ABSTRACT

Ph.D.   BIOCHEMISTRY

GREGORY KWAI-WAH KO
THE METABOLIC FATE OF PENTYLENETETRAZOL
IN THE RAT

Pentylenetetrazol administered to rats was
metabolized to a derivative. This derivative was
excreted in the urine along with unchanged pentylene-
tetrazol. Pentylenetetrazol and its metabolite can
be separated on paper chromatograms developed in
water-saturated isobutanol. Both substances, however,
are chromatographically inseparable with water as the
mobile phase. Distribution of tritium-labelled
pentylenetetrazol in the rat after intraperitoneal
injection indicated that it was readily taken up by
tissues, especially the liver. Perfusion of the
isolated rat liver with citrated blood containing
tritiated-pentylenetetrazol demonstrated that the
metabolite of this central nervous system stimulant
drug was formed in the intact liver. In vitro formation
of the metabolite by the perfused rat liver was inhibited
by SKF 525-A. No metabolite was formed, however, when the
agent was incubated with rat liver homogenates. A
simple column chromatographic method using the neutral
resin Amberlite XAD-2 was developed to enable isolation
of the metabolic derivative of pentylenetetrazol for
further chemical analyses.

THE METABOLIC FATE OF PENTYLENETETRAZOL
IN THE RAT

BY

GREGORY KWAI-WAH KO, M.Sc.

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ABBREVIATIONS

ANS	-	Autonomic Nervous System
b.w.	-	Body Weight
CNS	-	Central Nervous System
i.p.	-	Intraperitoneal
i.v.	-	Intravenous
PTZ	-	Pentylenetetrazol, Metrazol
H^3-PTZ	-	Tritiated-pentylenetetrazol
TPN	-	Triphosphopyridine nucleotide

To my parents and my wife

INTRODUCTION

Drugs of diverse pharmacological groups, capable of restoring the depressed medullary and other functions of the central nervous system (CNS) are usually referred to as "analeptics". The term "analeptic", from a pharmacological point of view, should apply to substances which stimulate the normal as well the depressed central nervous system (1), presumably by the same elementary mechanism (2). Certain analeptics are used in the treatment of CNS depression. Among them, pentylenetetrazol (Pentamethylenetetrazole, metrazol, cardiazol or leptazol) (PTZ) is the one most extensively studied. Being a convulsant drug, PTZ has some characteristic properties which aroused tremendous interest among neurophysiologists. Clinically, it is also used as an EEG-activating agent in the diagnosis of epilepsy, and as a convulsant in the therapy of schizophrenia. Most basic pharmacological problems concerning analeptic actions have been studied with pentylenetetrazol (PTZ).

I. PROPERTIES OF PENTYLENETETRAZOL

PTZ (Fig. la) is a fused ring 1,5 disubstituted derivative of tetrazoles, a class of nitrogen containing

heterocycles of which well over 300 derivatives have
been synthesized, and documented (3,4). The tetrazoles
are characterized by a five-membered, doubly unsaturated
ring consisting of one carbon and four nitrogen atoms,
whose basic structure appears below (Fig, 1b).

(8)

(9) H_2C (7)
(10) H_2C (6)
(1) N——C   (5)   (1) H—N————C—H (5)
(2) N   N   (4)   (2) N   N (4)
        N   N
       (3)   (3)
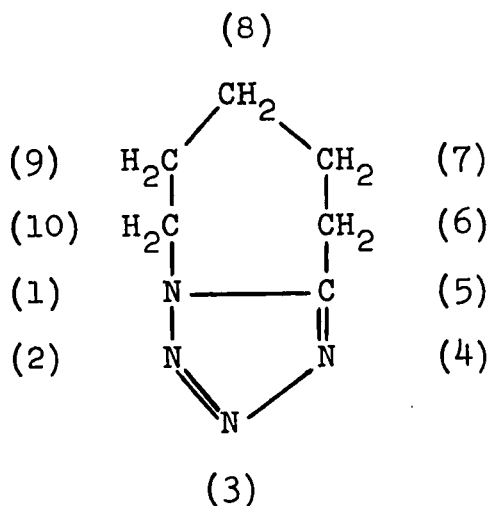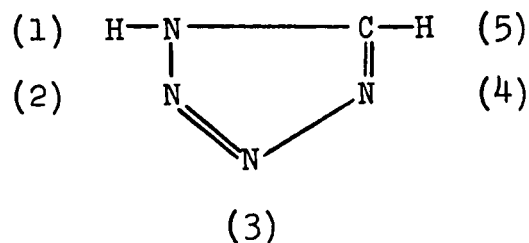
Fig. 1 a. PENTYLENETETRAZOL   Fig. 1 b. TETRAZOLE

    The tetrazole ring system can withstand a variety
of chemical agents and possesses considerable stability.
An interesting description of these and other five-
membered heterocycles in terms of the nitrogen system
of compounds has been described by Franklin and Bergstrom
(5).

Unsubstituted tetrazole is devoid of significant pharmacological action. Substitution of tetrazole produces compounds which are either stimulatory or depressive in action. PTZ is the only tetrazole derivative used therapeutically, although it is not the most active of these compounds.

Modification of the basic structure of PTZ alters its pharmacologic activity (6, 7). Introduction of a methyl (8), an isopropyl or a tertiary butyl group, particularly into position 8 of the PTZ molecule, greatly enhances its stimulatory effects on the CNS, although some of these derivatives are less effective as analeptics. Increasing the size of substituent groups, such as a secondary butyl or a tertiary amyl group, reduces or abolishes the stimulant action of PTZ (9). Thus, 8-cyclohexyl PTZ is a depressant rather than a stimulant drug.

PTZ is readily soluble in water and lipids while most of the other active derivatives are water-insoluble. The most significant property of PTZ seems to be its ability to form insoluble complexes with phenol-like substances (10). Also, the "proton-donor" nature of the tetrazole ring and of those PTZ derivatives substituted at position 5, compared with the weak "proton-acceptor" nature of the N-substitution, is noteworthy (3, 11).

One of the interesting properties of the tetrazole
derivatives is that stimulant compounds show slight or
no UV absorption. Tetrazole derivatives with depressant
action strongly absorb UV light. The substitution of
groups capable of low resonance conjugation with the
tetrazole nucleus, such as an alkyl group, at positions
1 and 5 are essential for central stimulant activity (12).
PTZ possesses stimulant action but also absorbs UV light
(13).

PTZ was first synthesized chemically by Schmidt in
1924 from cyclohexanone and a benzene solution of hydrazoic
acid in the presence of an acid catalyst (14). Since then,
there have been a number of patents filed, and several
variants in the original procedure were employed (15, 16,
17, 18). Thus, the interaction of cyclohexanone oxime,
phosphorous oxychloride, piperidine, and sodium azide are
claimed in one patent to form PTZ (19). In another,
p-toluenesulfonic ester of cyclohexanone oxime is treated
with hydrazine followed by diazotization to obtain the
compound (20).

When heated with hydrochloric acid in a sealed
tube, PTZ is decomposed to nitrogen, carbon dioxide,
and pentamethylenediamine (14). The proposed mechanism

involves preliminary formation of nitrogen, and a
pentamethyleneamine isocyanate, followed by hydrolysis
of the cyanate group.

$$\text{(structure)} \xrightarrow{\text{HCl}} N_2 + CO_2 + H_2N(CH_2)_5NH_2$$
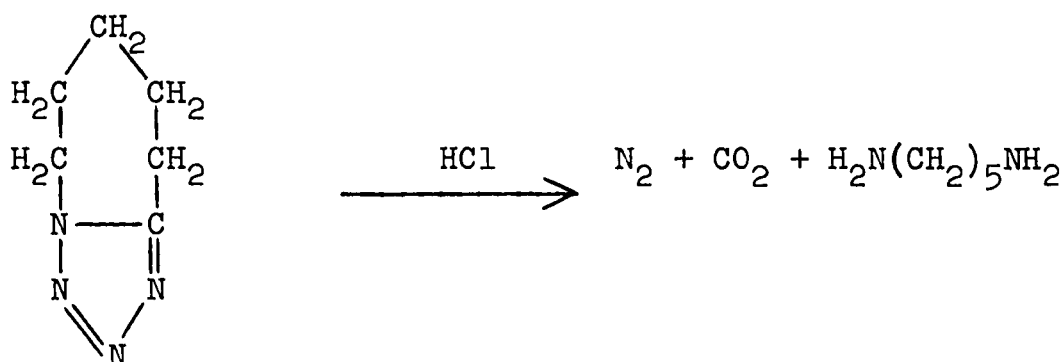
In the body, the biotransformation of this compound
is uncertain, and the drug is believed to be excreted
unchanged (21).

II. PHARMACOLOGICAL EFFECTS OF PENTYLENETETRAZOL

PTZ exerts a generalized effect on all the organs of
the body. Since the introduction of this convulsant drug
into clinical medicine in the late 1920's (22), the effects
of the drug in the following systems have been extensively
studied: blood pressure, respiration, and body temperature.
Hahn (2) has compiled an excellent review of these and
other studies of PTZ in vivo.

Recent work has shown PTZ affects, among other systems,
the sodium-potassium-activated ATPase in rat brain (23),
incorporation of P^{32} into brain phospholipids (24),

blood-brain-barrier permeability (25), phosphorylase

activity (26), and blood cholesterol level (27). The

effect of PTZ on the brain, however, is more severe (169).

The drug is capable of disrupting oxidative patterns of

the cells. It stimulates metabolism of the intact animal

brain leading to increased O_2 consumption and glucose

utilization (29). When administered to the body, the

convulsive response usually sets in within a few seconds

and ceases in a few minutes. Sollmann (30) described

the typical clinical reaction as follows:

"In two to five seconds after the
rapid intravenous injection, the patient
coughs; five seconds later, marked pallor
of the face; then intense blepharospasm,
a facial expression of fear and bewilderment,
the mouth is widely opened; then myoclonic
movements of the face, shoulder, arms, some-
times on one side, then well-marked rhythmic
clonus; and then some thirty seconds after
the injection, the tonic phase, with the
head retracted, the back arched, the arms
and legs extended, the wrist and fingers
flexed, the legs sometimes scissored, goose
flesh, apnea with marked cyanosis, rise of
blood pressure by 20 to 60 mm., erection and
ejaculation of semen. The convulsions in-
volve simultaneous and persistent contraction
of all muscles and resemble decerebrate rigidity.
After five to thirty seconds the tonus lightens
to a second clonic phase, first at the fingers,
finally at the legs, which lasts about twenty-
five seconds, with decreasing intensity. The
patient then relaxes, deep respiration resumes,
urine is passed. The patient, who has been
unconscious throughout the tonic phase, is now
confused, drowsy, probably asleep. After a
few minutes of rest there may be some further
relatively mild convulsive movements."

This description of clinical convulsion applies also to animals, except that the spasms are more prolonged with increase of dosage, and lead to asphyxial death.

There has been no general agreement as to the primary location of the convulsant action. However, PTZ has been shown to affect the higher centers of the brain, the autonomic nervous system and the spinal cord.

A. Action on Higher Centers

Evidence is available that PTZ stimulates the CNS at all levels (31). Stepwise removal of the brain tissue from medulla to cortex shows the decrease in convulsant activity is in direct proportion to the mass of the tissue removed (32, 33). This evidence suggests that for maximum activity, the entire area between the cortex and the brain stem may be involved.

Attempts to ascertain the site at which PTZ acts to elicit paroxysmal bioelectrical activity resulted in divergent conclusions. Some authors maintain the cortical site of origin of PTZ convulsion (34, 35, 36). A direct action of PTZ on the cortex was suggested to lead to changes in the EEG patterns (37, 38). Intravenous and subarachnoidal administration of the convulsant on

the cortical surface revealed the primary site of action
for PTZ to be the cortex (31, 39). Pollock and Gyarfas
(40) reported the paroxysmal activity was always first
seen in the thalamus and cerebral cortex. Ligation of
the basilar artery at midpontine region revealed a
greater sensitivity for PTZ by the higher centers than
the nuclei of the pons and medulla (41). Other inves-
tigators were unable to find a given area in the brain
in which paroxysmal activity could be consistently
recorded prior to the onset of generalized seizures
and they believed that bioelectrical activity occurred
simultaneously in all leads examined (42, 43). There
are still other indications that changes in EEG patterns
caused by the drug were due to anoxia, rather than the
effect of the drug on the brain (28, 44, 45). Straw and
Mitchell (46), upon examining the correlation of bio-
electrical activity and overt seizure pattern, concluded
that PTZ does not act at a consistent anatomic trigger
site to elicit paroxysmal bioelectrical activity.

The participation of the subcortical structures in
the effect of PTZ seizures was also stressed by some inves-
tigators (40, 47). The increased output of ACh from the
parietal cortex and the perfused cerebral ventricle was
thought to be due to activation of subcortical and
cortical cholinergic neurones (48). However, direct
stimulation of the cortex of the cat after pretreatment

B. Action on Spinal Cord

Experimental evidence has revealed that PTZ can also induce seizures in spinal animals (56, 57, 58). The sensitivity of the cord, however, is low for PTZ (43, 59, 60). Studies on the changes in electrical activity of the spinal cord after PTZ showed indeed the low sensitivity of the cord for the drug, and these changes in spinal activity were supposedly due to efferent impulses from supra-spinal centers (60).

On the contrary, there have also been reports of the unilateral supersensitivity of the spinal cord to PTZ after chronic lesions of the cord on the same side, and in acute spinal preparations if the centralateral frontal lobe or contralateral hemisphere has been re-moved previously (61).

The action of PTZ on the cord is easily antagonized by anaesthetics (56), especially in spinal animals (56, 62, 63). It has been claimed from observations in anaes-thetized intact cats that PTZ acts mainly on the motor reflex arc at a site associated with the terminations of the pyramidal tract (64). However, there are also other beliefs that PTZ acts on the sensory part of the reflex arc (65, 66).

More recent work showed that PTZ acts chiefly by stimulating excitatory synapses (67). The spinal neurones are affected by doses of the drug which cause convulsions in the intact animals. The excitatory effect of the drug is partially counteracted by stimulation of the neuronal system whose function is primarily inhibitory. Thus, Lewin and Esplin (68), suggested that PTZ uniformly stimulates excitatory and inhibitory neurones, and that convulsive manifestations are due to excitation of cerebral structures relatively unopposed by inhibition.

C. Action on Autonomic Nervous System

Direct stimulation of the autonomic nervous system (ANS) has been observed by direct injection of PTZ into the hypothalamus (69). Earlier, it was reported that PTZ convulsions affect both divisions of the autonomic nervous system (70, 71, 72) even in the presence of curare (73). The effect on the sympathetic system appears to be predominant (74). The preponderance of sympathetic effects was well demonstrated by organs with opposite autonomic innervation (71). In decapitated cats, nikethamide is more effective than PTZ on the spinal centers responsible for erection and ejaculation (62). These effects are believed to be mediated by the parasympathetic system.

In intact mice, ejaculation can be produced by the synergistic action of a barbiturate and one of several stimulants, especially nikethamide; the efferent pathways of ejaculation of the mice, however, are predominantly sympathetic (75, 76).

The effect of PTZ on the parasympathetic system can only be seen after convulsant doses of the drug, but even then it is still masked by sympathetic effects (71, 72). Parasympathetic centers are inhibited by small doses of PTZ (77). However, with therapeutic doses of the drug, the stimulant action of peripheral ganglion cells seemed to be greater on the parasympathetic than on sympathetic ganglia (78).

It has been reported that convulsant doses of PTZ facilitate the liberation of epinephrine from the adrenal medulla, and this release was thought to be partly responsible for the sympathomimetic response to the drug (74, 79). Other workers have also reported the facilitation of PTZ convulsions by relatively high doses of epinephrine (80, 81, 82). The mechanism of such facilitation is not known. Intravenous injection of PTZ into anaesthetized cats produced autonomic effects such as full retraction of nictitating membrane, piloerection, profuse salivation, etc. These effects are attributable to activation, either directly or indirectly, of autonomic centers (48).

PTZ inhibits cholinesterase (83). The high acetyl-
choline (ACh) levels of brains of narcotized animals
were lowered by PTZ administration (84, 85). This con-
vulsant drug also modified the in vitro synthesis of
ACh (86). Injection of PTZ into convulsive (ep) strains
of mice results in a 40% reduction in the ACh activity in
both the "free" and "bound" fractions (87). Changes in
the osmotically "labile" fraction of bound ACh in metrazol
induced convulsions were believed to be responsible for
the increased liberation of the choline ester (88). Intra-
venous injection of the drug increased the release of ACh
from the cerebral cortex of the sheep (89), and cat (48).
The ACh output was also elevated when the cerebral ven-
tricles were perfused with the convulsant drug (48). These
and many other observations seem to suggest that PTZ acts
via cholinergic mechanisms. The reports concerning the
action of atropine on PTZ-induced convulsions (43, 90)
contradict those on the effect of convulsions by PTZ on
the ACh content of the brain (84, 85). Although these
experiments do not justifiably conclude that PTZ-induced
central stimulation is due to cholinergic mechanism, it
has been suggested that PTZ, possibly by its analeptic action,
may restore the ACh metabolism in brain which had pre-
viously been depressed by anaesthesia (2). Parasympa-
thomimetic drugs with the exception of the anticholin-

esterase agent diisopropylfluorophosphate and of pilo-
carpine (91, 92) may enhance the development of PTZ-
induced convulsions (80, 93, 94).

III. THERAPEUTIC USE OF PENTYLENETRAZOL

A. In Barbiturate Poisoning

PTZ has enjoyed extensive clinical application.
Although its popularity seemed to be gradually replaced
by the availability of new drugs, its unique position in
pharmacological research remains unchallenged. PTZ is
one of the few analeptics whose life-saving effect against
depression of the CNS by anaesthetics has been firmly
established (95). It is necessary, however in these
instances, to approach the convulsant dose, and thus
the effective margin is not large (96). In barbiturate
poisoning, PTZ stimulates the CNS, resulting in increase
of respiration, blood pressure, and reflex activity (30).
In excessive doses, PTZ causes paroxysmal cerebral activity.
The drug is not as effective as picrotoxin against certain
cases of barbiturate poisoning, but is safer (1). PTZ is
also not very useful against fatalities from volatile
anaesthetics (97). Some investigators have doubted the
advisability of PTZ treatment in barbiturate poisoning, and
have all but given up this practice in favour of other
forms of physiologic management (98).

