additional co-factors or activators, provided sufficient K^+ ions are supplied in the buffer for maximal activation. (Shemin, 1968).

Materials and methods

δ-ALA was supplied by Sigma, Ltd., and kept refrigerated in a dark bottle.

Erhlich's aldehyde reagent, as modified by Mauzerall and Granick (1956) was prepared as follows:

1.0 gm. p-aminodimethylaminobenzaldehyde was dissolved in 35 ml. glacial acetic acid and 10ml. 60% (v/v) perchloric acid ($HClO_4$). The reagent was stored in a dark bottle and prepared fresh daily.

Fractionation of chick livers

The assay was performed on the 10,000 x g cytosol fraction of the liver as prepared by the method described under Cytochrome Oxidase - (Methods).

Assay procedure

The procedure adopted for the assay was that of Narisawa and Kikuchi (1966), modified slightly to give a final volume of 2.5 ml.

The assay mixture contained:-

δ-aminolaevulinate	20 µmoles
potassium phosphate buffer pH 7.4	150 µmoles
$NaHCO_3$	20 µmoles
$MgCl_2$	5 µmoles
Cytosol preparation	1 ml. (~10 mg. protein)

The incubation period was forty minutes, at which time the addition of 0.5 ml. 20% (w/v) TCA stopped the reaction and precipitated all protein.

After standing for 5 min., the incubation mixture was centrifuged at 3,000 x g for 3 min., and 1.0 ml. of clear supernatant aspirated for PBG determination. 1.0 ml. of Ehrlich's aldehyde reagent was added, and the tubes allowed to stand in a darkened room for 7 - 12 min. to permit full development of the unstable colour complex. Turbidity was removed by a brief centrifugation at 2,000 x g. The optical density was read in a Zeiss spectrophotometer at 553 nm.

Calculation of activity

E^{553}_{mM} for PBG = 62 (Mauzerall and Granick, 1956).

$3 \frac{\Delta E553}{62}$ (mg. Prot)$^{-1}$. t(hr.)$^{-1}$ = millimoles PBG/hr/mg Protein

In the comparative studies of in vitro experiments it was considered sufficient to calculate Δ E/mg/hr, provided that all assays were conducted identically.

Results

This soluble enzyme was assayed as a standard preparation with E.D.T.A. (0.1 mM) present. E.D.T.A. could not, however, be included in *in vitro* studies when Cd^{II} was added, since E.D.T.A. had a marked influence on the proportion of added cadmium that was available to bind the active site of the enzyme. E.D.T.A. was, therefore, excluded from all assays, except one, to which Cd^{II} was added.

The concentration of K^+ ions present in the assay medium played an important role in enzyme-activation, and K^+ was kept at a constant concentration of 0.15 M.

(i) *In vitro*

(a) With E.D.T.A. present in the reaction mixture.

Table 45 shows the effect of added cadmium chloride on the activity of ALA-dehydrase relative to a duplicated control sample. E.D.T.A. concentration was 0.1 mM for all tests.

TABLE 45: The influence of different concentrations of Cd^{II} on the activity of ALA-dehydrase in the presence of E.D.T.A.

Sample	Δ E553	Activity ($\times 10^3$)	Percent of Control
Control(No Cd^{II})	0.351	3.7	100
2×10^{-5}M Cd	0.375	3.9	107
4×10^{-5}M Cd	0.322	3.3	91
8×10^{-5}M Cd	0.339	3.5	96
4×10^{-4}M Cd	0.363	3.8	103

(b) Without E.D.T.A. present in reaction mixture.

More Cd^{II} ions were free in solution, and could exert a greater influence on the enzyme than when E.D.T.A. was present. Fig. 32 shows the protective influence of E.D.T.A. on ALA-dehydrase when intoxicated by Cd^{II}.

FIG. 32.

EFFECT OF CADMIUM in vitro ON
A.L.A.-DEHYDRASE ACTIVITY

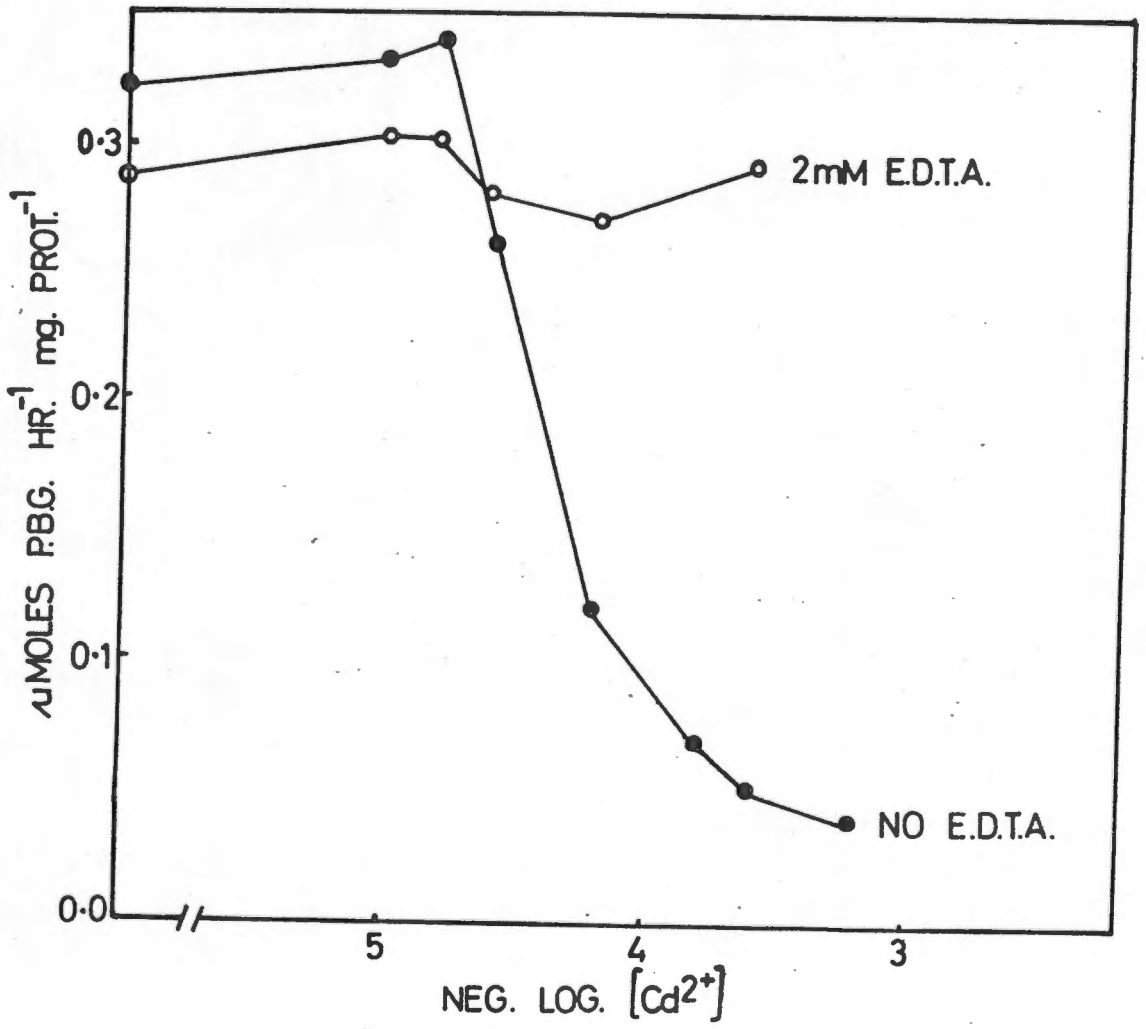


TABLE 46: The influence of different concentrations of Cd^{II} on ALA-dehydrase activity.

Sample	Δ E533	Activity ($\times 10^3$)	Percent of Control
Control (No Cd)	0.235	3.2	100
1×10^{-5}M Cd^{II}	0.243	3.3	103
2×10^{-5}M Cd^{II}	0.247	3.4	106
4×10^{-5}M Cd^{II}	0.207	2.8	88
8×10^{-5}M Cd^{II}	0.079	1.2	37
2×10^{-4}M Cd^{II}	0.050	0.7	22
4×10^{-4}M Cd^{II}	0.040	0.5	16
8×10^{-4}M Cd^{II}	0.028	0.4	12

The apparent mild stimulation of the enzyme was further investigated.

TABLE 47: The effect of low concentrations of Cd^{II} on ALA-dehydrase - (No E.D.T.A. present).

Sample	Δ E533	Activity ($\times 10^3$)	Percent of Control
Control(No Cd^{II})	0.417	6.9	100
5×10^{-6}M Cd^{II}	0.422	7.0	101
1×10^{-5}M Cd^{II}	0.426	7.1	102

There appeared to be no significant difference between the activity of the enzyme in the reaction mixture in the absence of Cd^{II}, and when it was added to a final concentration of less than 4×10^{-5}M.

