UNIVERSITÉ DU QUÉBEC À MONTRÉAL

EFFETS DU CL-20 CHEZ LA CAILLE JAPONAISE (*COTURNIX COTURNIX JAPONICA*) ET LA PURIFICATION D'UNE GST CAPABLE DE MÉTABOLISER LE CL-20

MÉMOIRE
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PAR
GHALIB KARIM BARDAI

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AVANT-PROPOS

Ce projet de maîtrise a été réalisé dans le cadre du projet de caractérisation d'un nouvel explosif, le CL-20, subventionné par le US Strategic Environmental Research and Development Program (SERDP, le projet CP1256). Développé par Thiokol Propulsion Inc., peu d'information existe sur les effets écotoxicologiques du CL-20. La présente étude est donc la première à évaluer les effets toxicologiques du CL-20 sur une espèce aviaire et à caractériser une enzyme présente dans le foie de caille pouvant biotransformer le CL-20.

La planification, le design, l'exécution et la rédaction des deux articles présentés dans le cadre de cette thèse ont été réalisés en majeure partie par moi-même. Cette thèse a été réalisée à temps partiel pendant que j'étais employé à la fois à l'IRB (Conseil national de recherches Canada – Institut de recherche en biotechnologie – groupe d'Écotoxicologie appliquée) et au CSUM (Centre de Santé de l'Université McGill). Dr Philip Spear m'a aidé dans le design des études de toxicité ainsi qu'à la révision finale des publications, conjointement avec Dr Geoffrey Sunahara. Les analyses du CL-20 ont été exécutées par le groupe de Chimie environnementale de l'IRB.

Deux articles, l'un étant accepté (Effects of Dietary Administration of CL-20 on the Japanese quail (*Coturnix coturnix japonica*); Ghalib Bardai, Geoffrey I. Sunahara, Philip A. Spear, Majorie Martel, Ping Gong, et Jalal Hawari) et l'autre ayant été soumis (Purification of a cytosolic GST from Japanese quail (*Coturnix coturnix japonica*) capable of biotransforming CL-20; Ghalib Bardai, Geoffrey I. Sunahara, Philip A. Spear, Stephan Grosz, et Jalal Hawari) sont les produits finaux de cette mémoire. Le premier article porte sur les effets toxiques du CL-20 sur le système reproducteur des cailles. Le deuxième article présente différentes hypothèses

expliquant les effets toxiques sur la reproduction des cailles et constitue donc une suite logique du premier article.

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AAP	army ammunition plants
ALD	approximate Lethal Dose
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AMY	amylase
AST	aspartate aminotransferase
BME	β-mercaptoethanol
BW	body weight
CDNB	1-chloro-2,4-dinitrobenzene
CE	crude extract
Chol	cholesterol
Cl	chloride
CL-20	hexanitrohexaazaisowurtzitane
CNS	central nervous system
CO	carbon monoxide
Cre	creatinine
Dbili	direct bilirubin
DPI	diphenyliodonium chloride
DTT	dithiothreitol
EA	ethacrynic acid
Glu	glucose
GSH	glutathione
GSSG	oxidized glutathione
GST	glutathione S-transferase
HCT	hematocrit
HMX	octahydro-1,3,5,7-tetranitro-1,3,5,7–tetrazocine
ISDN	isosorbide dinitrate
K	potassium
LAP	load, assembly and pack
LDH	lactate dehydrogenase
LOAEL	lowest observable adverse effects level
MAPEG	membrane-associated *p*roteins in eicosaniod and glutathione dependent metabolism
Mg	magnesium
Na	sodium
NADH	nicotinamide adenine dinucleotide
NOAEL	no observable adverse effects level
NTG	nitroglycerin

PBS	phosphate buffered saline
PCV	packed cell volume
PTH-aa	phenylthiohydantoin amino acid
PVDF	polyvinylidene difluoride
RDX	hexahydro-1,3,5-trinitro-1,3,5–triazine
ROS	reactive oxygen species
s-GSH	s-octylglutathione
Tbili	total bilirubin
TG	triglycerides
TNT	trinitrotoluene
TP	total protein
Uric	uric acid
UV	ultra-violet

RÉSUMÉ

L'hexanitrohexaazaisowurtzitane ou CL-20 est un nouveau composé énergétique présentement à l'étude pour fin d'utilisation militaire. Le RDX (hexahydro-1,3,5-trinitro-1,3,5–triazine) et le HMX (octahydro-1,3,5,7-tétranitro-1,3,5,7–tétrazocine) sont des contaminants couramment retrouvés dans le sol des sites militaires. La toxicité de ces deux derniers nitramines sur les espèces de vertébrés terrestres, tel que les mammifères, les amphibiens et les oiseaux, est de plus en plus documentée.

Le CL-20 est un nitramine polycyclique caractérisé par des liens N-NO_2, ce qui est comparable au RDX et au HMX qui sont deux composés nitramines monocycliques. Étant donné leurs structures chimiques similaires, nous avons émis l'hypothèse que les effets du CL-20 sur les oiseaux seraient aussi semblables à ceux du RDX. Au cours de la présente étude, nous avons évalué les effets toxiques du CL-20 sur un gallinacé, la caille japonaise (*Coturnix coturnix japonica*) exposée à des doses journalières et répétées de CL-20. En premier lieu, un test sub-aigu de 14 jours a été réalisé, durant lequel les oiseaux ont subi des gavages répétés pendant les premiers 5 jours suivis de 10 jours sans exposition au CL-20 (diluant seulement). Dans un deuxième temps, un test nutritionnel sub-chronique a été réalisé sur une période de 42 jours. Au cours de ces deux études, aucun effet toxique n'a été observé chez les oiseaux exposés au CL-20. Par contre, une augmentation du poids du foie, des niveaux du sodium plasmique et de la créatinine ont été observés chez les oiseaux ayant reçu les doses de CL-20 les plus élevées. L'analyse du plasma et de certains organes vitaux des cailles (foie, cerveau, cœur et rate) a montré que le CL-20 n'était pas présent dans ces tissus, suggérant que le CL-20 pourrait être biotransformé *in vivo*. Au cours de l'étude nutritionnelle sub-chronique, le poids des embryons a significativement et proportionnellement diminué en fonction de la dose de CL-20. Les embryons des oiseaux exposés au CL-20 présentaient plusieurs malformations congénitales (autant crâniennes que faciales), telles que des courbures du bec, l'hypertrophie du cerveau moyen et le développement d'un seul côté du visage couplé à une micro-opthalmie (comparaison non statistique avec les embryons contrôles). Le nombre d'œufs pondus par femelle tendait aussi à diminuer avec l'augmentation de la dose.