B. **In Convulsion Treatment of Psychiatric Depression**

PTZ has been used in psychiatric practice since 1935, particularly in schizophrenia, as a seizure inducing agent (99, 168). However, its effectiveness for this purpose is doubtful. Von Meduna (100), first conceived the idea of treating schizophrenia by inducing epileptoid convulsions, assuming clinically the two conditions to be mutually exclusive. PTZ is faster and safer than camphor in such treatment, but not as effective as insulin hypoglycemic shock or electroconvulsive therapy. Also, the treatment frequently resulted in minor complications such as temporary subluxations and dislocations and fractures. Nearly half of these patients have multiple compression fractures of vertebrae (101). For this reason, curare (102) or succinylcholine (103) is often administered along with the convulsant drug in the treatment of schizophrenia by convulsion therapy.

Von Meduna and Friedman (104) have extensively reviewed the therapeutic results of PTZ treatment of schizophrenia. This form of therapy has largely been replaced in modern medicine by newer and safer psychopharmacological drugs.

C. In Collapse

Initially, PTZ was introduced into medicine as a respiratory, cardiovascular stimulant. The respiratory stimulation is directly central, and not significantly dependent on carotid sinus chemoreceptors. Stimulation occurs after decerebration by a cut through the brain stem above the red nucleus (105). Convulsive doses of the drug show an initial drop in blood pressure followed by an increase (71). The rise in blood pressure is chiefly convulsive, but some stimulation of the vasomotor center occurs with subconvulsive doses (72, 106). Coronary vessels are mildly dilated (107), but the heart is not stimulated directly (108). Lueth (109) in 1938, reported beneficial effects of the drug on certain patients with complete heart block and the Adams-Stokes syndrome, and concluded that the action of PTZ was not on the heart, but apparently on the vasomotor and respiratory centers.

The stimulatory action of PTZ on the vasomotor and respiratory centers was observed clinically only when near convulsive doses had been administered. Its effectiveness as a sustaining agent in chronic cardiac and circulatory insufficiency and as a restorative in cardiovascular collapse remains to be proven (110).

D. <u>In Diagnosis of Epilepsy</u>

PTZ is used as a diagnostic aid in epilepsy. It finds valid use in EEG activation. Subconvulsive doses of the drug, alone or together with flashing light will often activate latent epileptogenic foci. These PTZ-induced convulsions are of value in characterising the underlying cerebral disorders in patients with proven epilepsy (111).

E. <u>In Geriatrics</u>

PTZ is currently being used in geriatrics. Daily administration of subconvulsive doses of the drug will bring about increased alertness and mental agility in the elderly depressed patients suffering from senile arteriosclerotic psychoses and related conditions (112, 113, 114).

IV. <u>METHODS OF ESTIMATION OF PENTYLENETETRAZOL</u>

The application of PTZ in clinical medicine is extensive. However, the chemical nature of the agent is such that satisfactory methods for its quantitative extraction and determination in biological materials are wanting. In many instances, the classical methods require indirect estimations which render them unreliable for the quantitative determination of the convulsant which usually is present only in small amounts in biological tissues. Even

when applied to pharmaceutical preparations, these methods
lack the necessary specificity and precision. Mercuric
chloride, for instance, was used to precipitate PTZ (115,
116). However, this mercuric chloride-PTZ complex is
not sufficiently insoluble to be used for the determination
of PTZ in biological tissues (117). Other agents, such
as cuprous chloride (118, 119) cadmium chloride (120),
phosphotungstic acid (121, 122), potassium bismuth iodide
(13), have also been employed as complexing agents with
PTZ. Nevertheless, they interact quantitatively with only
pure solutions of the drug. With biological extracts,
they are unsuitable since a pure precipitate cannot be
obtained. These procedures and those adopted by both
the United States Pharmacopeia (123) and National Formu-
lary (124), involve a gravimetric determination or a
filtration step.

To study the fate of PTZ in animals, Tatum and
Kozelka (117) devised a micro-distillation method for
the purification of the drug extracted from neutralized
tissue filtrates. This procedure is difficult to perform;
also treatment such as microdistillation may be too
drastic to isolate a metabolite of this compound.

There are other procedures reported for the
assay of PTZ. They include refractometry (125),
indirect polarography (126), complexometry (127)
and colorimetry (128, 129). None of these methods,
however, is adequate to study the metabolism of this
drug. Recently, PTZ in 0·1 N sulphuric acid was re-
ported to show maximum absorption at 265 mμ (13). In
1962, Kawamoto (130) conducted an investigation on the
metabolism of PTZ by gas chromatography. Again, treat-
ment of the compound to be characterized to high temp-
erature poses the question of applicability when the
chemical nature of the metabolite is being sought.
Earlier, Esplin and Woodbury'(21), used C^{14}-labelled
PTZ to study the fate of the agent in the rat. These
authors have been the only group to use tracer metho-
dology in studying the metabolism of PTZ in vivo.

V. METABOLISM OF PENTYLENETETRAZOL

The fate of PTZ in the body is not well known,
and few studies have been done to elucidate this prob-
lem. Because of the lack of precise and specific
methods for quantitative isolation and determination
of PTZ, the results of these studies concerning the
distribution, and fate of the drug are equivocal or
contradictory.

A. Absorption and Distribution

PTZ is rapidly absorbed from the gastrointestinal tract (13) and even more so from the oral mucosa (131). In fact, it is readily absorbed from all sites of administration. The observations of "early peak effect" following rapid injection of the drug led Werner and Tatum (132) to suggest that PTZ quickly enters the circulation. It is uniformly distributed in the blood, brain, muscle and liver and none of these tissues shows particular affinity for the drug (117). PTZ also leaves the blood rapidly (132) so that equilibrium with the other tissues is quickly established (117). This may explain the relatively short period of peak activity following its rapid administration.

B. Detoxication and Excretion of Pentylenetetrazol

The pharmacologic action of PTZ is short and not cumulative (113). In the rat, it has been shown that the drug is metabolized before being excreted (21). The rate of its detoxication in the rabbit was found to be directly proportional to the concentration of the drug in various tissues. This rate decreased as the drug concentration in the tissues decreased (117). Using their microdistillation method to estimate the concentration of PTZ in tissues, Tatum and Kozelka (117) conclusively showed

that parenchymatous injury affected the detoxication of PTZ. Earlier, by means of biological assays, Dills and Seeburg (134) similarly concluded that the liver played an important role in the metabolism of the drug in cats. Additional evidence was provided by Schiffman et al. (135), who showed liver damage significantly increased the potency of subcutaneously (but not intravenously) administered PTZ in mice and in rats. Fournier and Selye (136), however, showed rats with 75% of liver tissue removed had the same tolerance to subcutaneously injected PTZ as the control. Thereby, they maintained hepatic tissue was not the site of detoxication of metrazol.

Other organs thought to be responsible for PTZ detoxication included kidney (137) and gastrointestinal tract (122). Nevertheless, these beliefs were largely negated by nephrectomy and other studies (117, 134, 135). The role of the kidneys however, in the excretion of the drug became evident when a dose of radioactive PTZ in rats was excreted in an altered form (138). Elimination of the drug through the kidneys was suggested to be dependent on the formation in the liver of an unknown inactive intermediate compound which was re-transformed to an active substance in the urine (21). However, the excretion of unchanged PTZ through the kidneys has not

been excluded (139).

The identity of the excretion product is unknown.
Leppert (140), Graner and Santesson (141), and Schulte
(142) concluded in the dog and man, little if any, PTZ
is excreted unchanged by the kidney. A similar con-
clusion was also reached by other investigators (117)
working with other species (134). Hinsberg (122), was
able to demonstrate a part of PTZ in the feces of guinea-
pigs by bioassay, but not by chemical means. Recent
studies with gas chromatography also revealed no signi-
ficant changes in the amount of PTZ following incubation
with the liver homogenate (130). With C^{14}-labelled PTZ
administered to rats however, radioactive material could
be detected in the urine a short while after its adminis-
tration (21). Although this was assumed to be PTZ itself
because of its chromatographic behaviour, this sensitive
method did show, at least in the rat, that radioactive
material was excreted by the metrazol-treated rats.

STATEMENT OF PROBLEMS STUDIED

The pharmacological properties of pentylenetet-
razol are well known. There is little information,
however, regarding the fate of this compound in the
body. The kidney is believed to have little or no
effect in its detoxication (135). Although studies
with chemically poisoned liver (117) and hepatectomized
animals (21) suggested hepatic involvement in the re-
moval of this convulsant drug, direct evidence for the
metabolism of PTZ by any tissue has not been reported.

PTZ has been shown to affect acetylcholine (ACh)
metabolism in vitro (83, 86). It lowered the ACh con-
tent of nembutalized cat (84), and produced a decrease
in the level of brain acetylcholine following its
intraperitoneal administration into the animal (143).
However, at low concentrations, the drug was found to
have no effect on the release of ACh-like materials
from the brain particles (144). Even in high concentra-
tions in perfusion through cerebral ventricles, the
only effect which could be observed was shivering (145).
When PTZ was applied iontophoretically to the cells of
the cortex, rather variable degrees of excitation or of
facilitation of L-glutamate were seen in about 50% of
the cases (146). But intravenous PTZ clearly elevated

the excitability of cortical cells, making them respond
much more vigorously to glutamate.

It has been suggested that PTZ may be converted
to a derivative in the liver, which is responsible for
the convulsive activity observed in vivo (144). This
likely may be the case when in many instances, the
metabolic products of foreign substances in animals
are considerably more toxic than the original compounds
(147). This is particularly true in the case of picro-
toxin, also a central nervous system stimulant drug,
which is believed to have its effects mediated through
picrotoxinin (148, 149).

The present work is therefore undertaken to study
the fate of PTZ, and direct evidence will be presented
that this drug is metabolized in the liver.

METHODS

Male albino rats of the Wistar strain weighing 150 - 200 grams were used in all experiments. Blood donors for the perfusion experiment were of the same strain but weighed 250 - 300 grams. The animals were fed ad libitum with a Purina chow.

Pentylenetetrazol (metrazol) was purchased from Knoll Pharmaceutical Company, Cooksville, Ontario, Canada. Tritium labelled metrazol (H^3-PTZ) was prepared from metrazol by New England Nuclear Corporation, New Jersey, by exposure to tritium gas. The final active product had a specific activity of 650 μc/mg. Purity of the compound was constantly checked by paper chromatography. For injection purposes, the radioactive metrazol was mixed with appropriate quantities of non-radioactive carrier metrazol in saline to produce the convulsant effect.

All other reagents used were from commercial sources, either Baker or Fisher reagent grade products. They were used without further purification.

I. COLLECTION OF URINE FOR ANALYSIS AND FOR IDENTIFICATION OF PENTYLENETETRAZOL METABOLITE

Each rat (in groups of four) received a total of

18 mg of PTZ mixed with 0.04 mg of H^3- PTZ (26 μc)
intraperitoneally (i.p.) in two separate doses at
5 hour intervals. The dosage was enough to elicit
a slight convulsion in each animal. Urine collections
in metabolism cages began immediately and continued for
24 - 48 hours after the last injection, care being
taken to prevent mixing feces with the urine. The
collected samples were processed individually except,
when studying the identity of the metabolite in the
urine, they were pooled together. Immediately after
collection, the urine was dried by lyophilization.
Extraction of PTZ and its metabolite was done directly
on the dried residue.

II. EXTRACTION OF PENTYLENETETRAZOL AND METABOLITE FROM BIOLOGICAL MATERIALS

The extraction procedure used was essentially that
of Esplin and Woodbury (21). Dried tissue samples were
extracted with 5 volumes of 95% ethanol. The extract
was centrifuged at 5,000 x g for ten minutes in a re-
frigerated centrifuge and the supernatant dried in vacuo
in the cold. The ethanol extract was next treated with
5 volumes of acetone, centrifuged, and similarly dried.
The acetone residue thus obtained was re-extracted with
ether, and the supernatant, after being separated from the
precipitate, was dried by evaporation. After the final ex-
traction, the dried residue was re-suspended in a small volume

(usually 0.5 ml) of distilled water for chromatography
or other analyses.

A second extraction procedure consisted of ex-
tracting the urine successively with methanol, acetone,
and ethyl acetate in a manner similar to that described
above. It, however, revealed no significant qualitative
difference in the final results. Therefore, this second
system was employed mainly to supplement the one used
by Esplin and Woodbury (21) when tissue samples were
first handled.

In view of the report that PTZ readily entrains
in solvent vapours and sublimes which lead to considerable
loss during extraction (21), all extraction procedures
were performed in the cold, and in no case were the sam-
ples spread over a large surface.

III. PAPER CHROMATOGRAPHIC SEPARATION AND
      IDENTIFICATION OF TRITIUM-LABELLED
      METABOLITE

For separation and identification of H^3-labelled
constituents in the urine of individual rats, the dried
extract was suspended in small volume (usually 0.5 ml)
of distilled water. Aliquots of each sample were trans-
ferred to strips of Whatman No. 1 or No. 5 filter papers.

The chromatograms were developed by descending chro-
matography in water-saturated isobutanol or in dis-
tilled water (21). The R_F of pure PTZ on Whatman
No. 1 paper, labelled or non-labelled, is 0.80 in
the former system and 0.89 in the latter system res-
pectively. On Whatman No. 5 paper in isobutanol-
water, R_F of pure PTZ is 0.70. Exogenous PTZ added
to biological materials revealed no apparent variation
in R_F values obtained in both systems. PTZ on chroma-
tograms can be detected by a spray of acidic iodine
and potassium iodide solution (Lugol solution); it
appears light yellowish when stained with this solution.

With H^3-PTZ, the chromatograms were either radio-
scanned by passing the strips through a Packard radio-
chromatogram scanner (Model 7200), or cut into 0.5 x 2.5
cm strips and counted in a Packard Tri-Carb liquid
scintillation spectrometer (Model 3003). The phosphor
used was made up of 5 gms of 2,5-diphenyoxazole (PPO)
and 0.3 gms of p-Bis 2-(5-phenyloxazolyl)-benzene (POPOP)
in one liter of toluene. Maximum counting efficiency for
tritium in the Tri-Carb spectrometer was 35%, and correc-
tions were made for self absorption.

IV. COLORIMETRIC DETERMINATION OF PENTYLENETETRAZOL

The procedure for colorimetric determination of PTZ

by Daoust (150), was adopted to determine the PTZ content
in biological extracts, and to determine the efficiency of
the extraction procedure. This method is applicable to
samples containing 2 - 10 mg of PTZ.

V.   DISTRIBUTION OF TRITIUM IN THE TISSUES

Dissection of the brain tissue for this study was
according to Glowinski et al. (151). Liver tissue samples
were taken at random.

Rats were given a single dose of PTZ, equivalent
to 60 mg/kg body weight (i.p. and containing 65 μc H^3-PTZ
in each dose). At specified times after the injection,
the animals were sacrificed. Blood was collected in 0.2%
sodium oxalate in saline, then centrifuged to separate
the plasma from the cells. The brain and the liver were
next removed. For each sample approximately 50 mg (wet
weight) of tissue specimen was used.

After centrifugation, 0.1 ml of plasma was added
to 1 ml of 1 N Hyamine hydroxide solution (Packard Co.)
in a 5-dram Wheaton vial. It was then capped and heated
at 70° in a shaking water bath for 30 minutes to solu-
bilize the plasma protein. After heating, the vial was
cooled before introducing 0.5 ml of ethanol and 15 ml
of toluene scintillator to produce a homogeneous solution.

It was then counted for a sufficient period to give less than 2% counting error. Quenching effect was corrected by automatic standardization, and background was subtracted by the use of blank samples.

Brain and liver tissue samples were similarly prepared to analyse for radioactivity by converting into a liquid form with the Hyamine solution. The treatment of the tissue specimens was the same as for plasma with the following exceptions. Two ml of 1 N Hyamine solution was used for both brain and liver, and 1 ml of absolute methanol was added to the vial after solubilizing the tissue. The heating time at $70^{\circ}C$ was three hours for the brain, and 7 hours for liver before complete solution of the tissue was effected. 15 ml of the toluene-phosphor solution was then added for counting. The remaining tissues were pooled and freeze-dried. The dried tissues were homogenized in 5 volumes of ethanol and then treated as for extracting urine as described previously. Chromatograms were prepared containing aliquots of these extracts.

VI.   PERFUSION OF ISOLATED RAT LIVER

A.   Preparation of liver for perfusion

The technique used for perfusion of the liver was as described by Miller et al (152). It involves isolation

of the liver, and perfusion under optimum conditions
in an appropriate perfusion apparatus.

With the rat under nembutal anaesthesia, an abdo-
minal incision was made to provide maximal exposure.
The duodenal ligaments together with the bile duct were
tied off. Strings were attached to the inferior vena
cava. After tying the portal tributary, the portal
vein was rapidly cannulated. Then the ligature on the
inferior vena cava was tightened. Finally, the superior
vena cava in the thorax was also cannulated. The liver
was then removed from the rat, and placed on a platform
with the cannula protruding through a hole at the base
of the platform. The cannula in the portal vein was
now in position for connection with the oxygenated blood
supply. The total time of operation procedure was
usually within 10 to 15 minutes. The liver was covered
with moist cheesecloth to avoid drying during the per-
fusion.

B. Perfusion Procedure

Approximately 75 ml of blood was collected from
8 - 10 rats killed by decapitation under ether anaesthesia
and the blood flowed into citrate-dextrose-solution (CDS).
Twenty ml of CDS solution was used per 100 ml of blood.

Throughout the perfusion of the liver, the blood was oxygenated and humidified by passing through it a stream of gas composed of 95% oxygen and 5% CO_2, which had first been bubbled through a solution of physiological saline. The perfusion apparatus was kept in a cabinet at 37° C and humidity of approximately 100%. Blood entered the liver through the portal vein and left via the superior vena cava at a flow rate of 7 - 10 ml per minute. The drug to be perfused was first dissolved in 1 ml of saline, and then added to the main blood reservoir, and mixed thoroughly. The starting time of perfusion was reckoned as the time at which the drug was added. Aliquots of blood specimens were withdrawn from the reservoir at stated intervals, but a minimum of 60 ml of blood was always maintained in the reservoir for perfusion. A liver thus prepared can be perfused up to 5 hours.

C. Treatment of Perfusate and the Perfused Liver

Aliquots of blood samples removed during the course of perfusion, as well as the total whole blood at the end of the experiment were centrifuged at 2,000 x g for 15 minutes in the cold to separate the cells from the plasma. The plasma was immediately lyophilized, and extracted as described for urine samples.