At concentrations greater than 4×10^{-5}M, Cd^{II} exerted a powerful and progressive inhibitory effect on ALA-dehydrase.

(ii) In vivo:

The first results obtained were from chicks injected with 10 - 12 µg Cd^{II} at 12th - 14th day of incubation. ALA-dehydrase activity in the liver of these chicks did not differ significantly from normal, although the mean value was greater than the mean of control chicks assayed concurrently.

However, chicks subjected to a more drastic poisoning regime, viz. 16 - 20 µg intravenous cadmium on 15th - 16th day of incubation, had a pronounced change in activity when assayed for ALA-dehydrase.

In Table 48 the enzyme activities are given as a percentage of those of normal chick controls. Owing to the technical complexities inherent in the assay, there were considerable variations from day to day in the yields of porphobilinogen. The control for each Cd^{II} -poisoned chick was provided by the pooled liver of 4 normal chicks.

TABLE 48: The activity of cadmium-poisoned chick liver ALA-dehydrase as a percentage of normal values.

	No of samples	Activity as a % of normal
10-12 µg Cd^{II} on day 12	9	108 ± 8
16-20 µg Cd^{II} on day 16	4	70 ± 10

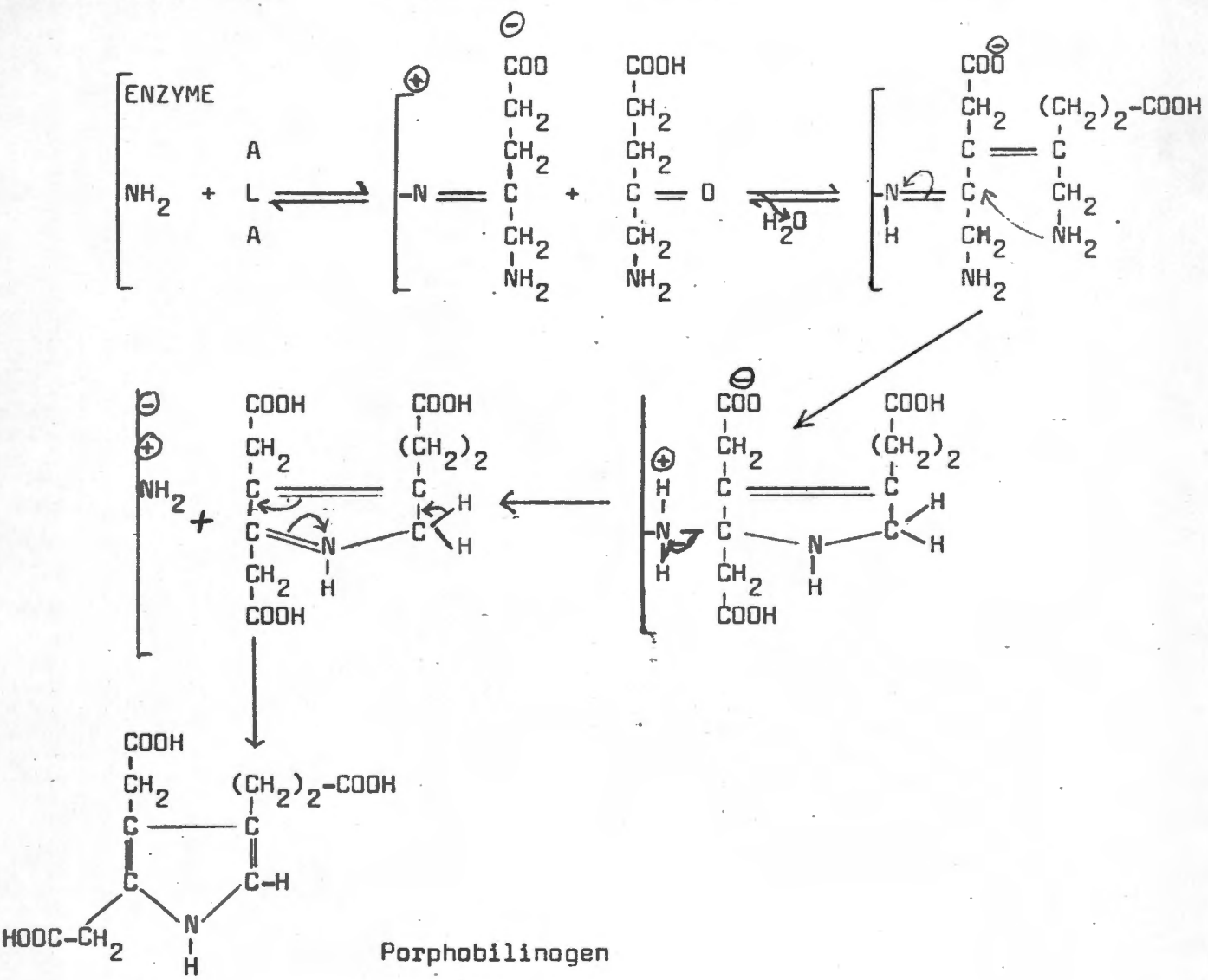
Each reading is the mean of 2 cadmium-poisoned chick livers.

Discussion

The observation that E.D.T.A. afforded protection to ALA dehydrase against relatively large concentrations of Cd^{II}, by chelating the metal, shows that the avidity of this enzyme for the metal was not as great as that of succinic or lipoamide dehydrogenase for Cd^{II}.

ALA dehydrase, particularly *in vitro*, was technically much easier to assay than was ALA synthetase and consequently the results were far more reproducible. The control values for pooled liver from 2 - 4 normal chicks varied less from day to day than did the ALA-synthetase results.

Fig. 33 : The postulated biosynthesis of porphobilinogen from 5-aminolaevulinic acid. (Shemin 1968)



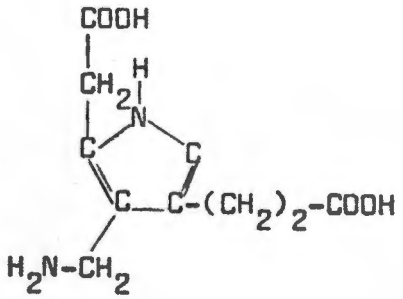
The chicks which were grossly poisoned were also investigated to determine the extent of any haematological disturbance they suffered as a result of Cd^{II} - poisoning ("General Investigations"). There was a high degree of correlation between the severity of anaemia and the inhibition of ALA-dehydrase in the liver of the chicks. This finding is suggestive of a similar inhibition of the ALA-dehydrase in erythroid cells of the bone marrow being responsible for the anaemia.

ALA-synthetase was assayed concurrently in the same liver tissue of poisoned chicks, but in the separated mitochondrial fraction. Activity of the enzyme in general followed the course of ALA dehydrase, but did not correlate as well with the degree of anaemia.

The active site of the ALA-dehydrase is known to include a primary amine which forms a Schiff base linkage between the enzyme and its substrate ALA.

The mechanism of the condensation reaction was postulated by Shemin (1968) as illustrated in Fig. 33.

It was essential that the molecular orientation of ALA be correct in the immediate vicinity of the enzyme, for an incorrect orientation of either molecule could, and did, lead to irregular pyrollic compounds. If the second ALA molecule was inverted, for instance, the resultant compound would have the following structure:-



ALA-dehydrase was observed to be inhibited in brain, liver, kidney and bone-marrow cells of rabbits poisoned with lead. (Gibson and Goldberg, 1970). The tissue most markedly affected was liver, in which the inhibition of the enzyme, relative to normal levels, was 38 - 56%. ALA-dehydrase activity in porphyric rabbits ranged from 160 - 170% of normal. Inhibition of the enzyme by lead was attributed to interference with thiol groups. (Gibson and Goldberg, 1970).