Ces résultats suggèrent que le CL-20 aurait un effet sur le foie des femelles adultes. Le développement des embryons d'oiseaux diffère de celui du fœtus de mammifères en ce sens que 90% de l'énergie requise par l'embryon d'oiseau provient de la β-oxydation des acides gras dérivés des lipides vitellins. Les lipides vitellins sont produits par le foie maternel et transmis aux oocytes en croissance, servant de source d'acides gras pour l'embryon. Ainsi, il est possible que les effets du CL-20 sur le foie maternel (via le métabolisme des acides gras et des composés xénobiotiques)

conduisent à des effets nuisibles sur le développement de l'embryon qui est intimement lié au vitellus maternel.

Notre hypothèse principale de travail est que le foie de caille pourrait contenir une ou des enzymes capable de biotransformer le CL-20. Nos résultats avec le cytosol total indiquent que la disparition du CL-20 est inhibée *in vitro* par l'acide éthacrynique ou un produit analogue au GSH, le s-octylglutathione, qui est une enzyme GST. La purification et la caractérisation de la glutathione S-transférase cytosolique (GST) a ètè effecutèe à partir du foie de caille. L'analyse partielle de la séquence N-terminale a montré que les deux classes de GST alpha et mu de la caille ont une homologie de 100% avec la GST du poulet. Cette enzyme purifiée a pu bio transformer le CL-20 en présence de la glutathione (GSH), un co-facteur obligatoire. Nos résultats suggèrent que la protéine purifiée a bio transformé le CL-20, tel que mis en évidence par la formation concomitante de nitrite (NO_2^-) et la disparition du CL-20. Nos résultats suggèrent que la biotransformation du CL-20 par GST *in vitro*, pourrait expliquer la faible toxicité et l'absence de CL-20 *in vivo*.

Mots-clés : caille japonaise ; CL-20 ; GST ; acide éthacrynique.

CHAPITRE 1

INTRODUCTION

1.0 Le CL-20: un nouveau produit chimique bientôt présent dans l'environnement.

Les composés polynitropolyaza-encagés de haute densité contiennent de l'énergie potentiellement élevée pouvant être utilisés en tant qu'explosifs et produits combustibles pour fin militaire. Présentement, les explosifs les plus utilisés sont le 1,3,5-trinitrohexahydro-1, 3, 5-triazine, connu sous le nom de RDX (Royal Demolition Explosive) et l'octahydro-1, 3, 5, 7-tétranitro-1, 3, 5, 7-tétrazocine, connu sous le nom de HMX (High Melting Explosive). Le hexanitrohexaazaisowurtzitane, connu sous le nom de CL-20, est un nouveau composé énergétique de la même classe chimique et qui a été synthétisé récemment par Neilsen et al. (1987, 1990). Le CL-20 est un nitramine hétérocyclique qui, comme le RDX et le HMX, contient des liens N-NO_2 fonctionnels qui caractérisent les propriétés thermiques et chimiques des explosifs. Le RDX et le HMX sont tous les deux des oligomères cycliques du méthylènenitramine, soit CH_2-N-NO_2,$(CH_2$-N-$NO_2)_3$ pour le RDX et CH_2-N-NO_2 pour le HMX, tandis que le CL-20 contient des unités répétitives de CH-N-NO_2 (Nielsen et al., 1998). Ainsi, ces trois composés énergétiques devraient réagir de façon similaire au niveau chimique et enzymatique. Par contre, le CL-20 présente une structure en cage tridimensionnelle, tandis que le RDX et le HMX ont des structures bidimensionnelles. Cette différence structurelle pourtant minime pourrait donner lieu à d'importantes différences au niveau des réactions chimiques et biochimiques et du type de produits de dégradation. Par exemple, il a été démontré qu'en conditions abiotiques et biotiques, un clivage initial d'un des liens N-NO_2 du RDX et du HMX affaiblit significativement les liens internes C-N, aboutissant au clivage subséquent de l'anneau et à la formation de produits gazeux. Par contre, dans le cas du CL-20

tridimensionnel, il a été suggéré que le clivage thermique des liens N-NO_2 et la formation subséquente de radicaux libres peut stabiliser les liens internes C-N grâce aux liens intermoléculaires et à leur polymérisation (Patil et Brill, 1991). La production et l'utilisation massives du RDX et du HMX ont conduit à une contamination importante du sol et des eaux souterraines (Hans et al., 1990 ; Myler et Sisk, 1991). Malgré le fait que le RDX et le HMX sont tous deux très peu solubles dans l'eau (45 et 5 mg/L respectivement), il est possible qu'ils soient lessivés à travers le sol et ainsi causer une contamination de l'eau souterraine. Le CL-20, étant probablement moins soluble dans l'eau, pourrait être également lessivé de la surface du sol vers la zone vadose et l'eau souterraine. Qui plus est, il a été démontré que le RDX et le HMX sont tous deux modérément toxiques aux microorganismes indigènes du sol (Gong et al., 2001 ; Sunahara et al., 2001), aux organismes aquatiques (Sunahara et al., 2001 ; Talmage et al., 2001), aux vers de terre (Robidoux et al., 2000, 2001) et aux mammifères (USACHPPM, 2001).