The erythrocytes of these blood samples were also freeze-dried, and the dried residue was treated similarly as described for other tissues. Analysis of this fraction showed there was no qualitative difference in the results between plasma and erythrocytes, and since the latter was difficult to handle, subsequent analysis was done on plasma only.

Following perfusion, the liver was cut into pieces, blotted with filter paper, and freeze-dried. The dried liver was then powdered, and extracted as described for plasma.

VII. INCUBATION OF H^3-PENTYLENETETRAZOL WITH LIVER HOMOGENATE

A. Preparation of Liver Homogenate

Rats were stunned and decapitated. The liver was removed from the animals, blotted with filter paper, and immediately homogenized in the cold in two volumes of chilled 0.2 M phosphate buffer, pH 7.4. Fresh preparations were used in each experiment.

B. Incubation with H^3-Pentylenetetrazol

Into each Erlenmeyer flask which contained 100 μmoles nicotinamide, 75 μmoles $MgCl_2$, 0.2 μmoles TPN, and 5 μmoles of PTZ (containing 145 mμmoles of H^3- PTZ,

IX. AN ATTEMPT TO ISOLATE THE H^3-PENTYLENETET-RAZOL METABOLITE FROM URINE OF THE RAT ON XAD-2 COLUMN

Each of five rats was given PTZ (18 mg, containing 13 μc of H^3-PTZ) intraperitoneally in two injections, and urine was collected as described earlier. The urine samples were pooled and filtered through sintered glass funnel to remove solid particles. The material retained on the funnel was washed with a minimum volume of water. The urine and washings were combined, and processed on an Amberlite XAD-2 (Rohm and Haas Co., Philadelphia) column which had a bed volume of 1 inch by 12 inches. The XAD-2 resin was packed in the column in distilled water, and washed with several volumes of methanol. Finally, large volumes of distilled water were passed through the column again and it was ready for use.

X. INCUBATION OF TRITIATED-PENTYLENETETRAZOL WITH CITRATED WHOLE BLOOD OF THE RAT

Approximately 10 ml of blood obtained from each rat by heart puncture was collected in citrate-dextrose-solution (CDS). Twenty ml of CDS solution was used per 100 ml of blood. The blood thus obtained was incubated with 0.04 mg H^3-PTZ (26 μc) at 37° C. At intervals of 60 minutes, aliquots (2ml) of the blood were removed from the incubation mixture. The blood was immediately

centrifuged in the cold at 2,000 x g for 15 minutes
to separate the cells from the plasma. The plasma
thus obtained was subjected to electrophoresis (2.3 V/cm)
on strips of Whatman No. 3 MM paper in barbital buffer
(pH 8.6) for 3, 6, 9, 12 and 15 hours in a Beckman
Electrophoresis cell (Durrum type, Model R - series D).
Radioactivity was located by scanning the strips in a
radiochromatogram scanner described earlier (p. 27).

EXPERIMENTAL
CHAPTER I
URINARY EXCRETION OF PENTYLENETETRAZOL METABOLITE IN THE RAT

A. CHROMATOGRAPHIC SEPARATION OF H^3-PENTYLENETETRAZOL
   METABOLITE IN THE URINE OF THE RAT

Cage collections of urine were made from rats each receiving a total of 18 mg of pentylenetetrazol which contained 0.04 mg H^3-PTZ (26 μc). Urine was collected from each rat for 24 - 48 hours after the last injection and processed individually. Urinary excretion of tritium following i.p. injections of H^3-PTZ is shown in Figure 2. Approximately 70% of the radioactivity had been excreted after the first 24 hours. Therefore, in subsequent qualitative experiments, urine was collected for 24 hours only.

After collection, the urine samples were freeze-dried immediately to avoid   losses due to entrainment and sublimation of metrazol, as indicated by Esplin and Woodbury (21), which may lead to contradictory results. Extraction of PTZ and its metabolite was done directly on the dried samples by treating them serially with 95% ethanol, acetone and ether, in the cold. Photometric determination shows approximately 70% of the drug can be recovered by this extraction procedure.

FIGURE 2

Cumulative Urinary Excretion of Total Tritium
Radioactivity after i.p. Administration of 26 μc
(0.04 mg) of H^3-PTZ and 60 mg/kg b.w. of Carrier
PTZ in Each Rat.

2
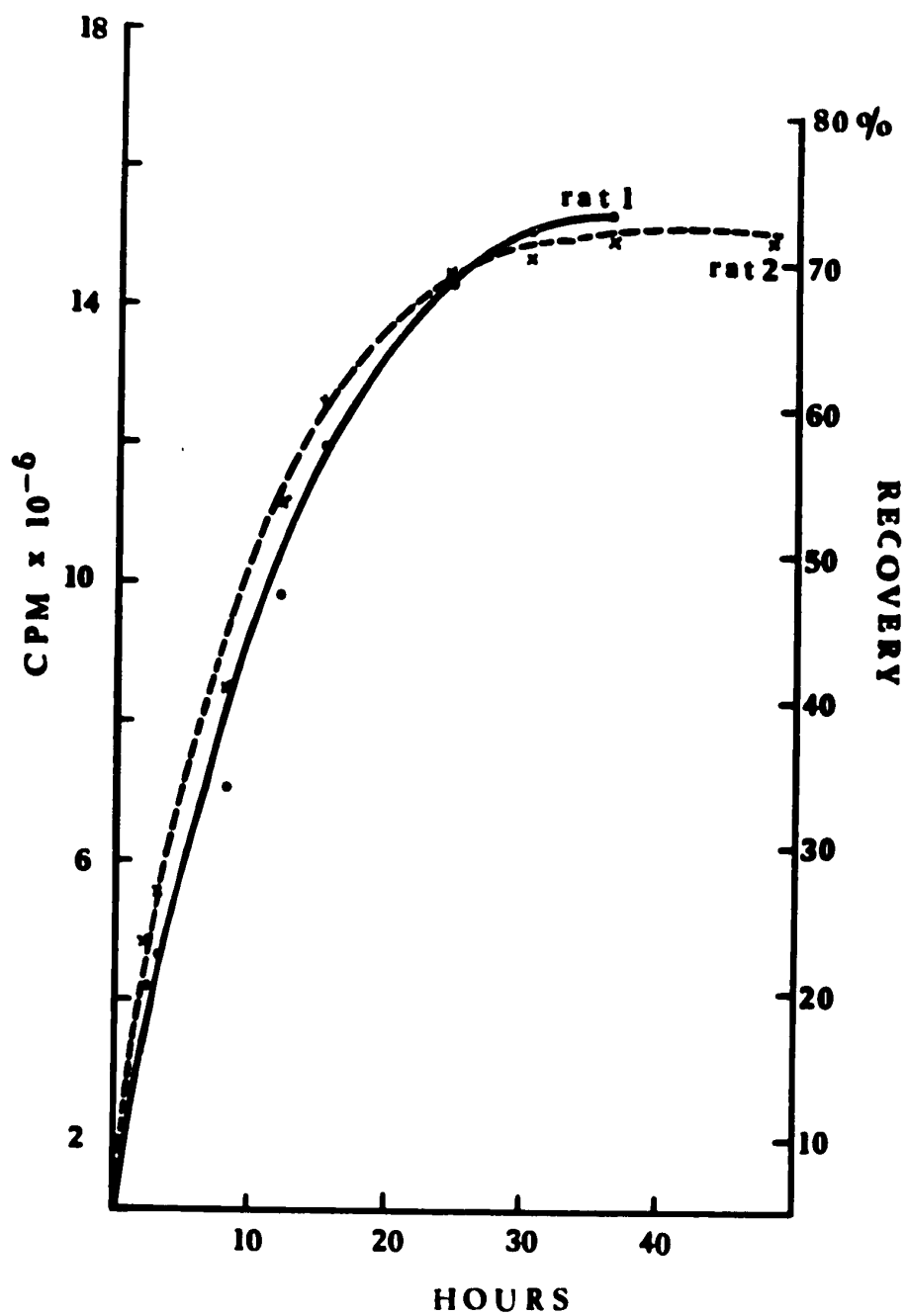
.Figure 3 shows the location of radioactivity on a paper chromatogram of the urine extract. This chromatogram was developed in water-saturated isobutanol on Whatman No. 1 paper. There are two major radioactive components in the urine of the rats receiving the H^3-PTZ. These two substances have R_F 0.64 (0.60 - 0.68) for Peak I and 0.78 (0.74 - 0.82) for Peak II. On Whatman No. 5 paper and the same solvent system, the R_F of these two substances are 0.60 (0.58 - 0.62) and 0.71 (0.68 - 0.74) for Peaks I and II respectively. Pure H^3-PTZ has R_F 0.80 in the isobutanol-water system (Fig. 3a). A minor component near Peak II was also observed. No R_F value was assigned to this component. Identical results were obtained from direct chromatography of whole rat urine shown later in Figure 7 (p. 50). However, when the same urine extract (Figure 3) or the whole rat urine (Figure 7) was chromatographed according to Esplin and Woodbury (21) in the water system, only one radioactive spot was found and this had R_F 0.84 (0.77 - 0.91). An example of such a radiochromatogram is shown in Figure 4; the R_F of pure H^3-PTZ in this system is 0.89.

B. TWO-DIMENSION PAPER CHROMATOGRAPHY OF URINE EXTRACT

Aliquots of urine extract from rats treated with PTZ (containing H^3-PTZ) were spotted at one corner on sheets of Whatman No. 1 paper. These chromatograms were developed two dimensionally as follows:

FIGURE 3

Radiogram of Extract of Rat Urine Collected after i.p. Administration of H^3-PTZ. The chromatogram was developed in water-saturated iso-butanol on Whatman No. 1 paper.

Full scale deflection: 3×10^2 cpm
Time constant: 30 sec.
Chart speed: 0.1 cm/min.

PEAK I

PEAK II
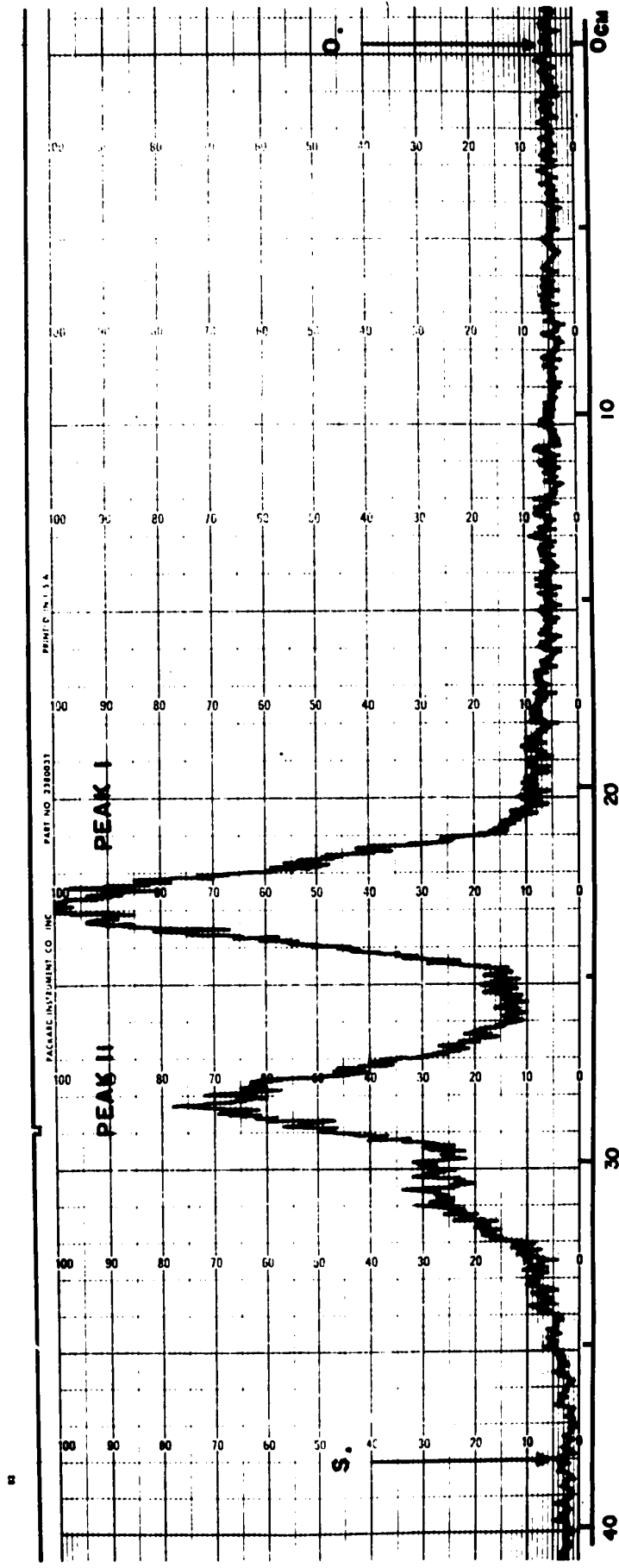
FIGURE 3a

Radio-scanning of a Paper Chromatogram of Standard H^3-PTZ Developed in Isobutanol-water on Whatman No. 1 Paper.

Full scale deflection: 3 x 10^2 cpm
Time constant: 30 sec.
Chart speed: 0.2 cm/ min.

39c
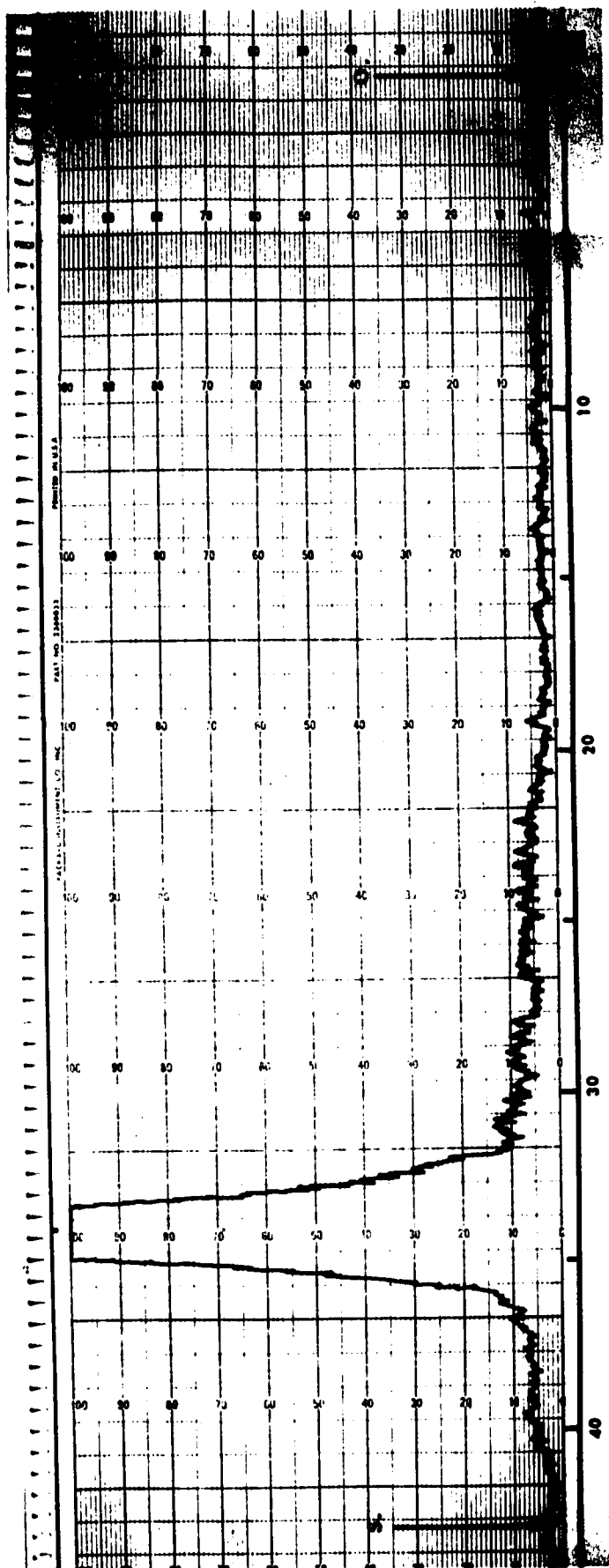
FIGURE 4

Radiogram of Extract of Rat Urine Collected
after i.p. Administration of H^3-PTZ. The chro-
matogram was developed in distilled water on
Whatman No. 1 paper.

Full scale deflection: 3×10^2 cpm
Time constant: 30 sec.
Chart speed: 0.1 cm/min.
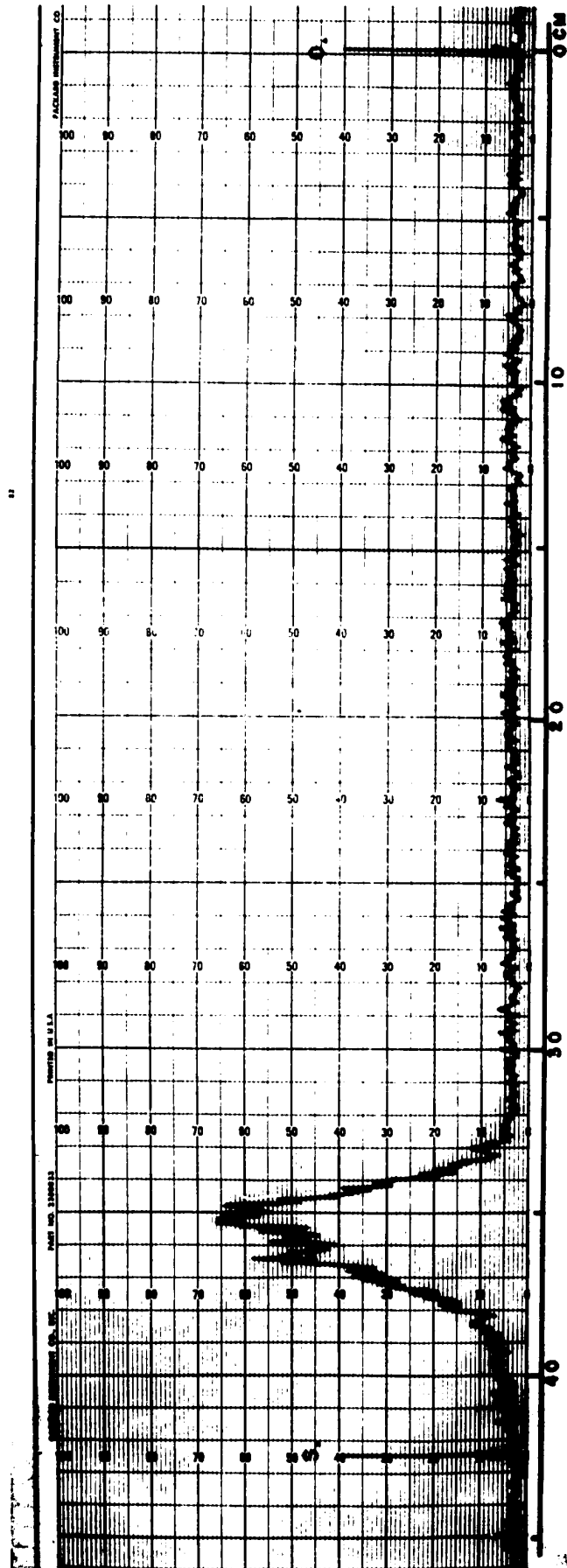
40a

A. The chromatogram was first developed in water. After drying, it was rechromatographed in water-saturated isobutanol at right angles to the first system. The band with R_F 0.77 - 0.91 in the first (water) system was then cut out for scanning of radioactivity.