The failure of less than 1×10^{-5} M Cd^{II} to inhibit the enzyme could imply that the first two or four thiol groups bound by cadmium do not participate in the active site, but as soon as Cd^{II} reached a concentration sufficient to attach itself to more thiols, there was an extremely rapid loss of enzymatic activity. (Fig. 32)

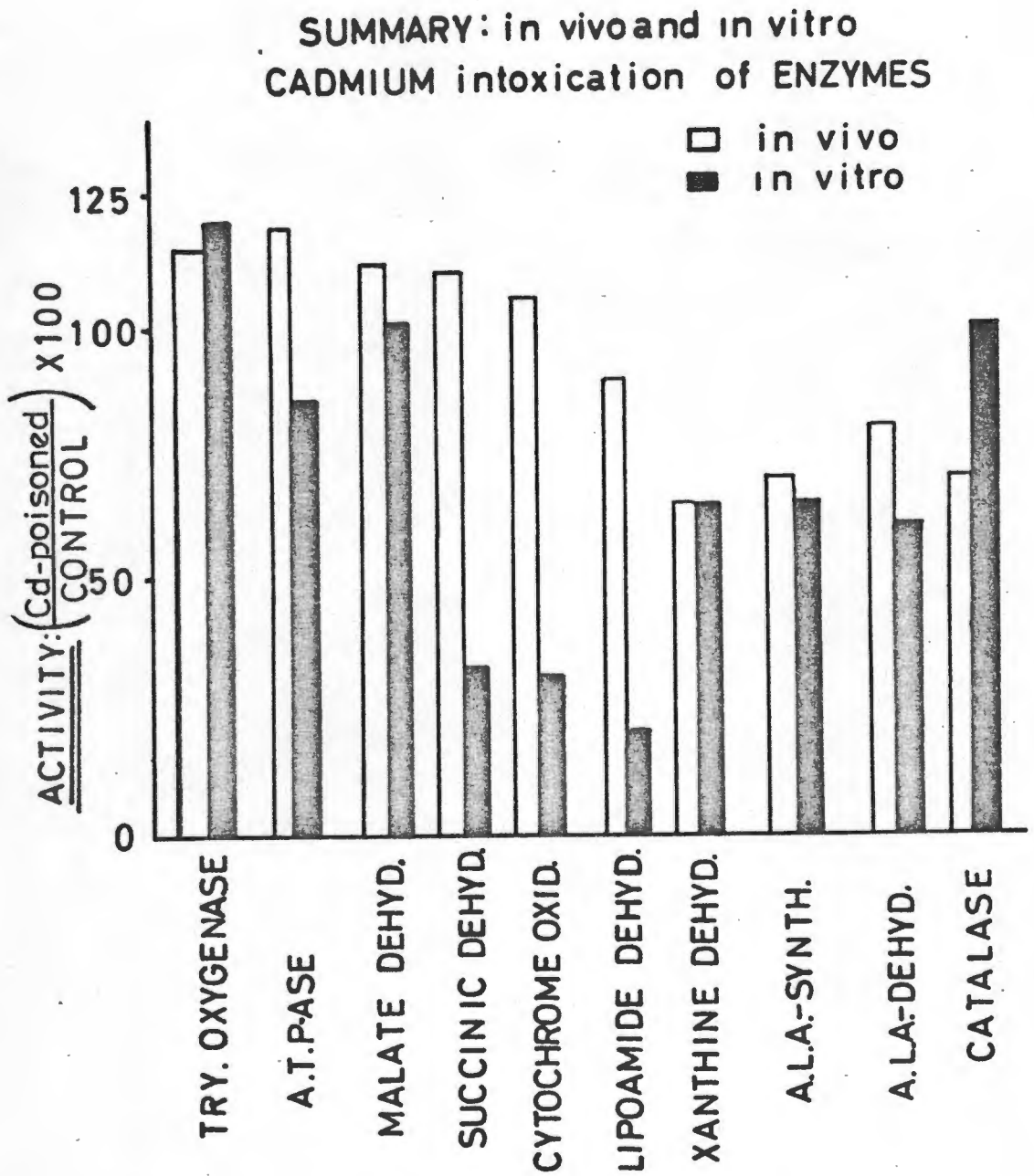
The very efficient protection afforded to the enzyme by E.D.T.A. supports this hypothesis, for were the active site directly susceptible to cadmium, E.D.T.A. could not prevent significant inhibition at the concentrations of

cadmium which were in fact tolerated by the enzyme.

In conclusion, it is clear from *in vitro* studies, and supported by *in vivo* determinations, that cadmium may exert a powerful influence on the reactivity and efficiency of chick liver ALA-dehydrase in expediting the conversion of ALA to porphobilinogen if the metal is present in sufficient concentration.

In this regard, purified ALA-dehydrase of the bacterium behaves differently. Nandi, Baker-Cohen and Shemin (1968) observed that the bacterial type was not influenced by any monovalent or divalent metal ion other than K^+. Such species differences are of particular interest from the viewpoint of bacterial evolution and adaptation.

FIG. 34.


SUMMARY: in vivo and in vitro
CADMIUM intoxication of ENZYMES
in vivo
in vitro
ACTIVITY: (Cd-poisoned / CONTROL) X100
125
100
50
0
TRY. OXYGENASE
A.T.PASE
MALATE DEHYD.
SUCCINIC DEHYD.
CYTOCHROME OXID.
LIPOAMIDE DEHYD.
XANTHINE DEHYD.
A.L.A.-SYNTH.
A.LA.-DEHYD.
CATALASE

PART IV

DISCUSSION

Experimental data on the enzymatic assays has been analysed in the relevant sections, but a number of additional points that have emerged during the course of this study remain to be discussed.

The chick embryo was chosen as an experimental model mainly because it was a closed system. It appeared to offer an opportunity to study the impact of cadmium on rapidly differentiating tissue cells, on interconversions; as for example, ovalbumin into serum albumin of the developing chick, and perhaps on the formation of aberrant low molecular albumins. Above all, it appeared to be a suitable model for a comparison of *in vitro* and *in vivo* interactions of cadmium and enzymes. These aspirations were to a great extent justified, it is believed, by the reproducibility of the experimental data and the sensitivity of the developing chick embryo to very low concentrations of added cadmium.

Methods of administering cadmium to the chick embryo included subcutaneous injection, injection into the crop, or intravenous injection. Several problems were encountered with subcutaneous injection, the chief one being the initial extremely high localization of the metal at the injection site, which was often responsible for tumour formation. Heath and Webb (1967) showed that cadmium exhibited a concentration gradient outwards from

the centre of the muscle tumour to the periphery. Dietary cadmium is poorly absorbed through the gastro-intestinal tract, as it has been shown that the absorptive mechanism for cadmium is not efficient (Sahagian, et al., 1967). In the present study, the recovery of cadmium from the liver was only 4% of that administered by injection into the crop of the chicks, whereas when chick-embryos were poisoned by the intravenous route, 60% of the administered cadmium was recovered from the liver. The intravenous administration of cadmium proved to be simple, rapid, and allowed a far more accurate estimate of the total amount of cadmium actually available for intoxication of the embryo.

A number of rather bizarre developmental changes were inflicted upon the developing chick by the introduction of Cd^{II}. Early embryos were extremely sensitive to the metal, as little as 4 µg being the $L.D._{50}$ for embryos up to 2 days of age. Larger quantities were tolerated by older and larger embryos, but whether tolerance was greater on the basis of Cd^{II}/body weight of embryo is not established. Loss of feather pigmentation, swelling of subcutaneous tissue, muscular weakness and blindness were some of the gross manifestations of the presence of cadmium in the developing chick at the time of hatching. No attempt was made to relate these aberrations to the

changes in the activity of the enzymes which were chosen for scrutiny. The opisthotonus redolent of the classical thiamine-deficiency and beri-beri of pigeons was not associated with elevated serum pyruvate, and not accountable in terms of *in vivo* inhibition of lipoamide dehydrogenase. It remains to be elucidated.

The intracellular distribution of cadmium had already been examined in several studies. When the tumours described by Heath and Parry were analysed for intracellular cadmium location, it was found that at least 60% of the cadmium was bound to the cellular nuclei, and that a very significant proportion of cadmium was actually bound to RNA and DNA. These findings were at variance with those of Cotzias *et al.* (1961 b) and with the observations of the present work. Whilst cadmium may be present at the tumour site in high concentration, possibly saturating the binding sites available in the cytosol and mitochondria, it appears more likely that the whole structure of the cell has become radically altered, and thiol-active proteins which are normally distributed throughout the cell, now appear mainly in the nuclear fraction. This could explain the hyperplastic state of the tumour cell.

Despite differences in the techniques employed for

determining cadmium concentration, the findings of Cotzias et al. (1961 b) on the intracellular distribution in hepatic tissue of poisoned rats correspond closely with the results presented here. Cadmium bound to the metabolically highly active mitochondrial fraction in the liver of acutely-poisoned chicks was only 20% of the total bound to the entire cell. Whether or not any of this cadmium actually reaches the inner compartment of the mitochondrion in which the dehydrogenases are situated is unknown, but in the light of present evidence, it appears extremely doubtful. This fact may offer some explanation for the apparent disparity often found when in vivo effects are extrapolated from in vitro observations. An attempt has been made in the present investigation to match in vitro and in vivo assays of activity of enzymes on the basis of equivalent cadmium concentrations. Differences observed should be evaluated with the realization that one is presently unaware of the exact conditions of pH, metal concentration and time of exposure of enzyme to the metal.

It is hoped that precise measurements of the quantity of Cd^{II} in cellular fractions may be achieved in future studies. Neutron activation appears to lend itself to analysis of nanogram quantities of cadmium and this analytical procedure would permit more collateral metabolic events to be followed.