La contamination des sols par des substances énergétiques est surtout retrouvée sur des sites militaires et est reliée aux activités de fabrication, d'utilisation et de la mise au rebut des munitions. Les installations d'entraînement militaire requièrent de grandes superficies de terrain vierge et à accès limité. Ces grandes superficies de terrain représentent des habitats de choix pour les espèces d'oiseaux migratoires et sédentaires. L'ingestion intentionnelle de sol est courante chez les oiseaux puisqu'elle aide à la digestion, ce qui constitue une voie d'entrée privilégiée des résidus d'explosifs dans l'animal. Le CL-20 étant moins soluble dans l'eau (solubilité dans l'eau = 3.5 mg/l @ 25°C ; log K_{ow} = 1.92 ; Monteil-Rivera, 2004), il pourrait être ingéré plus facilement par les oiseaux que le HMX ou le RDX. De plus, sa migration vers l'extérieur des sites contaminés se ferait plus lentement. Malgré le fait que les substances énergétiques représentent la source principale de contamination de milliers de sites militaires dans le monde, très peu d'étude ont porté sur la toxicité du CL-20

(Bardai et al., accepté) et du RDX (Gogal, 2002) chez les oiseaux. Ainsi, avant de commencer sa fabrication à grande échelle et son utilisation pour fin militaire, plus d'informations concernant les effets toxiques du CL-20 et son devenir environnemental sont requises. Il est également possible que l'utilisation militaire du CL-20 à grande échelle puisse conduire à une importante contamination écologique. Afin d'évaluer l'impact environnemental du CL-20, nous proposons d'effectuer une étude de toxicité sur les oiseaux.

1.1 RDX

1.1.1 Provenance et utilisation

Le RDX est un produit explosif communément utilisé dans la fabrication des détonateurs, des grenades, des bombes et d'autres produits d'artillerie militaire. Au niveau de sa structure chimique, ce composé (CAS no. 121-82-4) est un triazine trinitro-substitué dont la formule chimique est $C_3H_6N_6O_6$ (ATSDR, 1995). L'importance du RDX en tant que contaminant environnemental est reliée à sa grande utilisation sur les sites militaires et à sa toxicité potentielle sur la faune et les autres récepteurs écologiques.

1.1.2 Devenir environnemental, migration dans le sol et profil de toxicité

Durant les premières années de la Deuxième Guerre Mondiale, le RDX de qualité militaire (contenant environ 10% de HMX) a été largement utilisé en remplacement ou comme supplément du trinitrotoluène (TNT) dans la fabrication des obus, des bombes et des détonateurs. Les procédés de fabrication et d'assemblage alors utilisés ont conduit au déversement massif d'explosifs à l'état pur ou sous forme de mélange avec d'autres explosifs. Talmage et al. (1999) ont rapporté qu'une concentration allant jusqu'à 30 mg/L de RDX a été mesurée dans l'eau souterraine près de l'usine de fabrication de munitions de Milan (Milan Army Ammunition Plant). Des concentrations de 13,900 mg RDX/kg ont aussi été mesurées par Talmage

et al. (1999) sur ce site ainsi que sur d'autres sites militaires. Les propriétés physico-chimiques du RDX ayant une importance environnementale sont présentés au Tableau 1.0.

Tableau 1.0 Sommaire des propriétés physico-chimiques du RDX

Poids moléculaire	222.26
Couleur	Blanc
État physique	Solide cristallin
Point d'ébullition	Se décompose
Point de fusion	205-206 °C
Solubilité dans l'eau	38.4 mg/L
Log K_{ow}	0.87
Log K_{oc}	0.84-2.2
Pression de vapeur à 20°C	1.0 X 10^{-9}, 4.0 X 10^{-9} mm Hg
Constante de la loi de Henry	1.2 X 10^{-5}atm.m^3/mole

Références; ATSDAR, 1995; Talmage et al., 1999; HSDB, 2000

La photolyse est un processus important de dégradation du RDX puisque ce composé peut absorber fortement la lumière ultraviolette ayant longueur d'onde de 240 à 250 nm. Par ailleurs, la biodégradation anaérobie du RDX a été démontré à l'aide de plusieurs souches microbiennes et de cultures mixtes, ce processus de dégradation étant complété en moins de 5 jours. Lorsque le RDX est incubé en condition anaérobie en présence de boues usées et de cultures mixtes, la disparition du RDX est accompagnée de la formation de plusieurs métabolites tels que l'hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), l'hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), l'hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX), l'hydrazine, 1,1-diméthyl-hydrazine, 1,2-diméthyl-hydrazine, la formaldéhyde et le méthanol (McCormick et al., 1981, 1984).

1.1.3. Toxicité aviaire aiguë – exposition par voie orale

À notre connaissance, une seule étude a porté sur la toxicité du RDX chez les oiseaux. Gogal et al. (2001) a étudié la toxicité aiguë, sub-aiguë et sub-chronique du RDX chez le colin de Virginie (*Colinus virginianus*). Le RDX fut administré aux oiseaux par voie orale dans un diluant aqueux. Un mâle et une femelle par groupe ont reçu les doses suivantes : 125, 187, 280, 420, 630, 945, 1417, ou 2125 mg RDX/kg. Les doses létales approximatives (ALD) après 14 jours d'exposition étaient de 280 mg/kg pour les mâles et de 187 mg/kg pour les femelles.

1.1.4 Toxicité aviaire subaiguë – exposition par voie orale

Des groupes de six mâles et de six femelles de colins de Virginie ont été exposés pendant 14 jours à du RDX ajouté à la nourriture aux concentrations de 0, 125, 187, 280 et 420 ppm (Gogal et al., 2001). Les doses journalières calculées étaient de 10.8, 13.4, 22.3 et 26.3 mg RDX/kg poids corporel, respectivement.

Les résultats indiquent que la quantité de nourriture ingérée et le poids corporel ont diminué de façon inversement proportionnelle à la quantité de RDX administrée. Les ratios du poids de la rate / poids corporel des femelles et du poids du foie / poids corporel des deux sexes ont également été significativement affectés, ces deux ratios augmentant généralement de façon proportionnelle à la dose de RDX. Les effets hématologiques observés suite à l'exposition au RDX étaient une augmentation d'hématocrites chez les femelles, une diminution des protéines plasmiques totales chez les femelles, une augmentation des hétérophiles et une augmentation du ratio hétérophiles / lymphocytes dans le sang. La production d'œufs a diminué de façon inversement proportionnelle à la dose de RDX administrée. Les auteurs ont calculé une concentration sans effet adverse observé (NOAEL) de 8.7 mg RDX/kg/jour et une concentration minimale produisant un effet adverse observé (LOAEL) de 10.6 mg

RDX/kg/jour. Ces valeurs sont basées sur les diminutions du poids corporel et de la production d'œufs.