B. The chromatogram was prepared as above, except the systems used for development were: first dimension, water-saturated isobutanol; second dimension, water. After drying, the bands corresponding to R_F 0.74 - 0.82 and 0.60 - 0.68 in the first (isobutanol - water) system were cut out for locating the radioactive components.

The results obtained in these experiments are shown in Figure 5. It can be seen that the urine extract, first developed in water (Figure 5,a) which cannot separate the two tritium-containing substances, is resolved into two radio-components in isobutanol-water. The separation here is not as good as that previously shown in Figure 3. This may be attributed to the relatively short distance of development in the second direction.

When the extract, first separated into two components in isobutanol-water, as shown in Figure 5,b, was next developed in the water system, the R_F of both substances was almost unity.

FIGURE 5

Schematic Representations of Two-Dimension Paper Chromatograms.

a. First Dimension: Water
    Second Dimension: Water-saturated Isobutanol

b. First Dimension: Water-saturated Isobutanol
    Second Dimension: Water

Solvent Front

Solvent Front

Solvent Front

Solvent Front

DIMENSION 2 R$_f$

DIMENSION 2 R$_f$

DIMENSION 1 R$_f$

DIMENSION 1 R$_f$

Origin

Origin

0.0

0.5

1.0

0.0

0.5

1.0

a.

b.
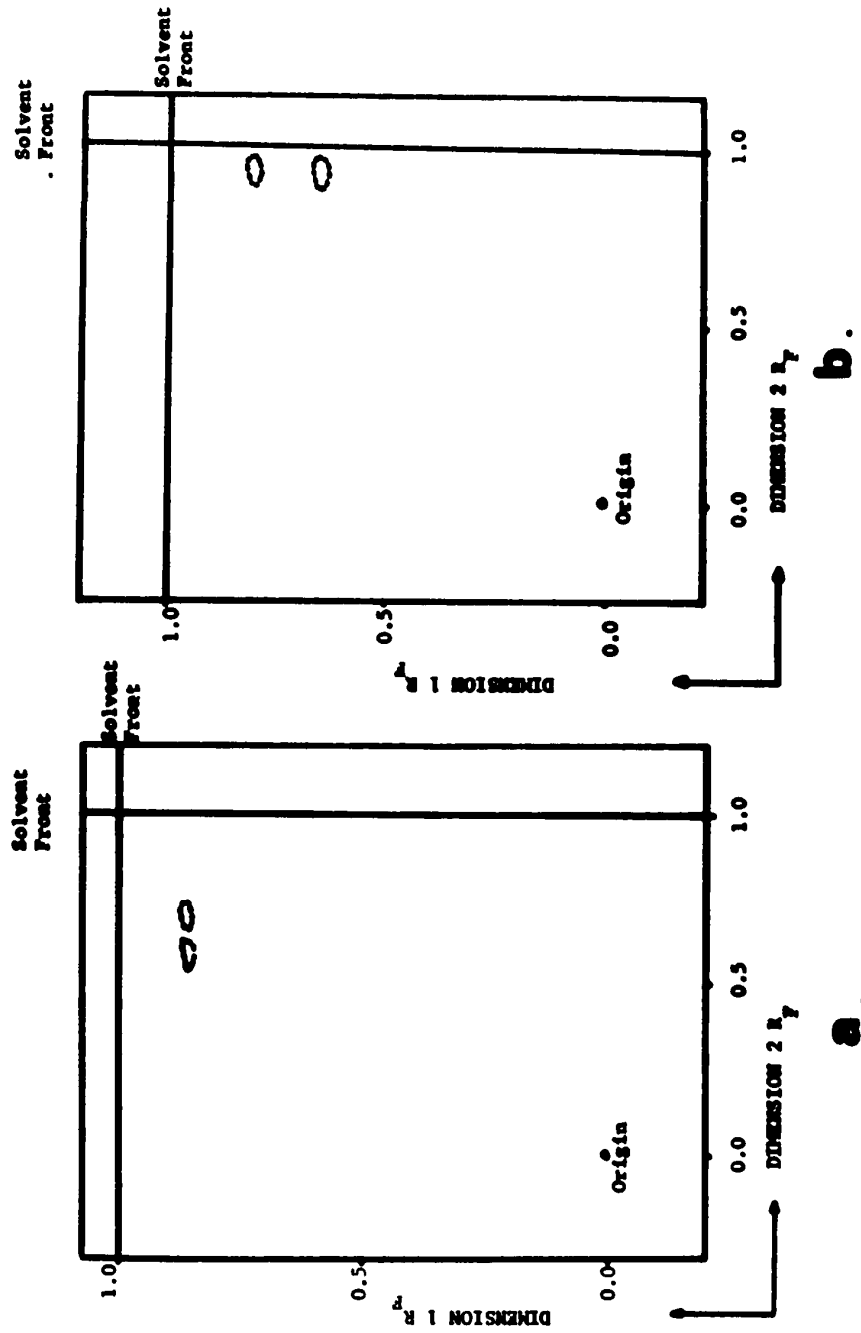
C. CHROMATOGRAPHIC IDENTIFICATION OF PENTYLENETETRAZOL IN RAT URINE BY ISOTOPE DILUTION

Further identification of the spot at R_F 0.78 (Figure 3) as PTZ was done by isotope dilution. A known aliquot of aqueous solution of H^3-PTZ was added to a known volume of extract containing labelled PTZ and the metabolite. Aliquots (20 μl) of this mixture were then chromatographed in both the isobutanol-water and the water systems. Also, the urine extract was mixed with non-radioactive PTZ and analysed chromatographically.

After development, the chromatogram which contained the radioactive constituents was cut into 0.5 x 2.5 cm strips, and counted in the scintillation spectrometer. It can be seen from Table 1 and Figure 6 that compared with samples devoid of exogenous H^3-PTZ, the radioactivity at R_F 0.78 was greatly increased whereas that at R_F 0.64 was correspondingly diminished. With non-radioactive PTZ, only one band, coloured yellow, was observed at R_F 0.79 when the chromatogram was sprayed with Lugol solution. Similarly, radiochromatogram developed in the water system showed increased activity at R_F 0.84. With non-radioactive PTZ, a yellow band was seen at R_F 0.87. It seems therefore that Peak II, with R_F 0.78 on chromatogram developed in isobutanol-water,

CHROMATOGRAPHIC DISTRIBUTION OF RADIOACTIVITY BETWEEN
TRITIUM-CONTAINING CONSTITUENTS* FROM URINE EXTRACT
BEFORE AND AFTER ADDITION OF LABELLED PTZ AND AFTER
ACID HYDROLYSIS OF THE URINE EXTRACT

SAMPLES	EXPERIMENT**	CHROMATOGRAPHY SYSTEMS		
		ISOBUTANOL - WATER		WATER
		PERCENT RADIOACTIVITY*** OF CONSTITUENTS AT		
		R_F 0.64 (Peak I-METABOLITE)	R_F 0.78 (Peak II-PTZ)	R_F 0.84
20 μl urine extract before addition of H^3-PTZ	1	52%	48%	94%
	2	55%	45%	93%
20 μl urine extract after addition of H^3-PTZ	1	19%	81%	90%
	2	25%	75%	92%
After hydrolysis	1	0.15%	99.85%	91%
	2	0%	100%	93%

*   Separation of constituents was achieved by paper chromatography in respective systems as noted on Whatman No. 1 paper.

**   Each experiment consists of pooled urine samples from two animals. Experiments shown here are typical of four runs all showing the same pattern.

*** Percentage was reckoned as that of total activity in Peak I and Peak II. Last column represents the fraction of the activity spotted on the chromatogram.

FIGURE 6

Radio-scanning of a Paper Chromatogram of
Urinary Extract of H^3-PTZ-Treated Rats with
Exogenous H^3-PTZ Added to the Extract Prior
to Chromatography in Isobutanol-water.

Full scale deflection: 1 x 10^3 cpm
Time constant: 30 sec.
Chart speed: 0.5 cm/min.
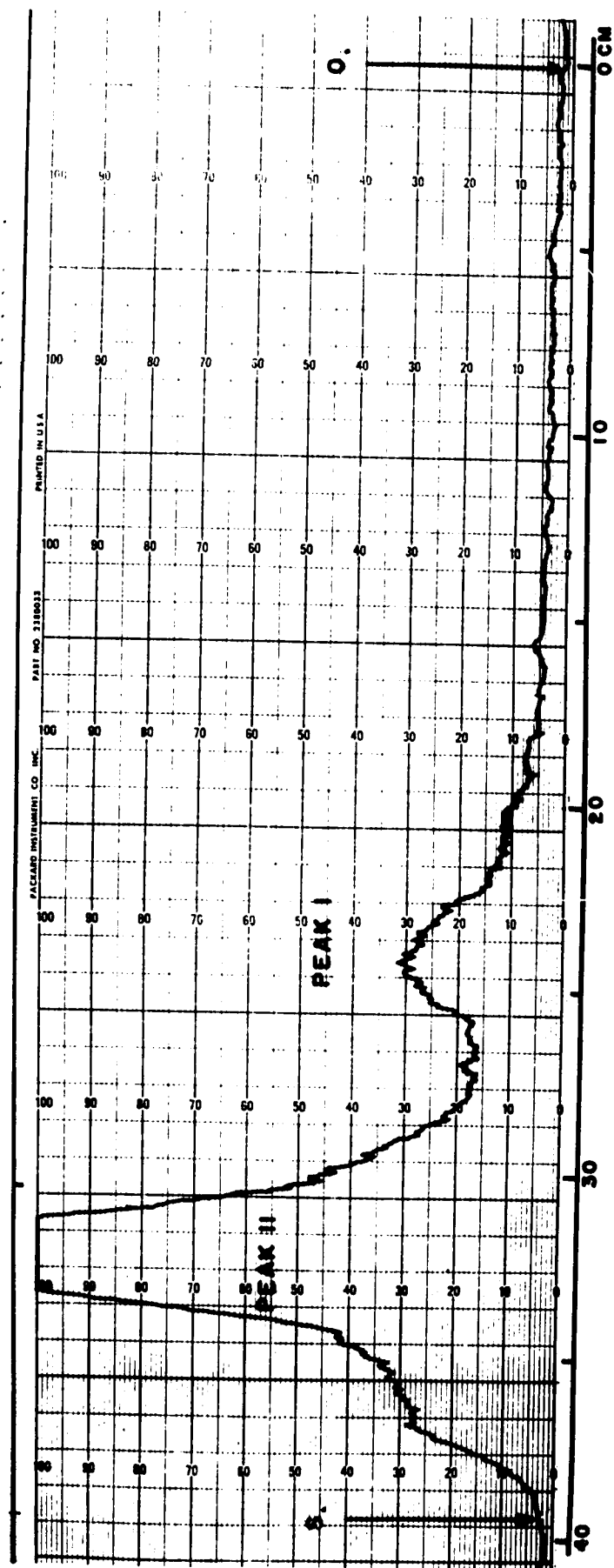
PEAK I

PEAK II

is not distinguishable from pentylenetetrazol.

The minor radioactive component of Peak II observed in Figure 3 was also present in the chromatogram as shown in Figure 6. This may be a minor metabolite or a precursor of the major metabolite in Peak I. However, when the isotopically diluted urinary extract was chromatographed in isobutanol-water, the activity levels in both Peak II and the minor component were increased. The level of radioactivity in Peak I was correspondingly decreased. It thus seems that the radioactivity level of the minor component is influenced by that in Peak II. This seems to indicate that this minor component may be due to tailing of the material on the chromatogram. This minor component was not observed consistently in all chromatograms developed in iso-butanol-water (compare Figures 3, 6 and 7 with Figures 11 and 13). Therefore its identity was not further investigated in the present study.

D. CONTROL EXPERIMENT

The possibility of tritium exchange or degradation of the drug during collection and/or extraction of urine was checked in the following manner.

H^3-PTZ was added to control urine, and incubated at 37° C for 24 hours. The whole sample was then subjected to the same treatment described above for the experimentals. These studies showed only one radioactive substance which was identified as PTZ when chromatographed in the two systems mentioned above.

E. CHEMICAL HYDROLYSIS OF URINE EXTRACT

HCl (1N) was added to an aqueous solution of the urinary extract. This mixture was then heated in a boiling water bath for 30 minutes. Rechromatography of this hydrolysate in isobutanol-water showed that the material of R_F 0.64 from the urine extract was hydrolysable by the acid. After hydrolysis, this material (R_F 0.64 from urinary extract) was chromatographically inseparable from PTZ. The distribution of radioactivity between the two tritiated substances after hydrolysis is shown in Table I.

It is apparent from these results that two major radioactive compounds can be detected in the urine of H^3-PTZ-treated rats. These two substances

behave differently on paper chromatograms developed in isobutanol-water system, but have the same mobility in the water system.

The percent distribution of radioactivity between the two radioactive compounds in the 24 hour urine sample, was obtained from the sum of radioactivity in the two peaks on chromatograms developed in the isobutanol-water system. Approximately 55 - 60% of the radioactivity appeared in Peak I (Metabolite) and 40 - 45% in Peak II (PTZ).

F. **INFLUENCE OF VARIOUS ROUTES OF ADMINISTRATION OF THE DRUG ON ITS METABOLIC FATE**

Bastian et al. (153) reported that the differential effects of drugs on the PTZ threshold, the extensor tonus and death in the mouse, readily distinguished by the intravenous method, were sometimes obscured when PTZ was administered subcutaneously. Little and Conrad (154) observed that the "clonic dose 50" (CnD_{50}) and "tonic dose 50" (TnD_{50}) of PTZ in mice were significantly higher on oral than on subcutaneous administration. The authors attributed this difference to a faster rate of absorption by the oral route coupled with a greater percent inactivation by the

liver. It is pertinent therefore, to study the effect of various routes of administration on the fate of PTZ in the rat. The drug (containing 26 μc H^3-PTZ) was given to the animals subcutaneously (75 mg/kg b.w.), intra-peritoneally (60 mg/kg b.w.) and intravenously (45 mg/kg b.w.). Urine was collected in each case and analysed as before. The results are shown in Table II.

TABLE II

INFLUENCE OF VARIOUS ROUTES OF ADMINISTRATION OF H^3-PTZ
ON THE DISTRIBUTION OF RADIOACTIVITY BETWEEN PTZ AND ITS
METABOLITE IN URINE

ROUTE OF ADMINISTRATION*	PERCENT** RADIOACTIVITY OF SUBSTANCES AT	
	R_F 0.64*** (Peak I-Metabolite)	R_F 0.78*** (Peak II-PTZ)
Intraperitoneal (60 mg/kg)	58%	42%
Intravenous (45 mg/kg)	61%	39%
Subcutaneous (75 mg/kg)	60%	40%

*   Three experimental runs were done on each route of administration all showing similar pattern.

** Percentage was calculated as that of total activity in Peak I and Peak II.

*** Chromatogram was developed in water-saturated isobutanol on Whatman No. 1 paper, and 20 μl of urine extract was spotted on each chromatogram.

It can be seen that there is no qualitative difference
in the metabolism of the drug due to various routes of
administration. Two major labelled compounds are present
in the urine in each case, and the percent metabolite formed
was approximately the same regardless of the route of
administration.

G. RATE OF EXCRETION OF TRITIUM IN THE URINE OF RATS
    DOSED WITH H^3-PTZ

Each of six rats was given 60 mg/kg b.w. of PTZ via
the i.p. route in one injection (containing 26 μc of H^3-PTZ).
Cage collections of urine were made at intervals as indi-
cated in Figure 8 up to 48 hours. The volumes of urine
excreted were estimated by weighing the containers before
and after collection, assuming a density of 1 gm per ml.
Radioactivity was then immediately assayed by adding ali-
quots (usually 10 μl) of urine to 4 ml of ethanol and 10 ml
of toluene-phosphor (containing 0.4% ppo, and 0.01% popop)
in the counting vials (155). Corrections for quenching
were made by automatic standardization in the spectrometer.
Aliquots of the same urine samples were transferred to
Whatman No. 1 paper for chromatography in water-saturated
isobutanol. The resultant chromatograms, of which Figure 7
is typical, showed that the same two radioactive peaks were
present in all samples collected at various time intervals.

FIGURE 7

Radiogram of Whole Rat Urine Collected Three
Hours after i.p. Administration of H^3-PTZ. The
chromatogram was developed in water-saturated
isobutanol on Whatman No. 1 paper.