The dosage of cadmium has been shown to be extremely critical, particularly when the chick embryo is young. There appears to be a good correlation between the estimated weight of the embryo and the lethal dose. The maximum non-lethal dose of cadmium rises relatively rapidly during the last week of incubation, as the body weight of the embryo also increases rapidly. The lethal dosage has been estimated as 0.5 - 1.0 μgCd/gm body weight of embryo. Toxicity is as high as this because all the cadmium is immediately transported to the liver, in contrast to the slow absorption over an extended period of time that accompanies subcutaneous or dietary cadmium-poisoning.

The chick does not appear to have a defined upper limit of incorporation of cadmium into cells. Cobalt, injected as Co^{60} into the yolk-sac of eleven-day old embryos, is taken up by a limited number of sites on the DNA of the embryo. The saturation point is reached at a Co^{60} dose of 10 μg per egg. (Menke and Sarif-Sarban, 1966). There appears however, to be no discernible limit to the total cobalt bound to yolk proteins, and absorbed therefrom into the chick for attachment to structural protein. It would prove very interesting, and important to the question of cadmium-poisoning as a whole, were it possible to discover how much intracellular ingested cadmium becomes bound to nucleic acid, and experiments along such lines are

currently proceeding. Intracellular cadmium becomes bound, interalia, to the "debris", which is the nuclear fraction plus contaminating mitochondria and cell membranes. How much of this is bound to nucleic acids is not yet certain, but of a total of 1.6 µg/g associated with the debris, probably less than 1 µg is bound by DNA and RNA. (Sutherland 1970).

Much experimental work has been reported on the competition between zinc and cadmium, and between zinc, cadmium and copper. The findings of Cotzias et al (1961 a) and Hill et al (1963) reveal that if the competitive process is allowed to proceed long enough, a zinc deficiency state is ultimately induced. In the present study it would seem that the chicks were not poisoned extensively enough to create this metabolic state.

It did appear that there was a decrease in growth rate subsequent to poisoning, and often the chicks were a day or more later in hatching than were the untreated controls. The symptoms of poisoning have already been described in the text, but it is of interest to note that the prealbumin peak of serum proteins was more prominent in the poisoned chicks than in the normals. This prealbumin is thought to be orosomucoid (Polson, 1970), and is present in embryonic blood. It diminishes with increasing age of the embryo, until it disappears a few days after hatching,. No evidence is available at the present time

to account for the persistence of this protein in the blood of poisoned chicks.

The general biochemical parameters agreed with those reported by Hill et al (1963) for young chicks fed cadmium for 3 weeks. Low values for P.C.V., total Hb, and M.C.H.C. are consistent with a diagnosis of microcytic, hypochromic anaemia, characterised by a smaller red blood corpuscle (low P.C.V.) and a lower mean corpuscular content. This anaemia, not found in man chronically poisoned with cadmium, but overt in lead-poisoning, could be anticipated on the basis of the in vivo changes in the two haem biosynthetic enzymes and in catalase, all of which must obviously become depleted by a deprivation of ferrihaem, unless the tissues give priority to the biosynthesis of holocatalase before that of haemoglobin.

The consistent non-appearance of minialbumin in the serum of poisoned chicks may be due to the fact that the single pulse dose of injected cadmium was insufficient to induce the hepatic lesion that is held responsible for this deranged metabolism in chronically poisoned animals. Kench, Gain and Sutherland (1966) have located the site of origin of minialbumin as being extrarenal, probably in the liver, since minialbumin has been observed circulating in the blood of poisoned nephrectomised animals. The appearance of minialbumin in the urine is contingent on later renal tubular damage by cadmium ions, which would allow passage of the protein into the urine.

Although as much as 60% of the injected pulse dose of cadmium finds its way into the liver cells and remains there during the period of development of the chick before hatching, it seems that conditions are not appropriate in this avian species for minialbumin formation. Further experimental work will be needed before one can conclude that low molecular albumins are not formed in the poisoned chick embryo, particularly in the light of the marked propensity of such proteins to aggregrate to molecules of conventional size.

Metallothionein, the interesting thiol rich protein first extracted from horse liver by Kagi and Vallee (1961) has more recently been purified by Parisi and Vallee (1970), and found to exist in the circulation as an α_2- macroglobulin. Metallothionein binds 40% of the zinc in serum; i.e. up to 77 µg zinc/gm of protein. It is feasible that pre-albumin detected in chick embryos may play a similar role to this protein.

The fact that cadmium is continuously absorbed, even in the presence of a considerable body load indicates that there is no metabolic control for the accumulation of toxic quantities of the metal (Cotzias et al 1961 b). Whether or not pre-albumin or metallothionein is responsible for binding and transport of Cd^{II} remains a question for future enquiries. Cadmium in the serum of industrial cadmium

workers has been observed to be widely distributed between the protein constituents, and a specific carrier of this metal has not been recognised. Rapid displacement of protein-bound zinc by the more powerful thiol binder cadmium, can occur, with the resultant rise in unbound zinc, as reported by Cotzias et al (1961 a).

The diversity of proteins offers metals a great variety of co-ordination centres and different metals can bind preferentially to a number of different ligands. This is not unique, since vanadium and other metals may avail themselves of unused binding sites on transferrin, although iron has prior claim on this protein. The metals which form the strongest Lewis acids, namely mercury, copper, lead and cadmium, rarely provide the catalytic centres essential for the activity of hydrolytic metalloenzymes (Vallee and Williams 1968).

The experiments on ostriches were undertaken primarily in order, through availability of larger quantities of material, to broaden the scope of the investigation. It was envisaged that a wider ranging programme of studies of enzymes and substrates would become possible. The ostrich unfortunately, is notoriously difficult to breed in captivity. However, experiments conducted on those ostriches which did thrive sufficiently gave results which tallied very well with the biochemistry of the chick, and were acceptable on that basis.

The influence of Cadmium on Enzymes

The basic format of all the cadmium-enzyme interactions described here was a comparison of the effects of the metal on *in vivo* activity of the enzymes with changes in the same enzymes *in vivo* under the influence of the metal in what was calculated to be a similar intracellular concentration. For example, a mitochondrial enzyme inhibited *in vitro* by 10^{-5}M Cd^{II} would be investigated in chicks poisoned with the metal to the degree whereby it was calculated that 10^{-5}M Cd^{II} would be attached to the mitochondria of chick liver cells. The *in vitro* measurements were made on pure enzymes, when commercially obtainable, or on homogenates cleared of extraneous cellular material by differential centrifugation.

The pure product was found to be more sensitive to cadmium than the cellular preparation in all enzymes dependent on a thiol group in the active site. According to Vallee and Williams (1968), cadmium ions show a predeliction for thiol bonds, with the result that these will bind cadmium prior to attachment of the metal to any other ligand. Thus, when cadmium is added to an homogenate which contains a large number of non-enzymic sulphide groups, the effective concentration of the cadmium relative to the enzyme being assayed will in all probability be less than when present with the purified enzyme alone.

The enzyme which proved most interesting in the light of metal-ion activation was adenosine triphosphatase. Mg^{II}-activated ATPase from rat kidney was more resistant to cadmium inactivation than was the Na/K-activated isozyme. At 1×10^{-4}M Cd^{II}, Mg^{II}-activated ATPase was not inhibited, whilst the Na/K-activated isoenzyme was depressed by 15%. This phenomenon stems from the similarity in properties of the two metal ions, Mg^{II} and Cd^{II}.

Apps (1968) has established that in order to form the ionised ATP^{4-} Mg^{2+} complex to achieve optimal ATPase activation the ratio of Mg^{II} to ATP must be 3:1. He studied the enzyme NAD-kinase, and concluded that Mg^{II} participates in a delicate metalloenzyme catalysis with 4 postulated binary complexes, E-NAD, E-Mg-ATP, E.Mg.ADP, E.NADP; and 2 ternary compexes, E.ATP.Mg.NAD and E.ADP.Mg.NADP, all in equilibrium with the free substrates NAD and ATP. NAD-kinase was also activated by zinc, cobalt and manganese.

Cadmium substitutes for Mg^{II} as the activating metal of the purified potato enzyme, apyrase. It does not exert a competitive inhibition on Mg^{II} activation of the enzyme, as reported by Schaub and Ermini (1969) for the mammalian enzyme, even when present in millimolar concentrations. From the work of Schaub and Ermini (1969) and Rifkin (1965) it does appear, however, that the mammalian enzyme is more sensitive to substitution of Mg^{II} by Cd^{II}, with a corresponding

depression of activity, than are the chick or potato ATPase.

The importance of metal-ion activation of the substrate emerged as an entirely new concept in this particular series of experiments.

Possible mechanisms of enzyme - metal - substrate interaction with regard to ATPase are as follows:-
(S = Substrate; M = Metal; E = Enzyme; P = Product)

$$S + M \text{ ----- } M - S \overset{+E}{\text{------}} E - S \text{ -- } E + M + P$$

or, less likely:-

$$E + M \text{ ----- } E - M \text{ ------ } E - M - S \text{ ---- } E + M + P$$

The initial action of Cd^{II} on a normally functioning system (E + S + Mg) is to compete with Mg^{II} for ATP. An ATP^{4-} -Cd^{2+} complex is formed, which activates the enzyme system to lyse ATP and set free inorganic phosphate.