1.1.5 Toxicité aviaire sub-chronique – exposition par voie orale

Cinq groupes de 10 mâles et femelles de colins de Virginie ont été nourri pendant 90 jours avec 0, 125, 187, 280 ou 420 ppm de RDX ajouté à leur nourriture (Gogal et al., 2001). Les doses orales journalières calculées étaient de 0, 10.8, 13.4, 22.3 et 26.3 mg/kg, respectivement. Les paramètres mesurés étaient les mêmes que ceux mentionnés pour l'étude effectuée sur 14 jours. Malgré le fait que les doses utilisées dans les études sub-aiguës et sub-chroniques aient été les mêmes, aucun effet adverse significatif n'a été observé. Par contre, des tendances apparentes ont été observées pour certains paramètres, tels qu'une diminution de la consommation de nourriture, une diminution des protéines totales, une diminution d'hématocrites et une diminution de la production d'œufs. Aucun effet grave n'a cependant été observé. Étant donné qu'aucun effet significatif du RDX n'a été observé suite à son exposition durant 90 jours, aucune concentration minimale produisant un effet adverse observé (LOAEL) n'a été rapportée.

1.2 HMX

1.2.1 Provenance et utilisation

Le HMX (High Melting Explosive) est un des nombreux composés déversés dans l'environnement au cours de la fabrication, de la manutention, de l'assemblage et de l'emballage des explosifs aux usines de fabrication de munitions militaires (AAPs) et aux autres installations militaires. Au niveau de sa structure chimique, ce produit (CAS no. 2691-41-0) est un composé hétérocyclique avec huit anneaux complètement nitrés dont la formule chimique est $C_4H_8N_8O_8$. Le HMX est aussi connu sous les noms de cyclotétraméthylènetétranitramine, octahydro-1,3,5,7-tétranitro, 1,3,5,7-tétrazocine et d'octogène. L'importance du HMX en tant que contaminant

environnemental est reliée à sa grande utilisation sur les sites militaires et à sa toxicité potentielle sur la faune et les autres récepteurs écologiques.

1.2.2 Devenir environnemental, migration dans le sol et profil de toxicité

Le HMX est un explosif plus puissant que le trinitrotoluène (TNT) et est utilisé comme détonateur d'arme atomique (fission) et comme matériau de base des explosifs plastiques et des combustibles de fusée (ASTSDR, 1997 ;USEPA, 1998). La fabrication du HMX est limitée à un seul endroit aux Etats-Unis, soit à l'usine Holston à Kingsport, au Tennessee. Jusqu'à 22 kg/jour ont été déversé dans les cours d'eau environnant cette usine via les eaux usées des usines de fabrication et de manutention. Des concentrations allant jusqu'à 3.36 mg/L ont été mesurées dans les effluents provenant de l'usine Holston (Talmage et al., 1999). Des déversements de HMX ont aussi eu lieu près des bâtiments où les munitions sont assemblées, entreposées ou testées. Des concentrations allant jusqu'à 5700 mg/kg ont été mesurées dans le sol de certains de ces sites (Talmage et al., 1999). Les propriétés physico-chimiques du HMX ayant une importance environnementale sont présentés au Tableau 1.1.

Tableau 1.1 Sommaire des propriétés physico-chimiques du HMX

Poids moléculaire	296.16
Couleur	Blanc
État physique	Solide cristallin
Point de fusion	276-280 °C
Solubilité dans l'eau	5 – 6.63 mg/L à 20-25 °C
Log K_{ow}	0.06, 0.26
Log K_{oc}	0.54
Pression de vapeur à 20°C	3.3 X 10^{-14} mm Hg
Constante de la loi de Henry	2.60 X 10^{-15}atm.m^3/mole

Références: ATSDAR, 1995; Talmage et al., 1999; HSDB, 2000.

Sa pression de vapeur et sa constante de Henry étant relativement basses (3.33 X 10^{-14} mm Hg et 2.60 X 10^{-15} atm.m^3/mole, respectivement), le HMX ne se volatilise pas vraiment dans l'air. Par contre, la dispersion aérienne du HMX adsorbé au sol et aux particules de poussière constitue tout de même une voie d'entrée de ce composé dans l'atmosphère (ASTDR, 1997). Ayant un coefficient de partition logarithmique du carbone organique-eau dans le sol relativement faible (log K_{oc} = 0.54), le HMX a une mobilité potentiellement élevée dans le sol et peut être ainsi lessivé jusqu'à la nappe phréatique. Des concentrations de 4.2 mg HMX/ L ont été mesurées dans les eaux souterraines de l'usine de fabrication de munitions de la Louisiane (Talmage et al., 1999). Tout comme pour le RDX, la photolyse semble être le processus de dégradation privilégié du HMX dans l'environnement, avec un taux de photolyse de premier ordre de 0.15 $jours^{-1}$ (USEPA, 1988). Ceci suggère qu'une concentration aqueuse de 0.5 mg HMX /L aurait une demi-vie de 4-5 jours lorsque le HMX est exposé à la lumière du soleil. Les produits de dégradation primaires de ce processus de dégradation sont les nitrates, les nitrites et la formaldéhyde. Par contre, les processus de biodégradation/biotransformation bactériennes ou microbiennes sont extrêmement faibles, même si la formation de 1,1-diméthyl hydrazine a été démontrée en condition anaérobie (USEPA, 1988).