Full scale deflection: 3×10^2 cpm
Time constant: 30 sec.
Chart speed: 0.5 cm/min.
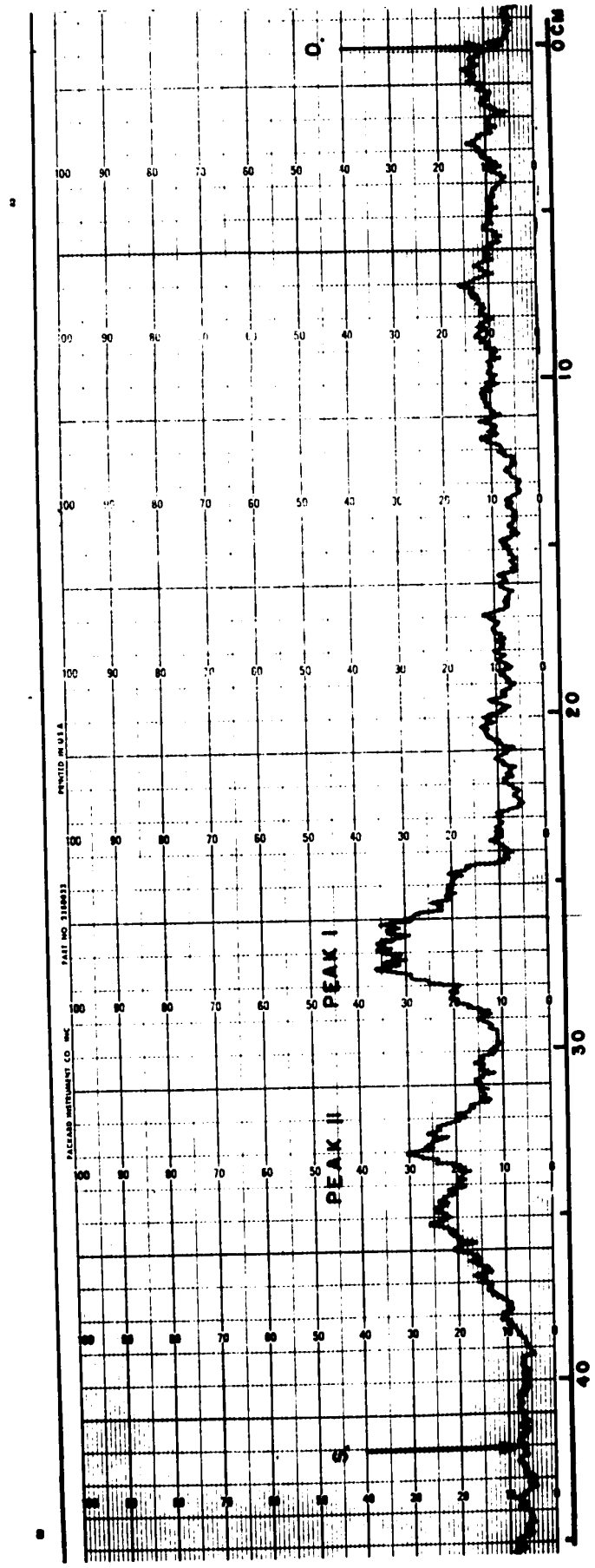
50a

The results from two of the animals used in this experiment are shown in Fig. 8. It is evident that the radioactive material was rapidly excreted in the urine. The peak excretion was two hours after administration of the drug, at the rate of approximately 2.40×10^{-6} cpm per hour in each of two **rats** observed. Thereafter, the rate of tritium excretion diminished sharply. At about the fifth hour, the rate of excretion increased again until the fifteenth hour which was followed by a steep decline. The reason for this sudden surge of excretion of radioactivity in this period is not known. Considerable amounts of tritium were still being excreted at 36 and 48 hours; at those times the rates were about 4×10^{-4} and 5×10^{-3} cpm per hour respectively. Cumulative percentage of excretion (Fig. 2) showed that approximately 60% of the dose administered was excreted in the first fifteen hours. Only a further 13% was excreted in the next 33 hours.

CHAPTER II

BLOOD AND TISSUE LEVELS OF TRITIUM IN THE RAT AFTER ADMINISTRATION OF H^3-PTZ

A. DISTRIBUTION OF TRITIUM RADIOACTIVITY IN THE TISSUES

Brain and liver tissues were excised from rats dosed with PTZ and H^3-PTZ. Blood was also collected from these animals, and all three tissues were analysed for radioactivity after being solubilized in Hyamine solution and mixed with the toluene-phosphor.

FIGURE 8

Rate of Urinary Excretion of Tritium Radioactivity after i.p. Administration of 26 μc (0.04 mg) of H^3-PTZ and 60 mg/kg b.w. of Non-radioactive Carrier PTZ to Each Rat.
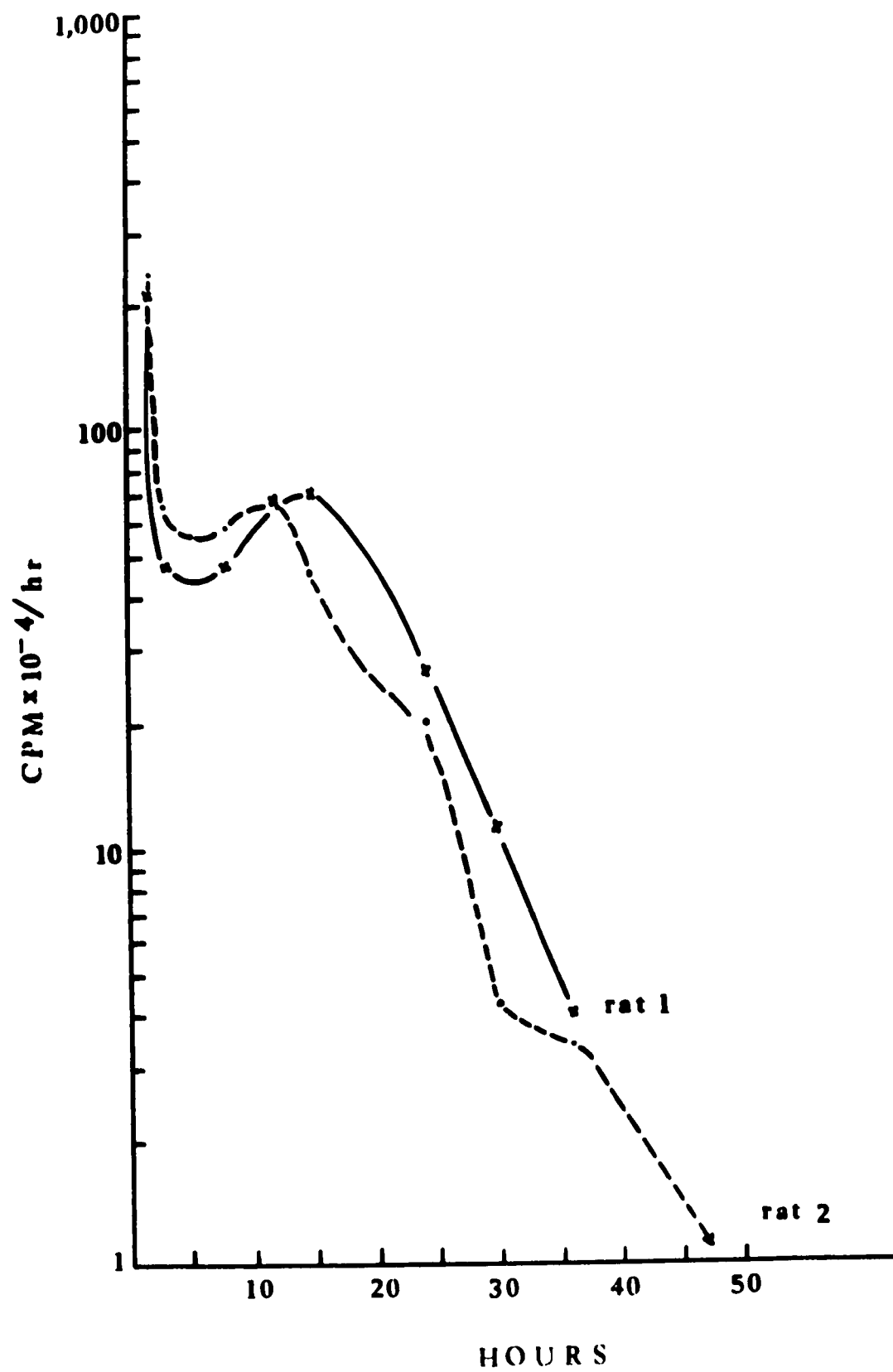
The distribution of radioactivity in four different
regions of the brain at various time intervals is summarized
in Figure 9. The relatively high levels of activity in the
four areas studied were found 30 minutes after injection of
the drug. However, the activity in the brain was rather
stable for three hours. No area of the brain showed
specific affinity for the drug.

Levels of radioactivity in brain, liver and plasma,
at different intervals after administration, are shown in
Figures 10 a and 10 b. The drug was rapidly taken up by
the tissues. One minute after administration all three
tissues had accumulated considerable amounts of radio-
activity. Within the first 30 minutes of the injection
of the drug, the radioactivity in the liver first reached
a peak at three minutes at which time convulsions were
observed in most animals. Two minutes later, both the
brain and plasma attained the maximum levels of radio-
activity.

The decline of radioactivity in the plasma was rather
sharp within the first 8 hours, but later on, it levelled
off. In the liver, as in the brain, the level of radio-
activity was rather steady up to 3 hours. During this
period, approximately 70% of the maximum level was still
present. Evidently, a second injection of a minimum con-

FIGURE 9

Distribution of H^3-PTZ in Different Areas
of the Brain.

C P M x 10^{-3} ± S.D./50 mg TISSUE (WET WEIGHT)

HOURS

CEREBRUM   CEREBELLUM   MIDBRAIN   HINDBRAIN

CEREBRUM   CEREBELLUM   MIDBRAIN   HINDBRAIN

HOURS

C P M x 10⁻³ ± S.D./50 mg TISSUE (WET WEIGHT)
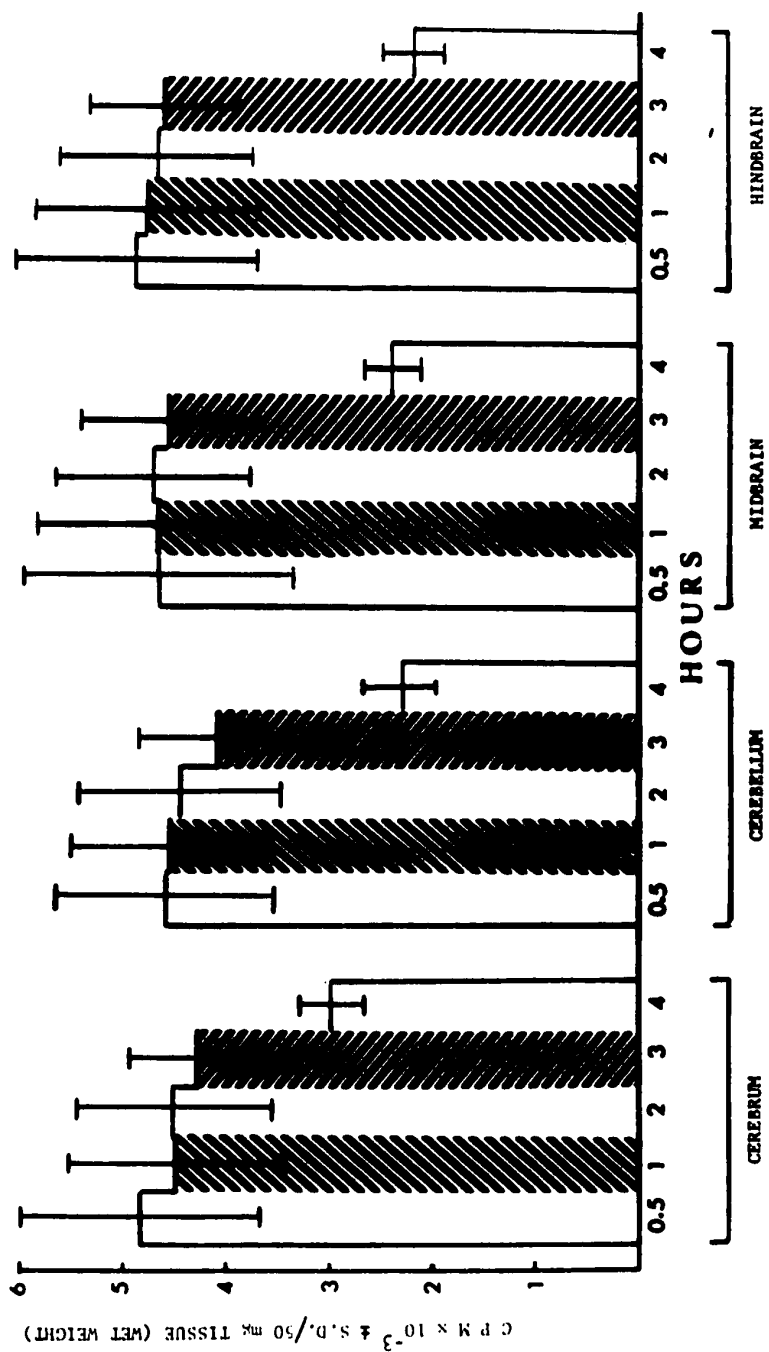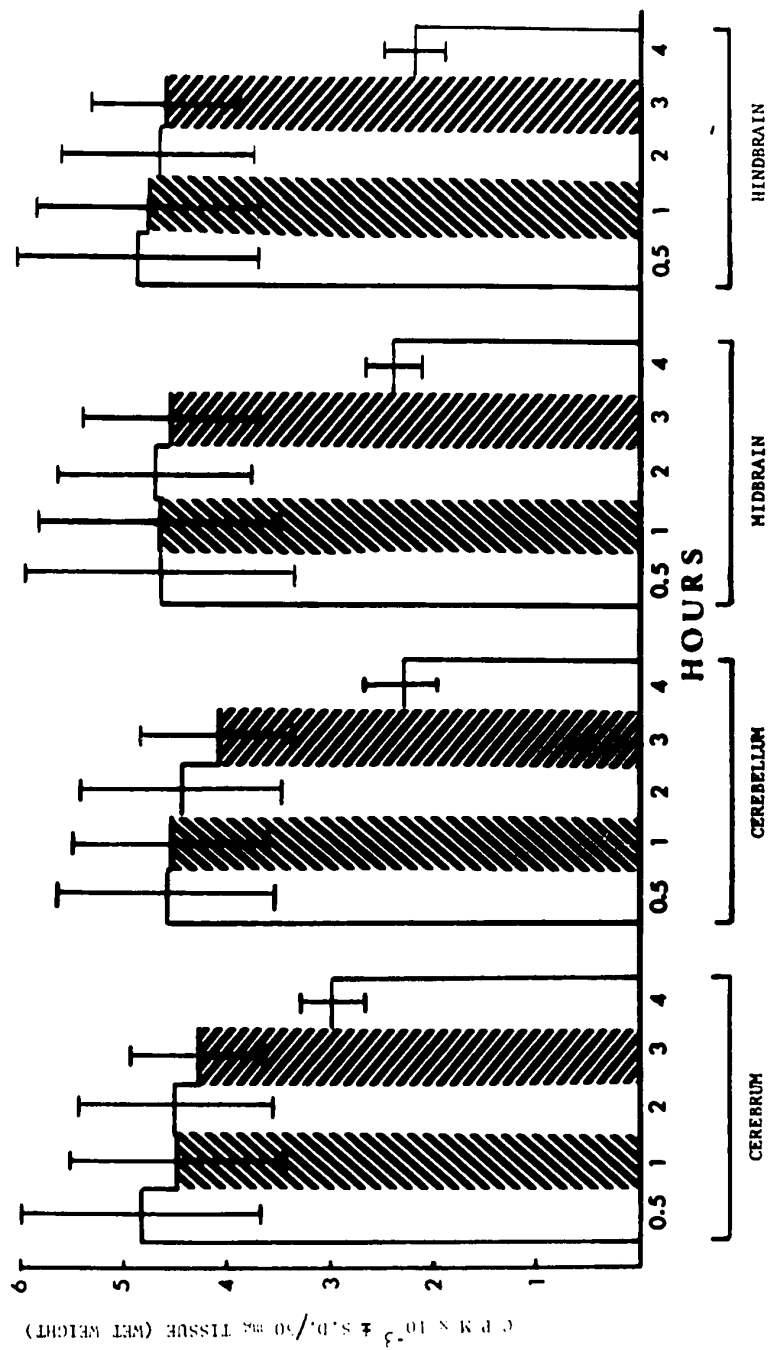
FIGURE 10a

Levels of Tritium in Brain, Liver and Plasma within 30 Minutes after i.p. Administration of H^3-PTZ into the Animals.

Brain and Liver: cpm/50 mg (wet weight)
Plasma: cpm/0.1 ml.