Above physiologically possible cadmium levels, the competition between Cd^{II} and Mg^{II} for the substrate could also indicate the possible competition of cadmium for enzyme-binding sites. Once cadmium is present in millimolar (or greater) concentrations, it tends to cause rapid, irreversible inhibition, behaving like a "classical" heavy metal (Ag^{I}, Hg^{II} or Pb^{II}). It poisons the enzyme by altering the quarternary structure. At this concentration, a rise in substrate concentration

will not produce a concomitant increase in reaction rate. However, ATPase was not inhibited by cadmium concentrations less than 5×10^{-4}M, and apparently the only action the cation had at such concentrations was confined to activations of the substrate ATP through formation of a Cd^{II} - complex. The presence of other ions and buffers may influence the inhibition of ATP-ase by Cd^{II}, as Mg^{II} has been shown to diminish the susceptibility of ATPase to mercurials (Lardy and Wellman, 1953).

At concentrations which may be encountered under physiological conditions, Cd^{II} may effectively replace Mg^{II} in the ATPase systems, whereas under similar circumstances, the very sensitive dehydrogenases would be entirely inhibited if the metal could exert full influence on these mitochondrial enzymes. (Racker 1965).

In vivo inhibition of enzymes involved in the de novo synthesis of the tetraporphyrin molecule, such as ALA-synthetase, and more particularly ALA-dehydrase, should ultimately manifest itself in the depression of total haem content in both circulating blood and in liver haemo-proteins. That this is so has been clearly demonstrated by Auerbach, Pieringer and Waisman (1959). In vivo experiments in the rat showed that catalase and tryptophan oxygenase were markedly depressed by

the ALA-dehydrase inhibitor, 3-amino - 1,2,4, - triazole.

The indifference of catalase to the presence of cadmium *in vitro*, and the significant lowering of the activity of the enzyme *in vivo* appear to warrant the conclusion that the synthesis of the enzyme is impaired *in vivo* in chicks poisoned with cadmium. Such a derangement of biosynthesis could implicate the formation of haem in the hepatic cytosol, or the formation of the apoprotein, and any part of the chain of biochemical events from nuclear DNA, messenger RNA, ribosomal RNA to transfer RNA and "pH5 enzymes", might be suspect. The appearance of minialbumin in poisoned mammals is indicative of an aberration in protein biosynthesis.

The demonstration of depression of ALA-synthetase was not as easily proven as was the depression of ALA-dehydrase in the cadmium-poisoned chicks. This was due mainly to the intricate and involved analysis of the former. Both enzymes possess active sites which are thiol dependent, and are consequently very prone to attack by divalent cations. Marked inhibition of these two enzymes was evident in the chronically poisoned chicks, as well as in acutely-poisoned embryos. The anaemia which developed was the initial indication of impaired haem biosynthesis. However, erythrocytic catalase was also markedly inhibited. Samples identical in haemoglobin concentration, when assayed for catalase, showed that activity of the red blood corpuscular enzyme was significantly less in

poisoned chicks.

Tryptophan oxygenase activity, as was the case with cytochrome oxidase, was not significantly altered by cadmium poisoning. This indicated that no step in biosynthesis nor any factor participating in the enzyme action was susceptible to the action of cadmium *in vivo* as far as this soluble enzyme was concerned.

Amongst other known requirements, copper ions are essential for these enzymes. The tissue pool of copper may become depleted during chronic cadmium poisoning, and some of the symptoms of cadmium intoxication simulate those of copper deficiency (Hill *et al*, 1963). It now appears that availability of copper for these enzymes assumes priority, and consequently there is no apparent lesion until a marked degree of copper deficiency pertains. That haem formation is impaired does not appear to influence tryptophan oxygenase activity appreciably in the newly-hatched chicks. Were the poisoning more severe, it is probable that some inactivation of tryptophan oxygenase would become apparent, due primarily to the inability to form the holoenzyme without the provision of haem.

The spectacular resistance *in vitro* of the soluble haem-containing (non-thiol) enzymes to cadmium is convincing evidence that such groups are stable, and do not exchange the iron atom for cadmium, nor do they

chelate with cadmium. Spectroscopic observations on cytochrome further confirm this finding, as there was no change in the absorption spectrum following addition of cadmium. Accordingly, it must be assumed that the thiol groups of cytochrome oxidase remain free to participate fully in reactions of the terminal oxidative pathway alongside the copper-containing haemoprotein moiety in what is a very complicated reaction mechanism.

The whole question of haem-enzymes and their activation or inhibition by metals is far from clear, as may be observed from the experiments conducted here on three such enzymes; catalase, tryptophan oxygenase and cytochrome oxidase. Each of these has a characteristic and distinctive catalytic function. Some controversy continues with respect to the exact valency state of the iron in the haem prosthetic group and to the role played by copper in tryptophan oxygenase. An interesting account of this topic has been compiled by Feigelson and Maeno (1966).

The decline in specific activity of tryptophan oxygenase during development of the chick embryo could be ascribed to overly rapid growth of the liver, outdistancing the rate of synthesis of enzyme, and possibly to a a decreasing demand for NAD(H), a catabolic product of

of tryptophan, as the embryo matures. NAD may also be available in the yolk constituents, which are absorbed at an increasing rate as the embryo grows older, especially in the final week of intraovular life.

The observed slight stimulation of the embryonic enzyme may be a reflection of the equally mild activation of tryptophan oxygenase *in vitro* with cadmium concentrations slightly above those to be found in the hepatic cytosol.

The results of all the studies undertaken during the course of this project indicate that there is more likelihood of a soluble enzyme conforming *in vivo* to the pattern that it manifests when assayed *in vitro* than there is for mitochondrial enzymes which do not follow the *in vitro* pattern when assayed in poisoned animals. This does not invariably apply, but certainly obtains more often than not. Teleologically, it is fortunate that mitochondria are protected against toxic agents such as cadmium, otherwise several essential functions would be curtailed abruptly, resulting in the immediate death of the chick. The protective mechanism afforded to mitochondrial enzymes *in vivo* is of the utmost importance, but is not fully understood. The fact that chelating agents, particularly dithiols, to some extent nullify the toxic effects

of added cadmium on testicular tissue (Gunn, Gould and Anderson, 1966), does to some extent explain the protective mechanism possessed by the cell against toxic elements. The binding capacity of the intracellular dithiols in the liver must be much larger than that needed to counteract the effects of a single pulse dose of cadmium. This is so because the mitochondrial enzymes of chronically poisoned chicks were not inhibited until the degree of poisoning was so extensive that widespread hepatonecrosis occured. At this advanced stage of intoxication, depression of enzyme activity becomes generalised due more to blanket inhibition of protein synthesis than to specific binding to active sites of enzymes such as cytochrome oxidase and succinate dehydrogenase.

Anomalous behaviour of the various haem biosynthetic enzymes, responsible for the porphyrias, has been studied in many cases of lead poisoning. The whole gamut of haembiosynthetic disorders embraces numerous primary malfunctions, of which the inhibition of ALA-synthetase and ALA-dehydrase are two of the major ones. Hernberg and Nikkanen (1970) have detected a significant depression of activity of ALA-dehydrase in normal people exposed to lead in the atmosphere. Their work revealed a close negative correlation between ALA-dehydrase activity and blood-lead concentration. The conclusion reached was

that present levels of environmental lead contamination can be responsible for a very significant biochemical disturbance. Cadmium has been shown to bring about a marked inhibition of *in vivo* ALA-dehydrase in rabbits (Gibson and Goldberg, 1970); and now it has been found that inactivation of the enzyme occurred in conjunction with microcytic, hypochromic anaemia in cadmium-poisoned chicks.

There is recent evidence to show that haem formation from δ-amino-laevulinic acid takes place in the cytoplasm, and that the haem to be incorporated into cytochromes is then transported into the mitochondria. A separate mechanism operates for the simultaneous synthesis of apocytochromes. Assembly takes place within the mitochondria (Schiefer, 1969). Inhibition of the holoenzyme by cadmium would not be possible unless cadmium was able to pass through mitochondrial membrane to bind intramural active thiol centres. Prolonged action of cadmium would be to inhibit the biosynthesis of haem sufficiently to impair cytochrome formation. It does appear, however, that prior to this, catalase is inhibited in this way. Possibly the cytochromes have preferential access to haem or the haem they need is synthesised *in situ* in chick liver mitochondria, in a location not accessible to Cd^{II} ions.

During the course of daily subcutaneous injections (0.1 mg cadmium), blood haemoglobin concentration fell by 16% in ten days. If the fall in Hb concentration continued at the above rate for six weeks there would have been a 64% lowering in the total haemoglobin.