1.2.3 Toxicité aviaire aiguë – exposition par voie orale

Une étude portant sur l'estimation de la dose létale approximative (ALD) a été réalisé avec 16 colins de Virginie (*Colinus virginianus*; Gogal et al., 2001). Les oiseaux ont été gavés par voie orale en utilisant un diluant aqueux et huit doses allant de 125 à 2125 mg de HMX/kg de poids corporel. Un oiseau de chaque sexe a été utilisé pour chaque dose testée. Une seule mortalité a été observée (une femelle exposée à 187 mg HMX/kg) après 6 jours de post-exposition. Il n'y avait pas de signe de toxicité aiguë. Une étude subséquente a été réalisée avec 8 oiseaux, soit 4 groupes d'oiseaux et des doses allant de 3188 à 10760 mg/kg. Une femelle exposée à 7173

mg/kg est morte après 12 jours de post-exposition. Aucun effet n'a pu être relié à l'exposition du HMX. La pureté du composé était de 98.5%. Une troisième étude a été réalisée en utilisant de l'huile de maïs comme diluant du HMX suivi d'une période de jeûne, mais aucune mortalité n'a été observée. Suite à l'autopsie des oiseaux, les auteurs ont indiqué que le jabot des oiseaux contenait tout le HMX introduit par gavage, suggérant que le HMX n'a pas été absorbé. Une étude sub-chronique présentement en cours semble confirmer qu'aucun effet nocif ait été observé chez les oiseaux exposés à 10,000 ppm dc HMX ajouté à la nourriture (Gogal, communication personnelle), suggérant que le HMX n'est pas vraiment disponible par absorption.

1.3 Caille japonaise

Les espèces et les sous-espèces du genre *Coturnix* sont des espèces indigènes présentes sur tous les continents sauf en Amérique. L'espèce *Coturnix coturnix japonica* a été intoduite en Amérique en 1870. Les caractéristiques des *Coturnix*, tels que leur capacité de produire 3 à 4 générations par année, en font un animal de laboratoire idéal pour des essais toxicologiques. Dans les meilleures conditions, la ponte des œufs débutent à l'âge de 35 jours, l'âge optimal étant à 50 jours, pour une moyenne annuelle de 250 œufs pondus par année. L'espèce *Coturnix* est relativement peu coûteuse à maintenir en laboratoire étant donné que 8 à 10 cailles peuvent occuper l'espace équivalent à un poulet. Les cailles sont des espèces de laboratoire dociles et plusieurs bases de données écotoxicologiques sont disponibles. Cette espèce est utilisée dans plusieurs tests de toxicité standardisés (ASTM 1997 ; USEPA, 1996a , 1996b ; OECD, 1984a, 1984b). Ceci indique donc que les données générés lors d'essais toxicologiques pourraient être utilisées dans des analyses de risques effectuées par des agences gouvernementales.

1.4 Métabolisme des drogues chez l'oiseau

Les oiseaux, comme les mammifères, ont la capacité de métaboliser les composés xénobiotiques tels que les drogues, les polluants, les pesticides ainsi que les composés endogènes (i.e. les stéroïdes et les acides gras), principalement à l'aide de l'enzyme hépatique cytochrome P450 (P450) et du glutathion *S*-transférase (GST) (Coulet et al., 1996 ; Maurice et al., 1991). Très peu d'information est disponible sur l'expression des isoenzymes GST présents chez la caille japonaise (Fouts et Pan, 1978 ; Dai et al., 1996), et aucune information n'est disponible sur le métabolisme des explosifs.

1.5 Les glutathion *S*-transférases

Les organismes vivants ont développé différents systèmes enzymatiques permettant une interaction avec les produits toxiques et les xénobiotiques. Ces enzymes sont divisés en deux systèmes enzymatiques, soit les enzymes de Phase I (réactions d'oxydation, de réduction et d'hydrolyse) et de Phase II (réactions de conjugaison).

Un des groupe d'enzymes de Phase II sont les isoenzymes de glutathion *S*-transférases (GST E.C. 2.5.1.18) que l'on retrouve chez plusieurs espèces vivantes, tels que chez les microorganismes, les insectes, les plantes, les poissons, les oiseaux et les mammifères (Hayes et Pulford, 1995). Les transférases sont impliqués dans différents types de réactions en catalysant la conjugaison du glutathion réduit (GSH) à des composés contenant des centres électrophiles via la formation de lien thioéther entre l'atome de soufre du GSH et le substrat (Chasseaud, 1979). Les glutathion *S*-transférases constituent un groupe complexe de protéines divisés en deux superfamilles d'enzymes possédant des activités enzymatiques complètement différentes (Hayes et Strange, 2000). La première superfamille présente chez les mammifères est divisée en huit familles classifiées selon leurs séquences respectives

et dénommées Alpha (α), Mu (μ), Pi (Π), Sigma (σ), Theta (θ), Zeta (ζ), Omega (ω) et Kappa (κ). La deuxième superfamille définie plus récemment est composée de transférases microsomales. Cette superfamille regroupe des protéines associées à la membrane impliquées dans le métabolisme de l'eicosanoide et du glutathion (en anglais, MAPEG). Chez les humains, la superfamille MAPEG est composé d'au moins six enzymes (Jakobsson et al., 2000).

Les GST solubles semblent être impliqués dans le métabolisme des xénobiotiques, des polluants environnementaux, des produits cancérigènes, de même que dans la détoxification des réactifs endogènes (Hayes et Pulford, 1995). Les enzymes MAPEG ne sont pas vraiment impliquées dans les réactions de détoxification mais plutôt dans la biosynthèse des leukotriènes et de prostanoïdes, qui sont des molécules lipidiques de signalisation endogènes (Jakobsson et al., 1999a).

1.5.1 Fonctions des enzymes glutathion *S*-transférases

Les GST sont généralement considérés comme des enzymes de Phase II jouant un rôle majeur dans la métabolisation des drogues. Ils contribuent à la survie de la cellule via le mécanisme de détoxification des composés xénobiotiques et catalysent la réaction générique suivante : GSH + R-X → GSR + HR. La fonction de cette réaction enzymatique est (1) de transporter le substrat près du glutathion (GSH) en liant le GSH et le substrat électrophile au site actif de la protéine et (2) d'activer le groupe sulfhydryl du GSH, permettant ainsi l'attaque nucléophile du GSH sur le substrat électrophile (R-X) (Eaton et Bammler, 1999).