55a

55a
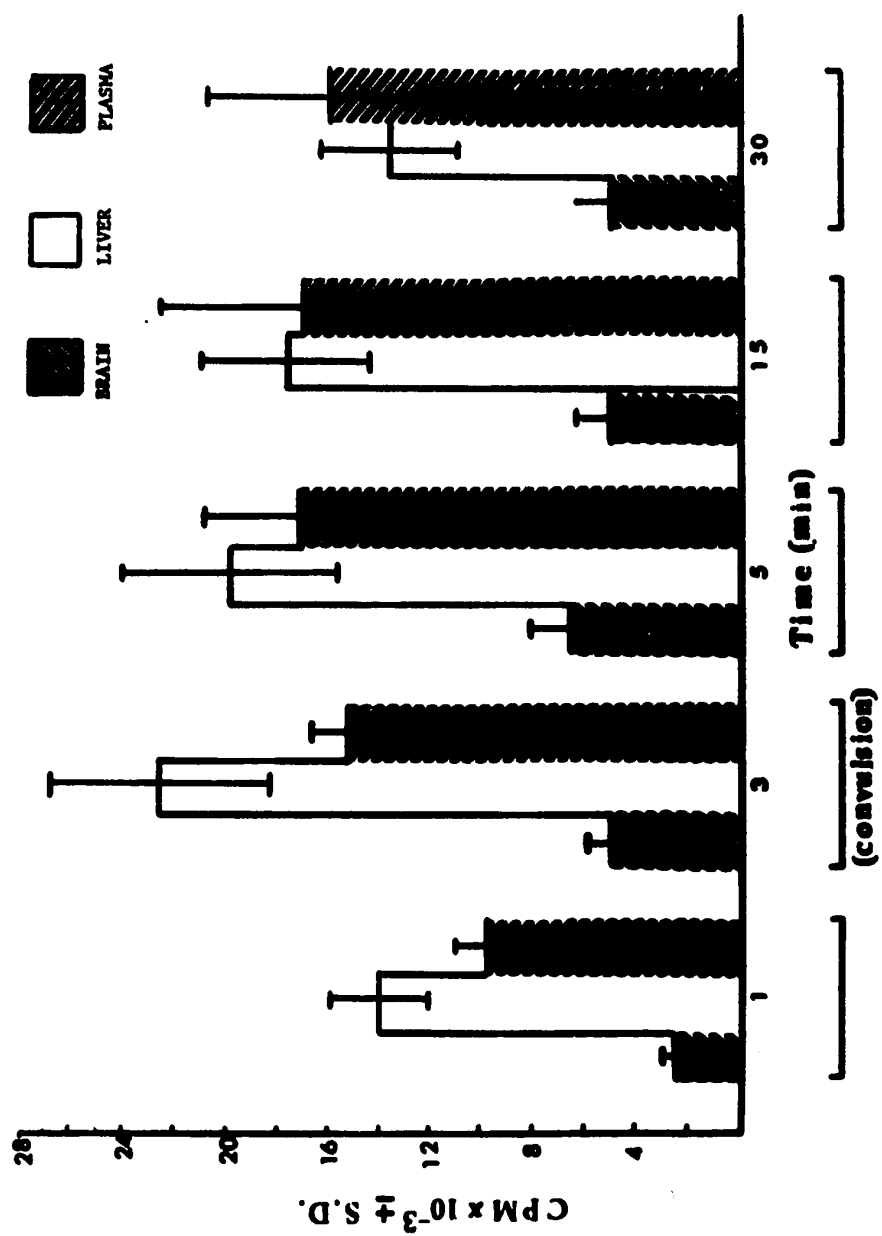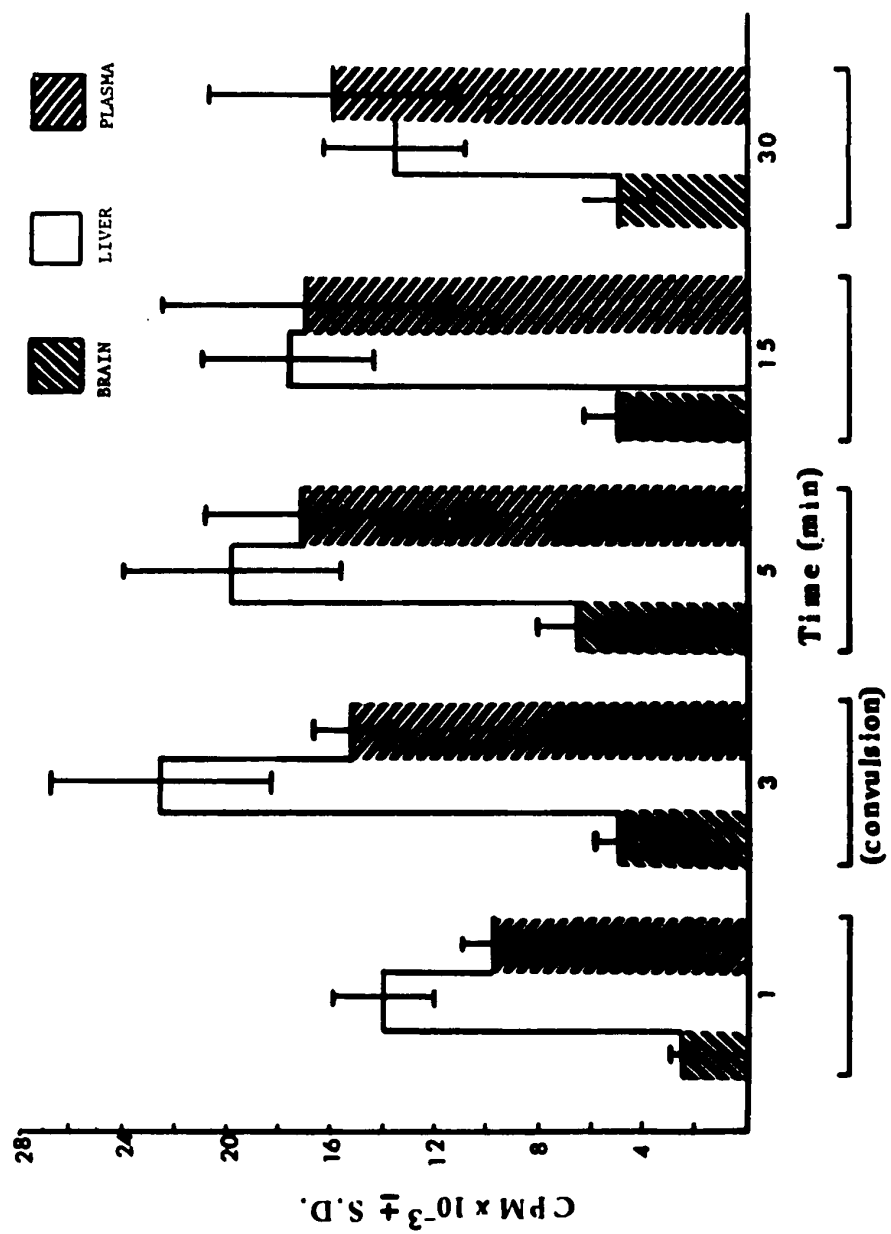
FIGURE 10b

Levels of Tritium in Brain, Liver and Plasma in H^3-PTZ-treated Rats.

Brain and Liver: cpm/50 mg (wet weight)
Plasma: cpm/0.1 ml.

56a

HOURS

CPM x 10⁻³ ± S.D.

BRAIN LIVER PLASMA
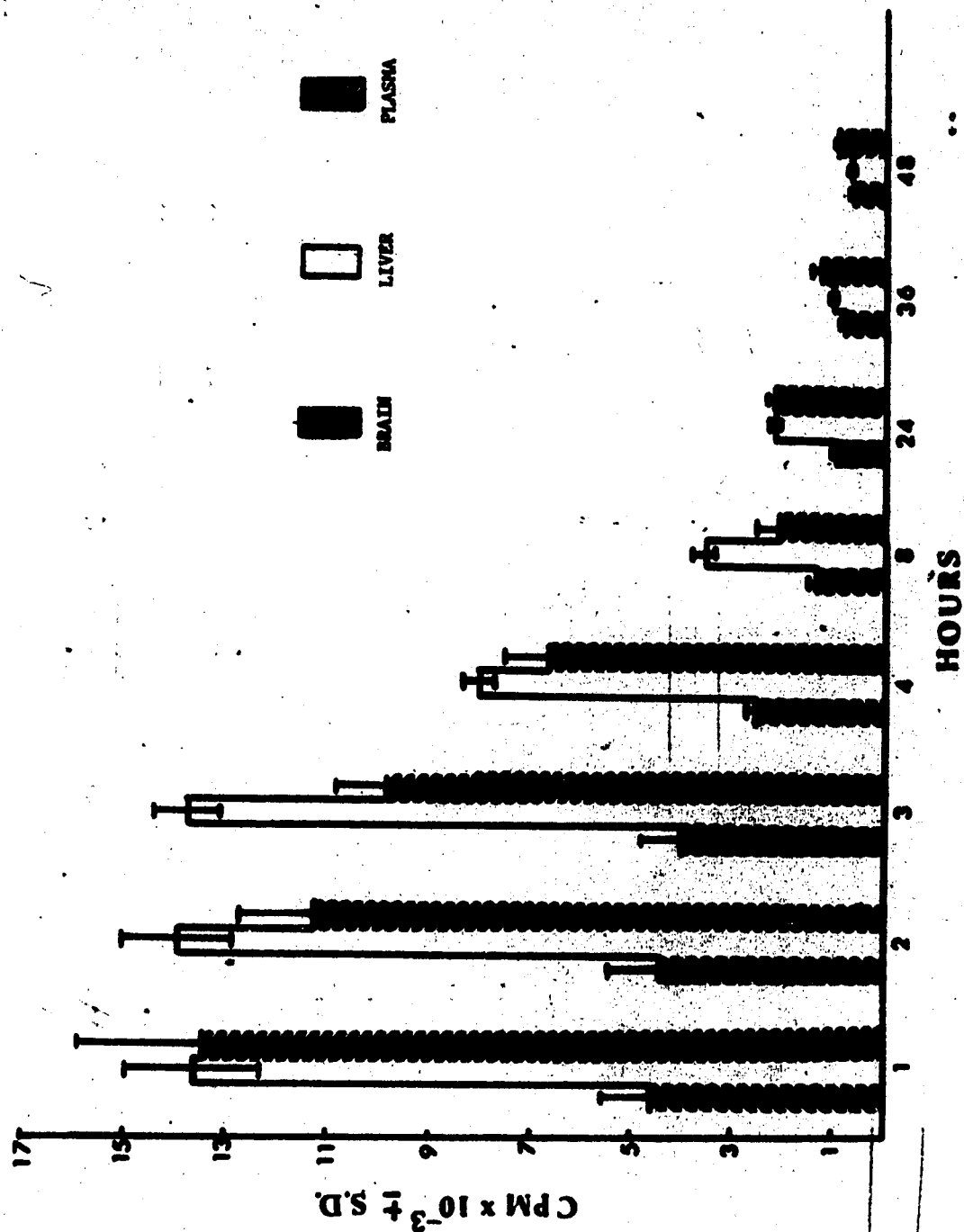
vulsion dosage at this time would have become lethal.
At 36 and 48 hours, sizable quantities of radioactive
material were still present in all three tissues which
likely explains the relatively low tritium in the urine
collected during this time.

B. RESULTS OF ANALYSIS FOR METABOLITE OF PENTYLENETET-
RAZOL IN TISSUES

The radiochromatograms of extracts of pooled brain,
liver and plasma are shown in Figures 11, 12 and 13 res-
pectively. The pools were made up of extracts collected
at each time interval. With brain, only one prominent
radioactive compound was located on chromatograms de-
veloped in isobutanol-water. A large peak was identified
as PTZ by its R_F value (0.80). The metabolite at R_F 0.64,
found in the urine was only barely observable in these
chromatograms. With liver, on the other hand, the same
radio-compound (R_F 0.80) corresponding to PTZ was still
present, but the metabolite (R_F 0.64) was now detectable.
The percent radioactivity of PTZ and its metabolite in
these samples, judging by the total activity spotted on
the chromatogram, was 80 and 20 respectively. The same
results were observed in the brain and liver samples
obtained from animals killed during PTZ induced convulsions.

FIGURE 11

Radiogram of Extract of Pooled Brain Tissues
from Animals Dosed with H^3-PTZ via the i.p. Route.
The chromatogram was developed in isobutanol-water
on Whatman No. 1 paper.

Full scale deflection: 3×10^2 cpm
Time constant: 30 sec.
Chart speed: 0.5 cm/min.

58a
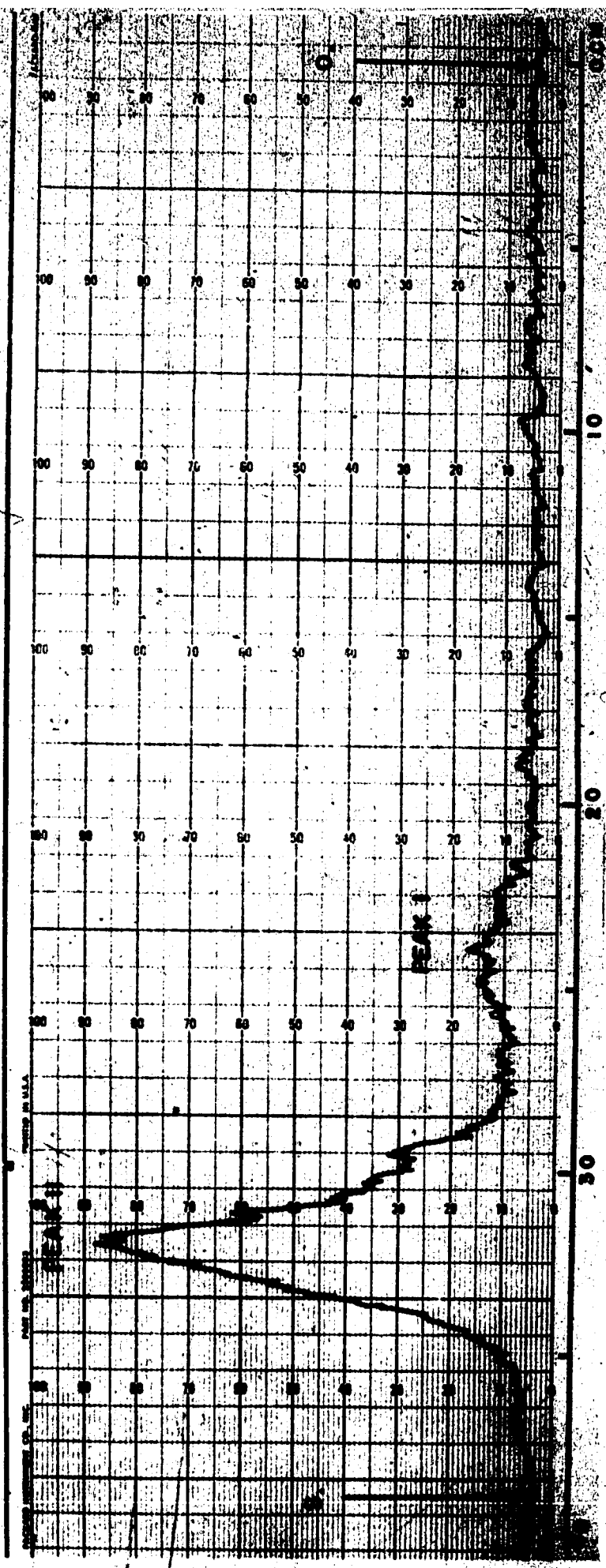
FIGURE 12

Radio-scanning of a Paper Chromatogram of Pooled Liver Extract after the Animals have been Dosed with H^3-PTZ. The chromatogram was developed in water-isobutanol.

Full scale deflection: 1 x 10^3 cpm
Time constant: 30 sec.
Chart speed: 0.5 cm/min.

59a

Figure 13

Radio-scanning of a Paper Chromatogram of Pooled Plasma Collected after Intraperitoneal Administration of H^3-PTZ to the Animals. The chromatogram was developed in water-saturated isobutanol.

Full scale deflection: 1×10^2 cpm
Time constant: 30 sec.
Chart speed: 0.5 cm/min.

60a
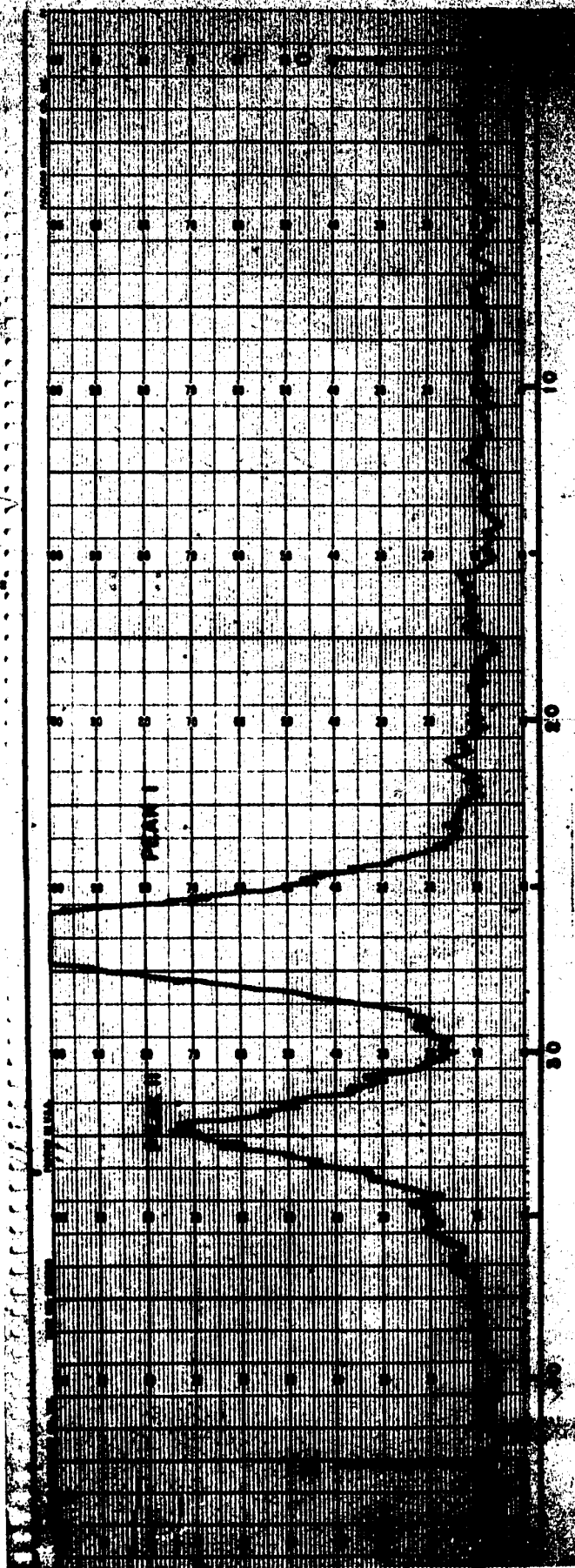
Plasma, obtained from blood collected on decapitating the rats, represented a pool of arterial and venous blood. Like the urine, analysis of plasma revealed two radio-compounds with about the same percentage radioactivity distributed between the two tritiated constituents being 45 and 55% for PTZ and its metabolite respectively.

CHAPTER III

PERFUSION OF ISOLATED LIVER TO STUDY BIOTRANSFORMATION OF PENTYLENETETRAZOL IN THE RAT

A. TIME COURSE OF PERFUSION

The preceding experiments provided evidence that PTZ is converted to a derivative in the body which is then excreted into the urine. Previous investigation showed that PTZ was metabolized by liver tissue (134, 135) since injury of this organ affected its detoxication (117). The data presented in Chapter II showed that the liver accumulated the greatest amount of radioactivity after administration of the labelled drug. To elucidate further the role of the liver in the biotransformation of this convulsant, the isolated rat liver was perfused with H^3-PTZ. At the end of the experiment, the perfusate and the liver were treated as described in Methods (see p. 32).

When the extract of the liver perfusate was chromatographed in water-saturated isobutanol and scanned for radioactivity, two tritium-containing constituents with R_F 0.78 and 0.64 could be detected on the chromatogram.

The results of these experiments are summarized in Figure 14. When 9 mg of PTZ (containing 26 μc of H^3-PTZ) were added to the perfusion medium, the resultant yields of metabolite were low and insignificant. When the liver was perfused for 4 hours, the yield of the tritium-labelled metrazol metabolite was consistently less than 10%. This yield of metabolite at R_F 0.64 was reckoned as percent of total activity spotted on the chromatogram.