This appears to be a remote possibility, since Klein (1968) has shown that turnover of porphyrins in the erythrocyte declines to a negligible rate after the first 10 days of its normal life span. It is not as yet known whether the life span of the corpuscles is adversely affected by cadmium. Relatively little of the absorbed cadmium remains in circulation to act upon the thiol groups in red blood cell membranes upon which the normal characteristics of fragility and permeability are so dependent. It transpired from present analyses that less than 5% of the cadmium injected at day 14 remained in the bloodstream at day 21. The rapid decline in the concentrations of serum proteins and of haemoglobin in chronically poisoned chicks between the third and fifth weeks of life does indicate a major inhibition of ALA-synthetase and ALA-dehydrase, or a massive increase in the rate of erythrocyte destruction. The anaemia and low catalase levels are consistent with these views. In contrast, lack of inhibition of cytochrome oxidase is unexpected

in view of both the sensitivity of the holoenzyme to cadmium *in vitro*, and to the *in vivo* inhibition of the haem biosynthetic enzymes.

The anomalous behaviour of mitochondrial enzymes towards cadmium is illustrated once again in malate dehydrogenase, which is remarkable in its resistance to lethal concentrations of cadmium. It was the only dehydrogenase in the present investigation that displayed such behaviour. The 14-SH groups of the enzyme (Varrone, *et al* 1970) were affected to the same extent by cadmium and by the oxidants I_2 and thyroxine. This enzyme is in extreme contrast to the sensitive dehydrogenases, particularly lipoamide dehydrogenase, on which the same concentration of cadmium had such a drastic effect.

Cytoplasmic enzymes, on the other hand, which were inhibited *in vivo* possessed similar sensitivities to cadmium *in vitro*. Catalase, which was resistant *in vitro*, was the exception. *In vivo* suppression of catalase probably originated in restriction of haem formation rather than in a shortage of apocatalase molecules. In general terms, intravenous cadmium in acute poisoning did not appear as a potent inhibitor of protein biosynthesis, although when present in sufficiently high concentrations it depressed all the

serum protein concentrations, particularly albumin.

Alterations in enzymatic activity could arise through the operation of a great number of contributory factors, some promotive, and others inhibitive. Overall changes in the morphological structure of the liver, as in hyperplasia of the cells of the parenchyma, biliary epithelium or connective tissue, the quantity of fibrosis, or intracellular changes in the numbers of mitochondria could obscure a simple stoichiometric relationship between enzyme and metal ion. In certain individual animals, ALA-synthetase may have diffused from the mitochondria into the cytoplasm,since heavy metal ions (Cu^{II}, Ag^{I}, Hg^{II}) have a well-known action on mitochondrial membranes through inactivation of thiol groups which are essential to the maintenance of permeability and active transport of such structures. In the presence of toxic concentrations of heavy metals, mitochondria swell extensively. (Photomicrograph of liver of Cd^{II}-poisoned monkeys, Gain 1965). The liver of the Cd^{II}-poisoned ostrich showed marked histological abnormalities, including bile stasis. (Plate 3).

Furthermore, in addition to a direct inhibitive action of cadmium on the enzyme as observed <u>in vitro</u> in the present investigation, one cannot exclude the possibility that biosynthesis of the enzyme may also be impaired to some extent. The magnitude of this impairment would depend on

which points in the synthesis of the enzyme were susceptible to the impact of Cd^{II}.

The intracellular level of cadmium is thus crucial in determining the ultimate response of the cell to the toxic effects of the metal. The lowest concentrations are tolerated by all but the most sensitive enzyme-sites, and thereafter the responses of various biochemical constituents begin to become influenced as the toxicity increases, until ultimately the essential mechanisms for sustaining life become grossly affected, and cellular lysis results.

The role of dithiols as intracellular protective forces is in providing additional-SH groups as metal chelating agents (Webb, 1966). However, in certain instances added thiols may be powerful inhibitors. This was notable with catalase, which was 20% inhibited with 4×10^{-5}M cystine, but was resistant to cadmium. There are 8 -SH groups in beef liver catalase, 4 of which are rapidly oxidised to disulphides, while the other 4 form mixed disulphides (Pihl, Lange and Evang, 1961). The reaction was spontaneously reversible, a maximal inhibition being achieved after 5 mins at $37^{o}C$.

The reactivity of -SH groups of proteins is usually less pronounced than in simple thiols, and such groups may be classified into reactive, sluggish or masked (Barron, 1951). If a protein is allowed to react with thiol reagent, the SH groups generally disappear at differing rates, some reacting

completely before others are affected.

The various thiol groups of sulphydryl reagents are not identical in function and structure, and may be differentiated as follows (Webb, 1966):

(A) The thiol group is at the active centre and is functional and labile, e.g. Lipoamide dehydrogenase.

(B) The thiol group is at the active centre but is non-functional, e.g. Cytochrome oxidase.

(C) The thiol group is vicinal to the active centre. When modified, a new structure is introduced into the enzyme, which may sterically or electrostatically modify the active site, e.g. Xanthine dehydrogenase.

(D) The functional -SH group is on the substrate or coenzyme, e.g. ALA-synthetase and Co-enzyme A.

Boyer (1959) emphasized that when the type of inhibition is being examined, there is often insufficient consideration given to secondary structural changes induced by reaction of thiol groups with the inhibitor.

Inability to reactivate a cadmium-treated enzyme by use of either chelating agents or by addition of sulphydryl groups may be due to one or more of several reasons, some of which are enumerated below:-

(A) The affinity of cadmium for the thiols in the protein may be so great as to be irreversible.

(B) Cadmium may have altered the tertiary structure of the enzyme to such an extent as to perturb or denature the enzyme.

(C) Cadmium may displace a prosthetic group or co-factor which must be replaced before activity can be restored.

(D) The added thiol reagent may inhibit the enzyme by promoting autoxidation, even though free thiol groups may initially reappear in the protein.

Knowledge of the role of the thiol group in enzymology and the effect a toxic metal like cadmium, has on it, are essential in the further understanding of protein chemistry. This thesis has attempted to clarify a few points with regard to metal-enzyme relationships in the young chicks, and has employed cadmium as a probe to investigate the intracellular deposition of metals.

The results indicate that it is not always possible to draw conclusions as to the behaviour of an enzyme, when in its cellular environment, by the reaction it displays in vitro when subjected to the same degree of metal intoxica-

tion.

These several aspects of the reaction of cadmium ions with thiol groups in enzymes are pivotal to our deliberations on the possible value of cadmium as a metabolic probe to locate enzymes within living cells. Inasmuch as thiols are often crucial to enzymic activity, the metal is a valuable tool in regard to the importance of thiol groups to any individual enzyme. This thesis has described experiments designed to procure more information on these cognate questions. The growing chick has proved to be a suitable model for this type of study, since it comprises a closed system, and injected cadmium is completely retained. As most of the metal was transported to the liver, mainly hepatic enzymes were investigated. By evaluating the change of activity of a pure enzyme which was brought about by cadmium *in vitro*, and by comparing the magnitude of this effect with the alteration in the enzymic activity elicited by a similar concentration of cadmium *in vivo*, it has been possible to demonstrate that mitochondrial enzymes in general are less freely exposed to cadmium within the cellular milieu than enzymes present in the cytosol. Categorical generalisations are not possible, since there are many factors operating *in vivo* which could not be allowed

for in the *in vitro* assay. The time of incubation of the enzyme with cadmium *in vivo* was obviously much longer than the 15 minutes employed to test the action of cadmium on the enzyme *in vitro*. This difference would favour more pronounced changes in enzyme activity *in vivo*, the converse, in fact, of the experimental findings. Irregularities of pH, cadmium distribution, and of other chemical constituents within individual cellular fractions or even component organelles may well have contributed anonomously but still significantly to the findings. Differences of activity of certain enzymes have been of such a magnitude as to justify clear-cut conclusions, e.g. succinic dehydrogenase is in a region of the mitochondrion inaccessible to cadmium. In other instances, e.g. tryptophan oxygenase, it has so far not been possible to assess whether the alterations are statistically significant or not. Without reservation, one can, however, conclude that cadmium has proved its value as a cellular probe, and the chick embryo has provided a convenient experimental system. Finally, this work has amply underlined the importance of the situation of an enzyme within the cell, in so far as its activity is liable to be changed by the presence of other factors, in this instance by cadmium ions.

PART V

SUMMARY AND CONCLUSIONS

(1) A method was evolved for the intravenous injection of exact quantities of cadmium into a developing chick embryo, and a screw-clamp apparatus developed to facilitate the operation.

(2) A poisoning profile was compiled of the rise in tolerated dosage of intravenous cadmium with age of the chick embryo. It was observed that the dosage tolerated increased from 7 µg at day 8 to 20 µg at day 16.