La fonction première du domaine N-teminal des GST est de fournir un site hydrophile pour activer le groupe sulfhydryl du GSH (GSH → GS•) et ainsi créer un nucléophile. Ce domaine N-terminal est la région la mieux conservée de tous les enzymes cytosoliques et contient le site de liasion au glutathion (G) et un site adjacent

(H) qui fournit un environnement hydrophobe permettant la liaison entre les composés électrophiles de structures différentes et les nucléophiles GS• (Armstrong, 1997). La conjugaison du glutathion augmente de façon importante la solubilité des métabolites par rapport aux composés mères. Les métabolites sont excrétés à l'extérieur de la cellule par le mécanisme de transport actif de la protéine résistance à des drogues multiples (rdm).

1.6 Conclusion

L' hexanitrohexaazaisowurtzitane (HNIW) ou CL-20 est un nitramine polycyclique de haute densité qui est présentement à l'étude pour fin d'application militaire. Le RDX et le HMX sont des explosifs utilisés depuis plus de 30 ans et représentent une source importante de contamination de milliers de sites militaires à travers le monde.

La contamination des sols par des composés énegétiques est surtout retrouvée sur les bases militaires et est reliée à la fabrication, la manutention et la mise au rebus des munitions. Les bases militaires requièrent de grande surface de terrain vierge à accès limité pour leurs activités opérationnelles et d'entraînement. Ces grandes superficies de terrain vierge constituent un habitat idéal pour la faune aviaire sédentaires et migratoires. L'ingestion intentionnelle de sol est courante chez les oiseaux puisqu'elle aide à la digestion, ce qui constitue une voie d'entrée privilégiée des résidus d'explosifs dans l'animal. Des études écotoxicologiques sub-chronique ont été réalisées avec le colin de Virginie. Le CL-20 étant moins soluble dans l'eau (solubilité dans l'eau = 3.5 mg/l @ 25°C ; log K_{ow} = 1.92 ; Monteil-Rivera, 2004), il pourrait être ingéré plus facilement par les oiseaux que le HMX ou le RDX. De plus, sa migration vers l'extérieur des sites contaminés se ferait plus lentement.

Ainsi, avant que ne survienne la production massive de CL-20, son utilisation pour fin militaire et son éventuel déversement dans l'environnement, il est nécessaire de déterminer le devenir environnemental et les effets toxiques de ce nouveau composé énergétique. Les données toxicologiques présentées dans le cadre de ce projet pourront aider les futurs gestionnaires de sites contaminés avec du CL-20 à prendre des décisions plus éclairées quant à l'utilisation de ce nouvel explosif dans l'environnement.

CHAPTER II

Effects of Dietary Administration of CL-20 on the Japanese quail (*Coturnix coturnix japonica*)

Ghalib Bardai,[1,2] Geoffrey I. Sunahara,[1,*] Philip A. Spear,[2]
Majorie Martel,[1] Ping Gong,[1,#] and Jalal Hawari,[1]

[1]Biotechnology Research Institute, National Research Council of Canada,
6100 Royalmount Avenue, Montreal, Quebec, Canada, H4P 2R2

[2]Centre de Recherche TOXEN, Département des Sciences Biologiques,
Université du Québec à Montréal, CP. 8888, succursale Centre-ville,
Montréal, Québec, Canada, H3C 3P8

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* Corresponding Author:

Geoffrey I. Sunahara, Ph.D.
Biotechnology Research Institute
National Research Council of Canada
6100 Royalmount Ave.
Montreal, QC, Canada, H4P 2R2
Tel: (514)-496-8030
Fax: (514)-496-6265
geoffrey.sunahara@cnrc-nrc.gc.ca

Current address: Analytical Services, Inc., 3909 Halls Ferry Road, Vicksburg, MS 39180, USA

2.1 Abstract

Hexanitrohexaazaisowurtzitane or CL-20 is an emerging highly energetic compound currently under consideration for military applications. With the anticipated wide use of CL-20, there is the potential for soil and groundwater contamination resulting in adverse toxicological effects on environmental receptors. Presently, there is a lack of data describing the toxic effects of CL-20 on avian species. The present studies describe the effect of CL-20 on Japanese quail (*Coturnix coturnix japonica*) modified from standard toxicity test guidelines. Firstly, a 14-day subacute assay was adopted using repeated gavage doses (0, 307, 964, 2439, 3475 or 5304 mg CL-20/kg body weight (BW)/d) for 5 d followed by no CL-20 exposure (vehicle only) for 10 d; and secondly, a subchronic feeding assay (0, 11, 114, or 1085 mg CL-20/kg feed) was followed for 42 d. During both studies, no overt toxicity was observed in the CL-20 treated birds. During the first 5 d of the subacute study, CL-20 exposed birds showed a dose-dependent decrease in BW gain; whereas increased liver weight, plasma sodium and creatinine levels were observed in birds receiving the highest dose tested. For the subchronic study, embryo weights were significantly decreased in a dose-dependent manner. Embryos from CL-20 exposed birds were observed to have multiple (both cranial and facial) deformities, beak curvatures, possible mid brain enlargement, and classical one-sided development with micro-opthalamia (non-statistical comparisons with control embryos). A trend towards decreased number of eggs laid per female was also observed. We conclude that CL-20 (or its degradation products) elicits few effects in adults but may affect avian development, although these preliminary findings should be confirmed.

2.2 Introduction

Hexanitrohexaazaisowurtzitane (HNIW) or CL-20 (Fig. 2.1) is a high-density polynitro compound that is currently under consideration for military application. CL-20 is envisaged to deliver 10 to 20% higher performance than octahydro-1,3,5,7-tetranitro-1,3,5,7–tetrazocine (HMX) and better performance than hexahydro-1,3,5-trinitro-1,3,5–triazine (RDX) (Nielsen *et al.* 1998; Geetha *et al.* 2003). RDX and HMX are common contaminants found in soil at military-related sites, and the toxicity of these nitramines to terrestrial vertebrate species such as mammals, amphibians and birds is becoming better known (Schneider *et al.* 1976; Levine *et al.* 1981; Talmage *et al.* 1999; Gogal *et al.* 2003; Johnson *et al.* 2004). Gogal *et al.* (2003) found the approximate lethal dose of acute oral RDX exposure in northern bobwhite (*Colinus virginianus*) to be 280 mg/kg body weight (BW) for males, and 187 mg/kg BW for females. The dose-dependent effects of RDX also included a decrease in food intake, body weight, and spleen and liver mass for 14 days but not for 90 days of exposure. Although the effects of CL-20 in plant and soil invertebrate species have been recently reported (Gong *et al.* 2004; Robidoux *et al.* 2004), there are no data available on the toxicity of CL-20 in birds.