Such low yields of radioactive metabolite in the above experiments may likely have been due to dilution by unlabelled metrazol. On the other hand, the isolated liver might have been unable to cope with this quantity of the convulsant drug which in vivo produced convulsions in rats weighing approximately 150 grams. Both factors were indeed valid, as shown in another series of experiments, when H^3-PTZ alone (0.04 mg, specific activity 650 μc/mg) was used as substrate for perfusion. As shown in Figure 14, the yield of the H^3-labelled metabolite at R_F 0.64 was greatly improved. When the liver was perfused up to three

FIGURE 14

Percentage* Yield of Tritium-containing Metabolite by Liver Perfusion of H^3-PTZ or H^3-PTZ plus Carrier PTZ at Various Time Intervals. Typical experiment of three runs in each series.

.----------. H^3-PTZ only

x——————x H^3-PTZ plus non-radioactive PTZ**

*   Percentage was reckoned as total activity spotted on the chromatogram, and corrected for 100% recovery.

**   Results erratic and insignificant (see Text).

63a

hours, the amount of derivative formed was more than 50%. This derivative is chromatographically indistinguishable from PTZ when developed in the water system, but is distinguishable in the isobutanol-water system. Typical scannings of the chromatograms of these experiments in both isobutanol-water, and water systems are shown in Figures 15 and 16 respectively.

B. METABOLIC INHIBITION BY SKF 525-A

SKF 525-A (β-diethylaminoethyldiphenyl propylacetate-HCl) is well known for its inhibitory action on the drug metabolizing enzyme system in the microsomes of the liver (156). Prabhu and Parikh (157) noted an enhancement of responses to PTZ in rats pretreated with SKF 525-A. It was therefore pertinent to observe the effect of this compound on the biotransformation of PTZ by liver perfusion.

Rats were given SKF 525-A (75 mg/kg i.p.) 30 minutes prior to anaesthesia for the subsequent removal of the liver. H^3-PTZ alone (0.04 mg, containing 26 μc) was used as substrate for perfusion. Results obtained are shown in Figure 17. After five hours of perfusion, there was one major tritium constituent found in the perfusate which chromatographically was identifiable as pentylenetetrazol.

FIGURE 15

Radiogram of Extract of Liver Perfusate. The liver was perfused up to three hours with H^3-PTZ as described in text. The chromatogram was developed in water-saturated isobutanol on Whatman No. 1 paper. The scanning was monitored on a logarithmic scale.

Chart speed: 0.5 cm/min.

65a

FIGURE 16

Radiogram of Extract of Liver Perfusate. The
chromatogram was developed in the water system on
Whatman No. 1 paper.

Full scale deflection: 1 x 10^3 cpm
Time constant: 100 sec.
Chart speed: 0.5 cm/min.
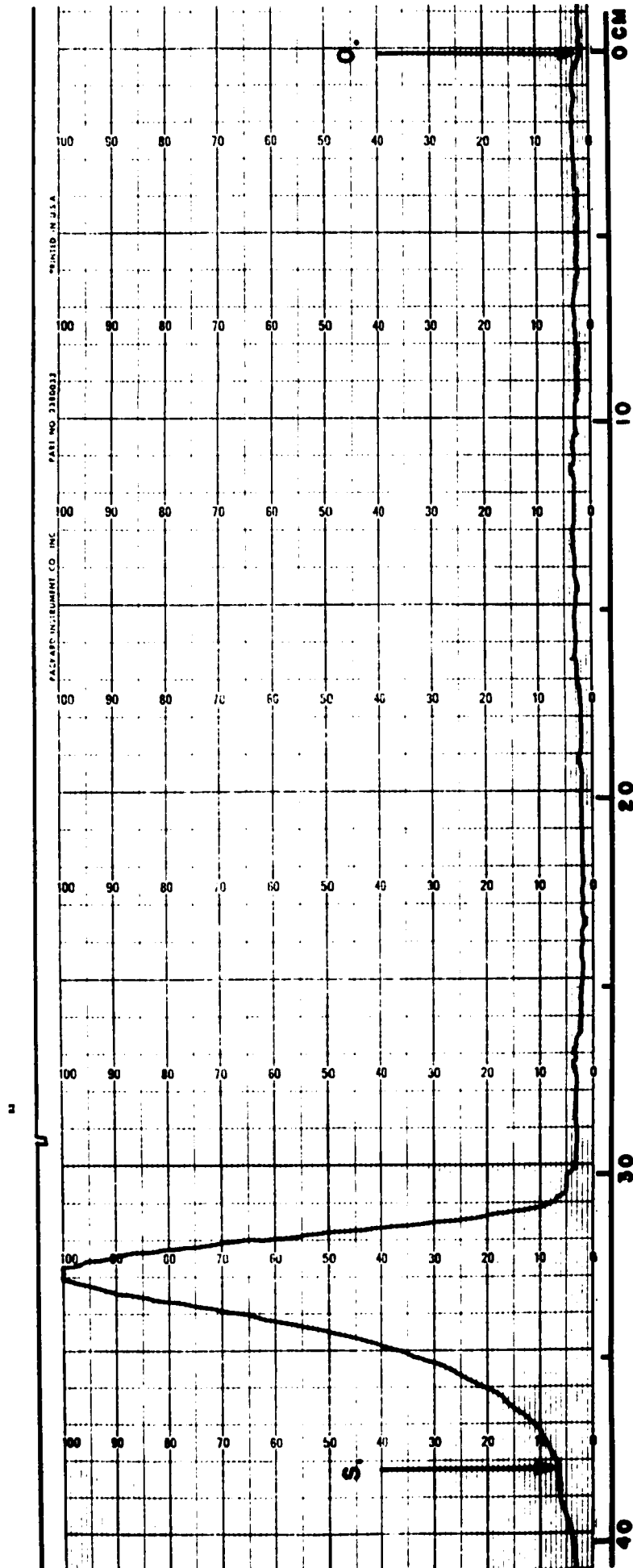
66a
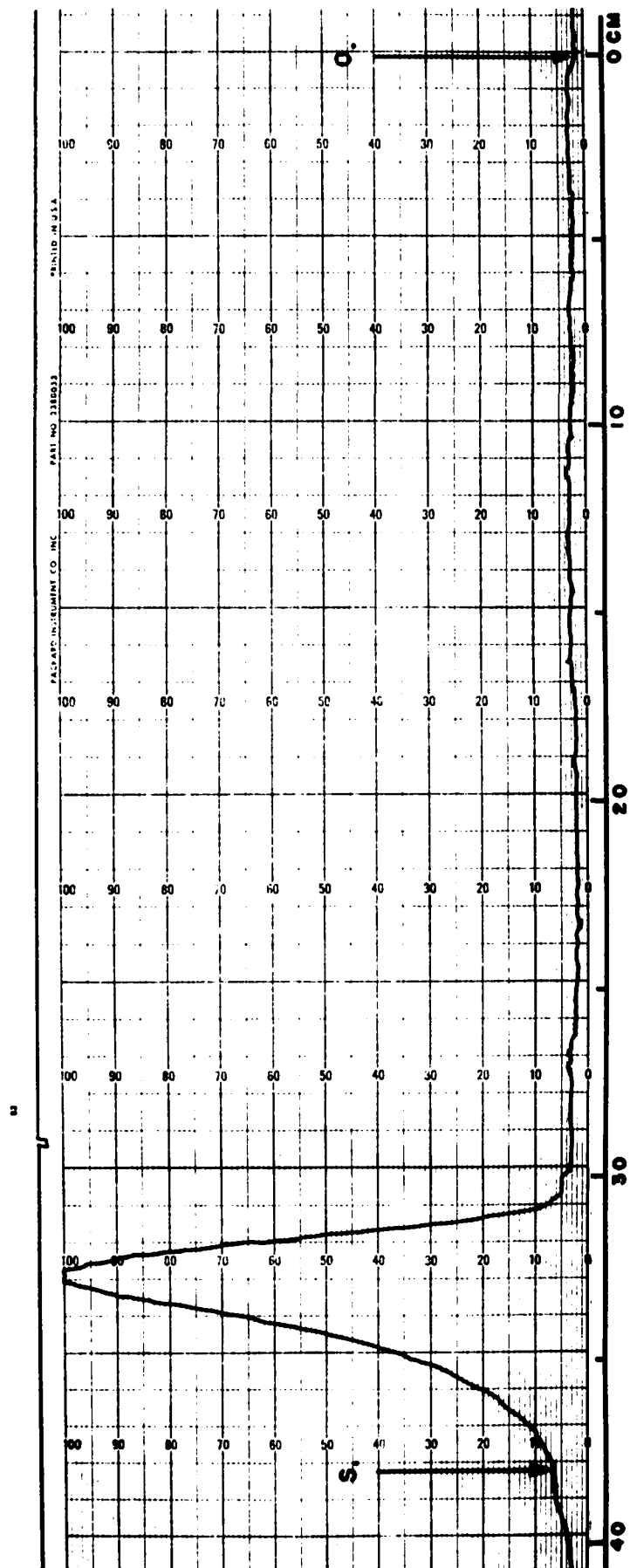
66a

FIGURE 17

Radio-scanning of a Paper Chromatogram of
Extract of Liver Perfusate in the Presence of
SKF 525 - A. The chromatogram was developed
in isobutanol - water on Whatman No. 1 paper.

Full scale deflection: 1×10^2 cpm
Time constant: 30 sec.
Chart speed: 0.5 cm/min.
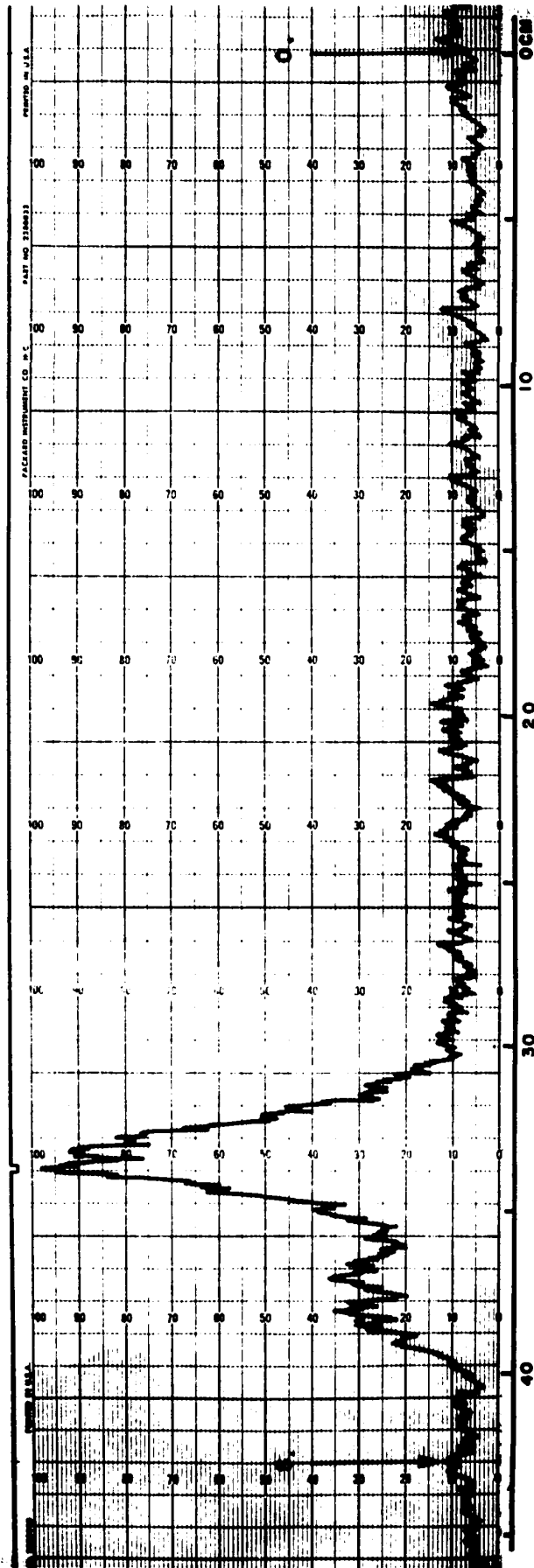
67a

The minor radioactive component near Peak II which appeared in some of the radiochromatograms of urinary extract (see Fig. 3, p. 39), was also present. Since this small peak seemed to be influenced by H^3-PTZ as shown in the Isotope Dilution experiment, it did not appear to be a second or minor metabolite.

Thus, under these experimental conditions, there was no major metabolite formed. It seems therefore that SKF 525-A inhibits the enzyme activity in the liver which is responsible for the metabolism of this convulsant drug in vivo.

C. INCUBATION OF TRITIATED-PENTYLENETETRAZOL WITH CITRATED WHOLE BLOOD OF THE RAT - CONTROL EXPERIMENT

Tritium-labelled PTZ was incubated with citrated whole blood of the rat at 37° C for various time intervals. After incubation, the plasma was separated from the cells, and subjected to eletrophoresis in barbital buffer (pH 8.6). Results of radio-scanning of the eletrophoretograms indicated that even up to 5 hours of incubation, there was no PTZ metabolite formed. These results seemed to indicate that the metabolite formed by perfusing the isolated rat liver with whole blood containing H^3-PTZ, was not due to any enzymes in the blood.

CHAPTER IV

INCUBATION OF H^3-PENTYLENETETRAZOL WITH LIVER HOMOGENATE

After incubation of PTZ with the liver homogenate, estimation of PTZ by means of gas chromatography revealed no significant changes in the amount of PTZ originally present (130). The results shown in Chapter III have demonstrated that perfusion of the isolated rat liver with the drug leads to its metabolic transformation. It is surprising that incubation of PTZ with the liver homogenate did not lead to similar observations. It has been suggested that a diversity of drug metabolic pathways possess a number of factors in common (158). A number of the liver enzyme systems which metabolize drugs are localized in the microsomes. These enzyme systems require O_2 and TPNH. It was therefore of interest to see whether liver homogenate fortified with these substances would produce similar results to those described with liver perfusion.

Tritium-labelled PTZ was incubated in air with rat liver homogenate supplemented with various substances as described in Methods (p. 33). After incubation, the reaction was stopped by quick-freezing and dried by lyophilization. After extracting successively with 95% ethanol, acetone and ether, the dried extract was re-suspended in water and chromatographed in isobutanol-water. After drying, the chromatogram was scanned for radioactivity, and the regions corresponding to R_F 0.60 - 0.68, and 0.74 - 0.82 were cut out and counted in a liquid scintillation spectrometer.

Results of radio-scanning showed only one substance to be present in all chromatograms and it was identified chromatographically as H^3-PTZ. The radioactivity detected in these two regions in terms of percentage of activity spotted on the chromatogram is shown in Table III. No significant changes in H^3-PTZ were observed in the band R_F 0.74 - 0.82 up to five hours of incubation. The radioactivity detectable in the region R_F 0.60 - 0.68 at five hours of incubation was negligible. Thus, there is no change in PTZ after its incubation with the liver homogenate.

TABLE III

INCUBATION OF PTZ WITH LIVER HOMOGENATE

SAMPLE*	PERCENT** DISTRIBUTION OF RADIOACTIVITY					
	CONTROL	1 HOUR	2 HOURS	3 HOURS	4 HOURS	5 HOURS
R_F 0.60-0.68 (METABOLITE)	0%	0%	0%	0%	0%	0.05%
R_F 0.74-0.82 (METRAZOL)	91%	92.5%	93%	95%	92.45%	90.5%

*   Chromatogram was developed in water-saturated isobutanol.

** Percentage was calculated as that of total activity spotted on the chromatogram. Typical of two experimental runs. Background count has been subtracted from values reported.

CHAPTER V

AN ATTEMPT TO ISOLATE THE H^3-PENTYLENETETRAZOL METABOLITE FROM URINE OF THE RAT ON XAD-2 COLUMN

A simple column chromatographic method using Amberlite XAD-2 resin, was developed to process large volumes of urine in order to isolate the metabolite for further chemical analyses.

After percolating the urine through the column,
100 ml of water was used to wash down the sample. Then,
methanol was used to elute PTZ and the metabolite.
Collection of fractions of the eluate commenced when
about half of the water-wash had passed through the
column. Aliquots (10 μl) of each fraction were counted
for radioactivity. The radioactivity in each fraction
is shown in Tables IV and V. When 130 ml of the filtered
urine (37.6 x 10^{-6} cpm) was applied to the column, the
total recovery of radioactivity was 71% (Table IV).
83% of this activity was contained in fractions 8 - 13.
In a second experiment, the recovery was much improved.
As shown in Table V, 83% of the radioactivity in the
urine (57 ml, 21.0 x 10^{-6} cpm) was collected in the eluate.
98% of this was found in fractions 4 - 10.

The fractions containing the most radioactive material
were pooled and dried in vacuo. The dried residue was
treated successively with 95% ethanol, acetone, and ether
as described previously (Methods, see p. 26). Finally,
after ether extraction and drying, the residue was resuspended

TABLE IV

RECOVERY OF RADIOACTIVITY IN FRACTIONS FROM THE
XAD - 2 COLUMN - EXPERIMENT 1

FRACTION	VOLUME (ml)	RECOVERY OF RADIOACTIVITY* cpm x 10^{-6}
Water washings	-	0.50
1	12.5	0.27
2	14.0	0.63
3	15.0	0.69
4	11.5	0.73
5	13.0	0.43
6	14.5	0.41
7	15.5	0.18
8**	12.2	1.17
9**	14.8	11.84
10**	12.7	6.28
11**	14.8	1.74
12**	15.0	0.61
13**	14.5	0.42
14-20 (pooled)	92.0	0.72
TOTAL	270 (approx.)	26.62

* 130 ml of urine (37.6 x 10^{-6} cpm) were applied.
10 μl aliquots were counted; total activity found in
fractions are given.

** Fractions 8 - 13 = 22.06 x 10^{-6} cpm (83% of total
recovered). Percent recovery = 71%.

TABLE V

RECOVERY OF RADIOACTIVITY IN FRACTIONS FROM THE

XAD - 2 COLUMN - EXPERIMENT 2

FRACTION	VOLUME (ml)	RECOVERY OF RADIOACTIVITY* cpm x 10^{-6}
1	19.8	0.05
2	18.2	0.06
3	19.0	0.07
4**	20.0	0.41
5**	19.7	3.86
6**	18.0	6.57
7**	16.4	2.08
8**	17.1	1.80
9**	19.0	1.32
10**	20.3	1.10
11	19.8	0.10
12	18.0	0.06
TOTAL	225 (approx.)	17.48

* 57 ml of urine (21.0 x 10^{-6} cpm) were applied.
10 µl aliquots were counted; total activity found in
fractions are given.