Cadmium administered by daily injections into the crop of a growing chick was not as efficiently absorbed into the animal. Only 4% actually became deposited in the liver, but ultimately much higher final concentrations of intracellular cadmium were achieved this way. Cadmium was extremely toxic when injected intravenously, and a single injection of 20 µg in the later stages of incubation was sufficient, if the chick managed to survive the initial shock, to delay hatching by up to 2 days.

(3) A series of biochemical investigations was conducted on day-old chicks poisoned with cadmium to ascertain whether or notthere was a significant change in basal metabolic activity.

(4) Poisoned chicks appeared weak, had a generalised oedema,

particularly of the limbs and neck, and were unable to walk. They were often unable to hatch by themselves.

(5) No significant increase in the concentration of minialbumin was detected in chicks poisoned either by acute or chronic cadmium administration.

(6) There appeared to be slightly elevated levels of urea and uric acid in the serum of chicks intoxicated by cadmium. The concentration of both blood glucose and serum proteins was depressed in poisoned chicks.

(7) The haematological system was markedly disturbed, with development of a severe microcytic, hypochromic anaemia. The proportion of haemoglobin in the deoxygenated form was high, presumably as a consequence of circulatory embarrassment in the chick.

(8) Several enzymes in the red blood corpuscles and in the liver were investigated in order to discover the effects of cadmium poisoning on the biochemical systems they represented. Catalase was observed to be inhibited *in vivo* both in the erythrocytes and in the liver, but was remarkably resistant *in vitro* to high concentrations of cadmium.

(9) Hepatic xanthine dehydrogenase and ALA-dehydrase, both of which resemble catalase in being located in the cellular cytosol, were inhibited *in vivo* and were equally sensitive *in vitro*.

(10) The specific activity of tryptophan oxygenase declined with increasing maturity of the chicks. The total activity of the enzyme in the liver, however, increased in proportion to the age of the embryo. The enzyme was not significantly affected by cadmium concentrations attainable *in vivo*, but was slightly stimulated *in vitro* at cadmium concentrations of 10^{-4}M.

(11) Mg^{II}-activated adenosine triphosphates in chick erythrocytes was stimulated *in vivo* by cadmium. This was also activated in the purified preparation Apyrase, in which cadmium substituted for magnesium whenever the concentration of the latter was less than 8 mM. *In vitro*, chick erythrocytic ATPase was inhibited to the extent of 20% by concentrations of cadmium attainable *in vivo* in poisoned chicks.

(12) Mitochondrial enzymes in general exhibited greater differences in activity between the control and cadmium-intoxicated samples when *in vitro* activities were compared with those *in vivo*. The primary rate-controlling enzyme

in haem biosynthesis, mitochondrial ALA-synthetase, was inhibited in severely poisoned chicks, but not to the same degree as was the soluble enzyme ALA-dehydrase. The synthetase was more sensitive to cadmium *in vitro* than *in vivo*.

(13) Cytochrome oxidase and succinate dehydrogenase were unaffected by cadmium poisoning *in vivo*, but were extremely sensitive to the metal ion *in vitro*. This difference clearly indicates that within the living mitochondria of the liver, these enzymes are out of contact with the Cd^{II} ions introduced into the chick. Lipoamide dehydrogenase was even more completely inhibited *in vitro* (30 - 50% at 5 μM Cd^{II}), and was slightly inhibited *in vivo*, and this mitochondrial enzyme also appears to be largely inaccessible to cadmium under the experimental conditions of this work.

(14) Kinetics of enzyme action indicated a non-competitive type of inhibition by cadmium. The inhibition was partially reversible, 40 - 60% of activity being restored to an *in vitro* enzyme system on addition of Cleland's reagent or E.D.T.A.

(15) Mitochondrial malate dehydrogenase was not sensitive to cadmium either *in vivo* or *in vitro*, but activity of the

soluble isoenzyme was slightly increased by cadmium-poisoning in chicks investigated *in vivo*. The enzyme was inhibited as strongly by oxidants such as I_2 or thyroxine as it was by cadmium. The two types of inhibition were additive.

(16) The avidity of cadmium for thiol groups was confirmed. The particular type or degree of inhibition provided information as to the nature of the active site in the enzymes brought into contact with cadmium, and shed light on the functional role of thiol groups in each enzyme.

(17) Ostriches were poisoned with cadmium, with less rewarding results, owing to the inherent difficulty in raising the embryos in an incubator. The biochemical effects of cadmium in this larger avian species were consistent with the data obtained from chicks.

(18) At least 60% of the cadmium administered intravenously to the developing embryo was recovered from the liver of newly-hatched chicks. Tissue cadmium was measured by atomic absorption spectrophotometry, following acid digestion of the tissues.

(19) The intracellular distribution of cadmium was determined in the liver of poisoned chicks. The greatest

concentration of cadmium was found in the cytosol, followed by the mitochondrial, microsomal and finally nuclear fractions. Sixty percent of the cadmium in hepatic tissue was located in the soluble fraction of the cell-free homogenate. The concentration of cadmium in the various fractions was 4- to 6-fold higher following a régime of chronic poisoning than was achieved by a single intravenous pulse-dose of cadmium.

(20) The developing chick embryo proved to be an extremely useful biological model, and cadmium a useful metabolic probe in the search for information on the intracellular location of enzymes, and on the role of their functional groups which react with cadmium. In turn, the response of such enzymes to the presence of cadmium ions aids in the understanding of metal-protein interactions. In the final analysis, greater insight should be gained into the relationship between the atomic structure of cadmium and its biochemical behaviour.

(21) Much work is still to be done in this field, and there are many questions as yet unsolved. Investigation of the intracellular action of cadmium is continuing.

PART VI

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PART VII

HISTOLOGY

Photographs are of tissues fixed in neutral buffered formalin and stained with haematoxylin and eosin.

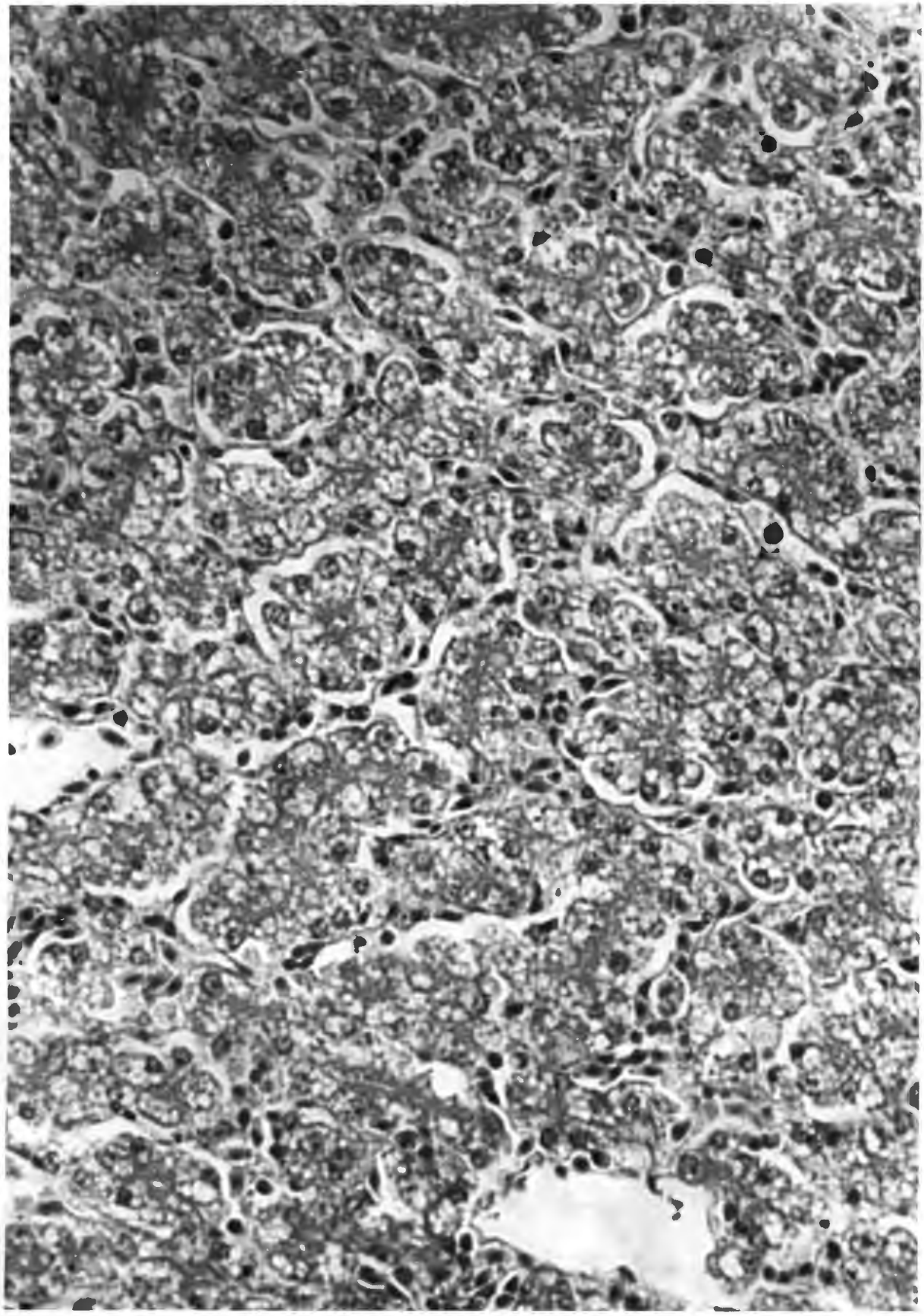
Plate I. Normal ostrich liver

The parenchymal cell cytoplasm contains numerous small vacuoles. Many nucleated red cells are present in the sinusoids.