CL-20 is a polycyclic nitramine characterized by having N-NO_2 bonds, similar to the two monocyclic nitramines RDX and HMX. Based on these structural similarities, we hypothesized that the effects of CL-20 to birds will be similar to those of RDX. In the present study, we investigated the toxic effects of CL-20 on the gallinaceous test species, the Japanese quail (*Coturnix coturnix japonica*) exposed to repeated daily doses of CL-20. We selected this species because it is amenable to laboratory toxicity testing, and species of this order can have significant dietary exposure to soil based on their foraging and behavioral habits. This species are used in many standard toxicity testing regimes for standard avian toxicity testing (ASTM 1997; USEPA 1996a, 1996b; OECD 1984a, 1984b). Data generated using these standardized toxicity test

methods will be useful for the ecotoxicological risk assessment of birds exposed to CL-20.

2.3 Materials and Methods

2.3.1 Chemicals and reagents. CL-20 (CAS# 135285-90-4) in the ε form (purity > 95%) was obtained from ATK Thiokol Propulsion (Brigham City, Utah, USA). Acetonitrile and acetone (high performance liquid chromatography, HPLC grade) were obtained from EM Science (Darmstadt, Germany). All other chemicals and reagents were of the highest grades of purity available, and were obtained from Anachemia (Milwaukee, WI, USA). Deionized water was obtained using a Zenopure Mega-90 water purification system. All glassware was washed with phosphate-free detergent, rinsed with acetone, and acid-washed before a final rinse with deionized water.

2.3.2 Raising of test species. Fertilized Japanese quail (*Coturnix coturnix japonica*) eggs (n= 70 for subacute study, and n= 120 for subchronic study) obtained from a local breeder (Couvoir Simetin, Mirabel, PQ, Canada) were placed in an environment-controlled incubator at 37 ± 1°C (SD), 65 ± 5% relative humidity, and turned 180° every fourth hour. Three days before hatching, rotation of eggs was stopped and the relative humidity raised to 70%. Eggs were grouped at the center of the incubator, on a 5-mm mesh until they hatched. When dry, quail chicks were transferred to a heated brooder (35°C achieved using an electric resistance element), under constant illumination with red incandescent light to prevent eye damage. Birds were fed a diet of powdered Turkey Starter (Purina Mills, St Louis, MO, USA) and tap water *ad libitum*, until 14 days of age. Quail were then maintained under controlled conditions of temperature (22.0 ± 3.0°C), relative humidity (50.0 ± 6.0%), and lighting (14:10 h, light:dark cycle).

2.3.3 Experimental design. The subacute study (Fig. 2.2A) described in this article was modified from the USEPA (1996a) and OECD (1984a) guidelines. Forty-four 2-week old quail were weighed, banded, and observed for any abnormalities. A blood sample (1 ml) was taken from the jugular vein (described below) and each bird was then randomly assigned to six groups (n = 7-8 per group, 44 total). Animals were housed together in stainless steel cages (61 cm width x 76 cm length x 41 cm high; 10-mm mesh floor) (2-3 individuals per cage) and were uniquely identified by a leg band. Two days later, birds were gavaged daily for 5 d with pulverized commercial feed (Ralston Purina Turkey Grower) containing CL-20. Briefly, the feed was pulverized using a hand-held blender until a granular consistency was formed, before the addition of CL-20. The CL-20 contaminated feed was prepared daily, and the doses were adjusted to the body weight that was taken daily during the dosing period (1% of BW). To deliver the contaminated feed, the desired quantity was transferred into a 5 ml syringe, and then 1 ml water was added to the syringe to form a slurry. A 10-gauge stainless steel curved feeding tube equipped with a Luer Lock fitting (Becton Dickinson, Canada) onto the syringe was inserted down the esophagus to the crop. The slurry was administered gently to ensure minimal regurgitation. The calculated doses delivered (mg CL-20/kg BW) were: 307 ± 1, 964 ± 8, 2439 ± 12, 3475 ± 5, and 5304 ± 53. A second blood sample (from 1 to 1.5 ml) was collected 5 d after completion of the dosing. At the end of the experiment (Day 14 of study), a third blood sample (5 ml) was procured and the organs (heart, brain, liver, and spleen) were weighed and were frozen at –80°C for residual CL-20 analysis. The somatic index (%) was calculated as the wet organ weight (g) per 100 g body weight.

The subchronic study (Fig. 2.2B) described in this article was modified from the USEPA (1996b) and OECD (1984b) guidelines. The lighting period was changed (16:8 h light: dark cycle) to provide optimal reproductive conditions when the quail were 2 weeks old. The birds (n = 48) were then randomly assigned to each of the four dose groups (described below) in triplicate. Each exposure was conducted in

triplicate, each group of 3 females and 1 male were housed together in stainless steel cages (described above). Quail were uniquely identified by a leg band. During this period, close surveillance was required to monitor any signs of increased aggression. Individuals injured or demonstrating incompatible behavior relative to the group were replaced. Eggs were collected and incubated during the pre-exposure period to verify fertility and thus ensure that only proven breeders were included in this study. Two days prior to the start of feeding CL-20 diets when quail were 54 d old, a blood sample (2 ml) was taken from each bird. The diets were prepared by mixing CL-20 (previously dissolved in acetone) with commercial feed (Ralston Purina Turkey Grower) to achieve nominal concentrations of 10, 100, and 1000 mg CL-20/kg feed. The vehicle control diets were treated with similar quantities of acetone without CL-20 as described above. Acetone was allowed to evaporate, and the CL-20 feed mixture was then placed in glass jars and stored at 4.0 ± 2.0°C in the absence of light. Measured concentrations of CL-20 in the feed were analyzed using HPLC (11 ± 1, 114 ± 26, and 1085 ± 52 mg CL-20/kg feed corresponding to the target exposure groups of 10, 100, and 1000 mg/kg BW, respectively), and were found to be consistent throughout the study. All feed was provided daily *ad lib*. Daily feed consumption was estimated as the difference between initial and final feed weight plus the quantity of unconsumed pellets recovered from the litter pans, which was determined daily for the first 2 weeks of the study, and then every alternate day.