** Fractions 4 - 10 = 17.14 x 10^{-6} cpm (98% of total
recovered). Percent recovery = 83%.

in a small volume of ethanol and spotted on papers for chromatography in the isobutanol-water system. The regions with R_F 0.60 - 0.68 and 0.74 - 0.82 on the chromatograms were cut out and exhaustively extracted with several volumes of methanol. The methanolic extracts were dried <u>in vacuo</u>, and the residues obtained were used for analyses.

CHAPTER VI

ELEMENTAL ANALYSIS OF PENTYLENETETRAZOL METABOLITE EXTRACTED FROM RAT URINE

The PTZ metabolite, obtained from XAD - 2 column, was dried over anhydrous P_2O_5 in a vacuum dessicator at room temperature. It was then analysed for its elemental composition. The following results were obtained: C, 21.7%, 22.1%; H, 3.1%, 3.4%; N, 14.0%, 14.2%; and S, 8.9%, 9.1%

Similarly, the other substance (Peak II - PTZ) extracted from paper chromatograms as described in Chapter V, was analysed. The results were as follows:

Calculated: C, 52%; H, 7%; N, 41%.

Found: C, 52%; H, 7%; N, 40%.

               C, 51%; H, 7%; N, 42%.

This was further proof that the substance at
Peak II, obtained from paper chromatograms developed
in isobutanol-water, was the CNS stimulant drug
pentylenetetrazol.

DISCUSSION

The major problem in studying the metabolic fate of PTZ in animals is the lack of suitable methods for its estimation in body fluids and tissues. The non-specific nature of most of the complexing agents used to precipitate pure PTZ in solutions, makes it difficult to separate this compound from the biological phase. Also, the low sensitivity of some of these methods of estimation, such as that of Daoust (150), makes them unsuitable for use in metabolism studies where small quantities of the drug are used.

Esplin and Woodbury have used a tracer compound to examine the metabolism of PTZ in the rat (21). These authors concluded that the agent was metabolized in the liver before being excreted. However, they were unsuccessful in their attempts to demonstrate this metabolite in the urine of the rat after _in vivo_ administration of PTZ. The results obtained chromatographically led them to assume that the PTZ derivative reverted to PTZ, its original form, either in the urine itself or during the process of identification. Since PTZ is so easily precipitated by complexing with ions such as mercuric, cuprous or cupric, it is unlikely that the drug is excreted unchanged in the urine of the rat. Although similar conclusions were reached by other workers (140, 141, 142) both in dog and in man, the absence of PTZ from the urine after parenteral administration of the drug is of course, no proof that

the metabolite is not formed _in vivo_. In as much as chromatographic homogeneity in any one solvent system does not constitute final proof of identity, it is possible that the compound recovered from the rat urine by Esplin and Woodbury (21) was a mixture of metrazol and its metabolite. Such an assumption is warranted by their finding of two crystalline species when a solution of $HgCl_2$ was added to an aqueous solution of crystals obtained by microdistillation of urine extract. No relative proportions of these two crystalline species were given. It is therefore not known if they formed the $HgCl_2$ complex to the same extent. In addition, they estimated the final product obtained as a complex represented only about two percent of that initially present in the urine sample before extraction. It is probable that the large fraction lost contained also PTZ in a metabolically altered form which may or may not have the same affinity for $HgCl_2$ as does the unchanged PTZ.

The present investigation revealed that PTZ administered to the rat either intraperitoneally, intravenously or subcutaneously is excreted partly unaltered and partly as a metabolite (Table II). Metabolism of PTZ therefore appears to be qualitatively the same in spite of different routes of administration. Thus, the difference between the observations of Esplin and Woodbury (21) and that reported here cannot be due to choice of route of administration of the compound.

The existence of the metabolite became evident when
the urinary extract of the experimental animals was chro-
matographed in a water-saturated isobutanol system, but
not in a water system. The former system revealed two
major radioactive components in such an extract (Fig. 3),
whereas the latter could not separate these constituents
(Fig. 4). Additional evidence was obtained when paper
chromatograms were developed two dimensionally in the two
systems mentioned (Fig. 5). The identity of the minor
component near Peak II was not attempted. It may be the
precursor of the major metabolite in Peak I or it may have
been a minor metabolite of PTZ. Results from the Isotope
Dilution experiment (p. 43) seemed to indicate that it
may likely have been due to tailing of material on the
paper chromatogram. These results demonstrate that PTZ
in the rat is partly metabolized to a derivative and is
excreted along with unchanged PTZ in the urine.

The isolation of the metabolite in the urine extract
is not an artifact created by processing the samples. The
presence of this metabolite can also be demonstrated in
the unprocessed urine sample (Fig.7). Furthermore, such
possibilities as tritium exchange and degradation of the
labelled drug during collection of urine have been care-
fully examined as shown in "Control Experiment" in Chapter
I. PTZ alone was present in these control experiments.

The identity of one of the two substances in the

urine as PTZ was established by isotope dilution (Table
I), by paper chromatography (Fig. 6) and by elemental
analysis (Chapter VI). Peak II on chromatograms develop-
ed in isobutanol-water was shown to be PTZ itself, and
Peak I was a metabolic derivative of this CNS stimulant.

PTZ and its metabolite were rapidly excreted as
shown in Fig. 7. Two hours after the rats received the
labelled drug, a sizable quantity of radioactivity has
been excreted into the urine. No attempt was made to
study the excretion of radioactivity in the urine prior to
2 hours, since catheterization of the animals would have
been necessary. However, it may be anticipated from the
levels of tritium in the plasma (Fig. 10b), that a con-
siderable quantity of the radioactivity should be cleared
into the urine within an hour after dosage.

In agreement with Esplin and Woodbury (21), 70% of
the radioactive material given intraperitoneally was re-
covered in the urine in the first day. The total recovery
only rose to about 73% by 48 hours. Half of the radio-
activity injected was excreted in the urine in approxi-
mately 12 hours.

In the present study, feces were not examined for
the metabolite. Esplin and Woodbury (21) have shown
that the excretion of C^{14}-pentylenetetrazol in the feces was

erratic and insignificant.

Because of the nature of the label used, it was not
possible to trace the radioactivity in expired CO_2 in
the present work. This too has been shown to be negli-
gible in PTZ metabolism (21).

The excreted materials were always composed of PTZ
and its metabolite. The exact percentage of each of these
two substances at each specific period of urine collection
has not been investigated. The relative proportion of
radioactivity between the two substances in the 24 hour
sample was found to be approximately 40-45% for PTZ and
55-60% for the metabolite. If the animals metabolized
both labelled and non-labelled PTZ indiscriminately, which
should be the case, one could assume that 55-60% of the
PTZ might have been excreted as a derivative in 24 hours.

The data presented in Figures 10a and b agreed well
with the report of Tatum and Kozelka (117) that PTZ is
very readily absorbed from all sites of administration.
One minute after i.p. administration of the tritiated
drug, all three organs studied had accumulated considerable
quantities of radioactivity. This rapid absorption and
distribution of the agent was probably due to its great
solubility in water and lipoids. It is recognized that

organic bases generally penetrate cells as the un-
dissociated molecule, which is the more lipid-soluble
form. Acidic conditions such as that in the upper
intestinal tract would ionize the compound and depress
its absorption. Since PTZ is just as readily absorbed
from gastrointestinal tract (13, 131), as in other
locations, it seems that the drug is not ionized during
the passage from the site of administration to the blood
stream. This may mean the drug is absorbed from the
tissues unchanged.

Pentylenetetrazol, like most organic bases, after
being absorbed, leaves the circulation and localizes in
high concentrations in parenchymatous tissues (159). The
drug was believed to be concentrated in the liver after
absorption, thus affecting cholesterol synthesis (160).
Experimental evidence shows, within a few minutes after
absorption, the liver exhibits radioactivity relatively
in excess of that present in the plasma (Fig. 10a). The
brain also has the ability to concentrate this convulsant,
but to a much lesser degree than hepatic tissue. Since
plasma levels of radioactivity were several times higher
than cerebral levels, any blood trapped in the CNS would
tend to elevate the radioactivity of the organ. The same
should apply to liver tissue.

The uniform distribution of radioactivity among selected areas of the brain (Fig. 9) indicated that PTZ does not localize in any part of the organ. The large variation in the distribution data (Fig. 10a) observed within 30 minutes of administration could be due to sampling which was obtained when there was a rapid fluctuation of activity levels. Moreover, peak brain levels do not occur at the same time in all animals.

It was estimated that in rabbits, it took six seconds for PTZ to reach the brain (43). The present work shows the rat brain attained the highest level of radioactivity at five minutes; two minutes after convulsions were seen in most of the PTZ-treated animals. The levels of the drug found in the brain are relatively minute in terms of either the dosage administered or the levels of the drug in other organs. The uptake of the activity by the liver was much higher than that by the brain. The radio-activity in the liver was 3 - 5 times higher than in the cerebral tissues within the first few hours after dosage. Concentrations of drugs in brain tissue are generally low in proportion to other tissues (161, 162). This small concentration of PTZ in the cerebral tissue indicates a high potency of the compound. A large dosage is necessary when the agent is quickly metabolized, as only part of the absorbed material can elicite the effect.

liver was also sensitive to SKF 525-A inhibition.
Earlier, it was reported that the action of PTZ in rats
in the presence of SKF 525-A was enhanced (157). Thus,
it seems that the metabolism of PTZ _in vivo_ is an enzy-
matic process. This system, similar to other drug meta-
bolizing enzymes, is susceptible to SKF 525-A inhibition
(165). Further experimentation is necessary to eluci-
date the nature of this enzymic system.

Contrary to other findings, Fournier and Selye (136)
claimed that hepatic tissue was not involved in the de-
toxication of PTZ. From the distribution of radioactivity
in the tissues (Figs. 10a, b), it is clearly shown that
the liver takes up enormous quantities of the radioactive
material. Furthermore, only a fraction of the administered
drug is needed to produce a seizure in the intact animal.
It is therefore likely that the rest of the drug after
absorption is stored in the liver where it is rapidly
destroyed. The surgical method of Fournier and Selye (136)
removed only 75% of the liver. The remaining 25% could
still act as a storage for the convulsant drug which might
then be slowly released from the tissue after its sub-
cutaneous administration. This may explain the same tolerance
to PTZ of the hepatectomized and control animals reported
by these investigators.

Results of incubation of PTZ with liver homogenate
agreed with the work of Kawamoto (130) in that no meta-
bolite was detectable under such experimental conditions.
This is inconceivable when the agent was shown to be
transformed to a metabolite by perfusion through an iso-
lated liver. However, other studies have shown that the
in vitro metabolism of strychnine required a thorough
study on pH optima and the supplementation of various
cofactors (166). It may be the same for PTZ and these
data are necessary if the enzyme activity responsible
for the destruction of this convulsant poison in vivo
is to be localized.

An attempt was made to characterize the metabolite.
The small size of the dose of PTZ required to elicit a
pharmacological response without fatal consequences has
necessitated the use of large volumes of urine samples
to obtain the metabolite. Consequently, with the existing
extraction procedure, the material finally isolated was
not entirely free from other substances. The yield was
also far from quantitative. In addition, it was diffi-
cult to concentrate an enormous volume of urine. The
sample did not stay frozen during lyophilization. This
may have led to losses of material.

The neutral resin Amberlite XAD-2 is currently
used for the isolation of many steriods and their con-
jugates. An outstanding value of this resin is the
ease of processing large volumes of sample (170). Since
methanol is used as the eluting solvent, it greatly
simplifies the subsequent concentration and extraction
procedure. Furthermore, there is no great loss of
the substances to be isolated. The recovery (71%) in
the first experiment was not satisfactory, and the
recovered radioactivity seemed to scatter around the
main elution peak. This could be due to the large volumes
of urine percolated through the column which exceeded
its capacity. Later, when a smaller volume of urine
(57 ml) was used, the recovery (83%) was substantially
increased.

Elemental analysis of the PTZ metabolite finally
obtained (Chapter VI) showed that there was 9% sulphur
in the sample. Assuming that PTZ is sulfated in the liver
of the rat before excretion, the simplest form would be a
sulfate ester. The formation of this sulfate ester would
require prior hydroxylation of the molecule. Thus, the
simplest derivative might be a sulfate ester of hydroxy
pentylenetetrazol. A theorectical sodium salt of
hydroxy pentylenetetrazol ($C_6H_9N_4SO_4Na$) would have the

following percentage composition: C, 28.0; H, 3.5;

N, 22.0; and S, 12.5. The values actually obtained

for the metabolite were: C, 21.7, 22.1; H, 3.1, 3.4;

N, 14.0, 14.2; and S, 8.9, 9.1. A possible site for

sulfation in pentylenetetrazol is carbon No. 6 in the

PTZ molecule. Thus, a hypothetical hydroxy pentylene-

tetrazol sulfate would have the following structure:
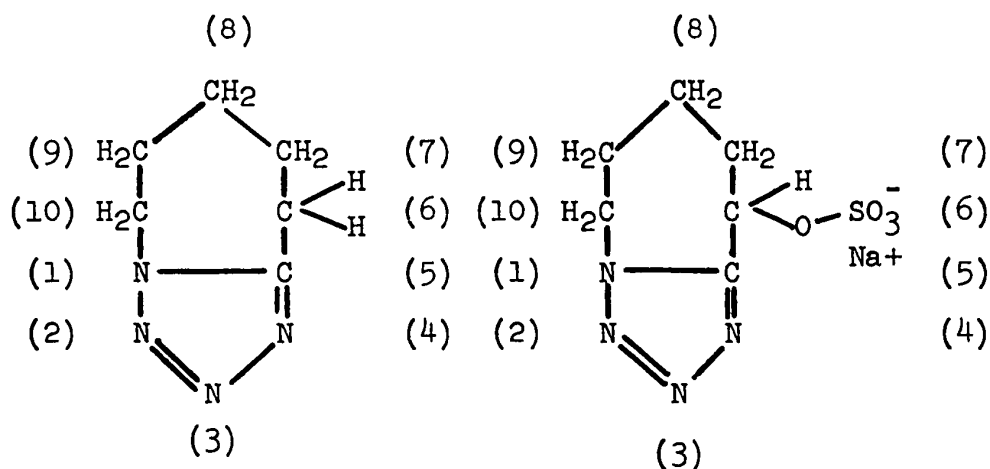
PENTYLENETETRAZOL   SODIUM SALT OF HYDROXY PENTYL-
ENETETRAZOL SULFATE
(HYPOTHETICAL)

Considerable discrepancy still exists between the

theorectical composition of PTZ-sulfate and that obtained

from analysis of the metabolite. Such poor agreement may

be attributed to the following two reasons:

1. Impure sample - this is very likely as the sample

invariably contained about 15-20% ash after its combustion

at high temperature.

2. Contaminant - other metabolites of different com-

position may likely be present in this fraction. Further

purification of the metabolite obtained is needed.

The biological action of PTZ has been suggested to be mediated through a metabolic derivative (144). The present studies have suggested that this derivative is probably a sulfated pentylenetetrazol. The pharmacological potency of this metabolite has not yet been investigated because methods for the isolation and purification of the metabolite are still being developed. From nephrectomy studies, however, this PTZ derivative was assumed to be biologically inactive (21, 136).

The present data on the distribution of radioactivity in both the liver and the brain also warrant such an assumption of an inactive PTZ metabolite. The activity in both tissues rose simultaneously indicating the drug reached both tissues at the same instance. If the convulsant drug must be activated in the liver before it can exert its convulsant effect, there would have been a lag in the appearance of radioactivity in the brain. Since this was not observed, it seems that only PTZ can ascribe the role as the seizure-producing agent.

Results of analysis of metabolite in these tissues revealed that there was only one compound in the brain which was proven chromatographically to be metrazol. The liver, however, contained both metrazol and its metabolite; most of it being metrazol itself. It appears

that metrazol after absorption, is stored in the liver
to be metabolized. When the drug is biotransformed to
a derivative, it is eliminated via the circulation into
the kidney where it is excreted in the urine.

In a comprehensive review of the factors affecting
drug action, Brodie and Hogben (167) have defined the
pharmacologic importance of metabolic alteration.
Generally, metabolites were seen to be more polar and
less lipid-soluble than the parent congeners. The de-
creased lipid solubility of metabolites tends to prevent
their reabsorption in the kidney tubules. Passage of
the metabolite across the blood-brain-barrier would also
be hindered or prevented due to their low lipid solubility.
The absence of the PTZ metabolite in the brain when there was
a high concentration of it in the blood, indicated that the
metabolite may have little or no access to the CNS.

On the other hand, biotransformation, in some instances,
may be essential in the activation of certain drugs (147).
One example, picrotoxin, was mentioned previously (148, 149).
Hence, additional quantitative data and kinetic studies
need to be conducted with regard to the rates of formation
of the metabolic product before the significance of this
PTZ metabolite in relationship to that of the parent

compound can be assessed. More rigid proof requires that pharmacologic tests be performed on the PTZ metabolite. Such experiments must await the isolation of this compound and the chemical synthesis of the proposed PTZ derivative. Until then, the evidence presented here cannot be regarded as indicating that the pentylenetetrazol metabolite is pharmacologically inactive.

SUMMARY

(1)   Pentylenetetrazol administered to rats was found
      to be metabolized to a derivative which was excreted
      in the urine along with unchanged PTZ.

(2)   Separation of PTZ and its metabolite can be
      achieved by paper chromatography in water-saturated
      isobutanol. However, these two substances cannot
      be separated when paper chromatograms are developed
      in distilled water.

(3)   Distribution of tritiated-pentylenetetrazol and
      analysis of the metabolite in tissues revealed that
      only PTZ was present in the brain. Both the liver
      and the plasma contained PTZ and its metabolite in
      different proportions.

(4)   In vitro perfusion experiment showed that the meta-
      bolite is formed in the rat liver and the formation
      is susceptible to SKF 525-A inhibition.

(5)   The probable chemical nature of the metabolite and
      its pharmacological significance are discussed.

CLAIMS TO ORIGINALITY

1. Pentylenetetrazol (metrazol) administered to rats was metabolized to a derivative which was excreted in the urine along with unchanged pentylenetetrazol.

2. Pentylenetetrazol and its metabolite can be separated by paper chromatography in a water-saturated isobutanol system, but not by a water system.

3. Perfusion experiments showed that the metabolite is formed in the liver of the rat, and the formation is susceptible to SKF 525-A inhibition.

4. A column chromatography method using Amberlite XAD-2 resin was developed to isolate the pentylenetetrazol metabolite.

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