Plate II. Normal liver

Plate III. Cadmium poisoned liver

The general architecture of the liver is maintained and there is no evidence of frank cellular necrosis. There is, however, widespread biliary stasis and a large intracellular bile plug occupies a dilated bile canaliculus in the centre of the field. Cells in the right half of the micrograph have large cytoplasmic vacuoles which contain a granular material similar in staining characteristics to that of the bile plug. Only occasional nucleated red cells are seen in the sinusoids.



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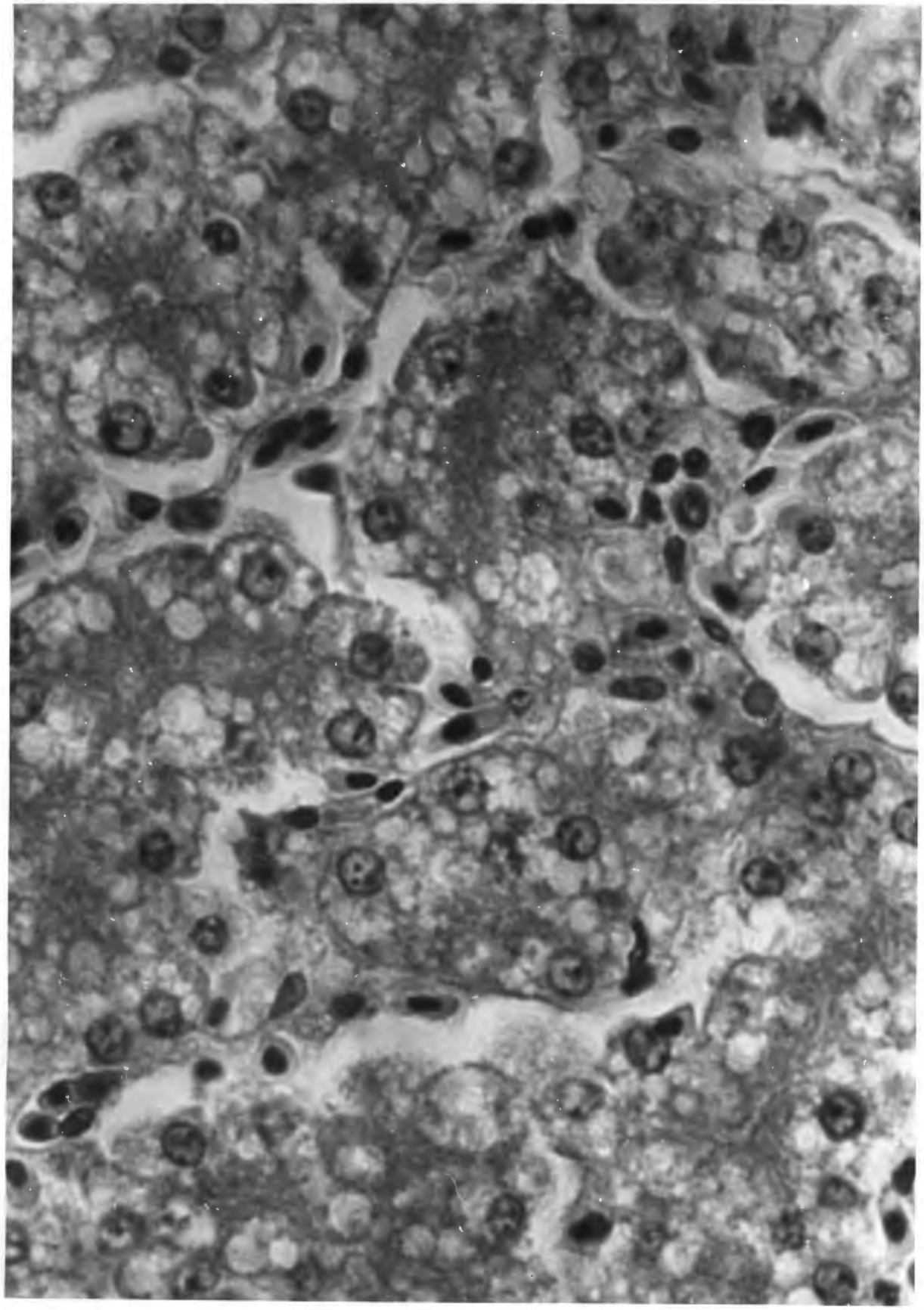

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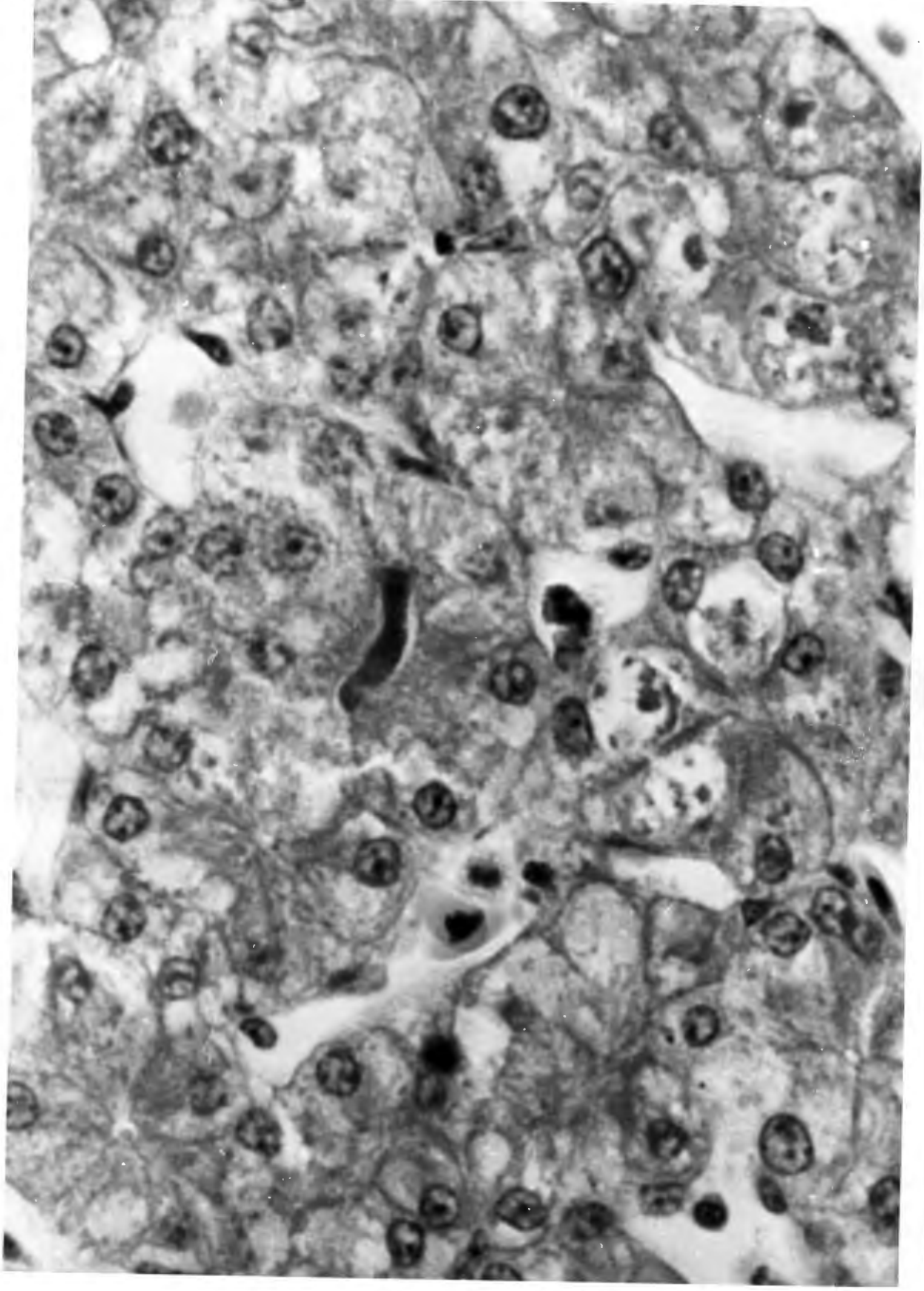

III