A subsample of eggs (based on total eggs collected, n= 15-20 per day) were collected and frozen at -80°C; the remaining eggs (n= 10-12 collected per day) were stored in a refrigerator (16 ± 1°C and 60-70% humidity) for 5 d. Eggs of the latter group were then incubated at 37.8°C and 60% relative humidity. Following eight days of incubation, the eggs were cooled to 4°C, and embryos were then removed and weighed. Embryos were then preserved in capped vials containing 10% formalin, and were stored at room temperature in the dark until further evaluation of developmental effects. Embryos were evaluated according to the Hamburger-Hamilton system (1951)

for normal stages of chick embryo development. At the end of the 6-week exposure period, the adult birds were sacrificed and examined for gross lesions. Liver, spleen, heart, and brain were excised, weighed, frozen in liquid nitrogen, and then stored at -80°C prior to residual CL-20 analyses.

For both studies, birds were monitored daily for changes in health or disposition (*i.e.*, alertness, appearance). For the subacute study, body weight gain was measured daily for the duration of the gavaging phase, and then at study days 10 and 14. Body weight and feed consumption for the subchronic study were measured daily for a 2 week period, and then every alternate day. At the end of the experiments, birds were anesthetized (2-5% isoflurane), and euthanized (90% CO_2 / 10% O_2). Moribund animals were treated in the same fashion. Manipulation and handling were in accordance with the Canadian Council on Animal Care guidelines (1984).

2.3.4 Blood collection and analysis. For clinical chemistry and hematological analyses, female quail blood was collected from the jugular vein with a 27-gauge needle and 3-ml syringe containing 100 µl of a 1% EDTA solution in saline. Males were not considered for this study because of the low number of males per exposure group (1 male per 3 females per replicate, 3 replicates per exposure group). Blood was thoroughly mixed and 250 µl was removed for whole blood analysis. Plasma was prepared in 1.5 ml Eppendorf tubes centrifuged at 13,000 x g for 5 min, and was used either fresh for clinical chemistry analysis to screen for biochemical and pathological abnormalities, or was stored at -80°C for plasma residual CL-20 analysis. Quail plasma was analyzed to determine its clinical chemistry profile (Beckman LX20 Pro, Rochester, NY) which included sodium (Na), potassium (K), chloride (Cl), glucose (Glu), creatinine (Cre), total protein (TP), phosphorus (PO_4), total bilirubin (Tbili), direct bilirubin (Dbili), uric acid (Uric), magnesium (Mg), cholesterol (Chol), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase

(ALP), amylase (AMY), lactate dehydrogenase (LDH), and triglycerides (TG). For hematological analysis, a drop of anticoagulated blood was placed on a microscope slide and a smear was made. The blood smear was fixed in methanol, stained with modified Wright-Giemsa and the leukocyte differential was performed where 100 leukocytes (lymphocytes, heterophils, monocytes, basophils, and eosinophils) were enumerated. Heterophil to lymphocyte ratios were calculated to investigate stress. For hematocrit (HCT) determination, a non-heparinized hematocrit capillary tube was filled with anticoagulated blood. The bottom of the capillary tube was sealed with a clay plug, and centrifuged for 5 min at 3000 rpm, and the HCT was determined as the ratio between total capillary volume and the packed cell volume (PCV).

2.3.5 CL-20 extraction from quail tissue. CL-20 extractions were performed according to USEPA Method 8330A (USEPA 1998) with the following modifications. For CL-20 exposure studies, whole organs taken from test animals were immediately homogenized on ice in 7 ml cold acetonitrile using a Polytron tissue homogenizer (30 s for brain and spleen, or 1 min for heart and liver) in Teflon tubes. Following overnight sonication in the dark at 8°C (60 Hz; Branson 3200, Danbury, CT, USA), samples were centrifuged at 4°C for 10 min at 10,000 x g. To 5 ml of the decanted supernatant was added an equal volume of a $CaCl_2$ (90 mM)-$NaHSO_4$ (1.6 mM) solution, and the resulting mixture was vortexed for 30 s and stored at 4°C for 2 h to precipitate proteins. The resulting solution was filtered through a 0.45 μm membrane prior to HPLC analysis (described below). To increase the sensitivity of the above method, the supernatants of different tissue samples (liver, heart, brain, and kidney) were also concentrated. Each supernatant (5 ml) was evaporated to dryness in a Speed Vac (Savant, Hicksville, NY, USA). To the precipitate was added 500 μl acetonitrile and vortexed for 1 min, after which the $CaCl_2$ - $NaHSO_4$ solution was added as described above. For the control recovery studies, whole organs (liver, brain, spleen, or heart) were taken from non-treated quail

and were spiked with varying concentrations of CL-20 prior to being immediately frozen at -80°C for 24 h. The organs were then thawed in cold acetonitrile, and CL-20 was extracted as described above.

2.3.6 Extraction of CL-20 from the test diet. Acetonitrile extractions of test diet were also performed using the USEPA Method 8330A (USEPA 1998). One ml of 1.6 mM $NaHSO_4$ prepared in water was added to two grams of feed and vortexed for 10 s. To this was added 10 ml (for feed containing 10 mg CL-20/kg feed) or 20 ml of acetonitrile (for feed containing 100-1000 mg CL-20/kg feed), prior to vortexing for 1 min.

2.3.7 HPLC analysis. In brief, a ThermoFinnigan chromatographic system composed of a Model P4000 pump, a Model AS1000 injector, and a Model UV6000LP photodiode-array detector (λ=230 nm) was used. A Supelcosil LC-CN column (250 x 4.6 mm, 5 µm particles; Supelco, Bellefonte, PA, USA) was used for separation with a column heater set at 35°C. The isocratic mobile phase consisted of methanol/water (70/30, v/v) delivered at 1.0 ml/min. The sample volume injected was 50 µl with a 14-min run time. The limit of quantification was 0.05 mg/L. Relative standard deviation for the instrument precision was < 1.3% for concentrations equal or higher than 0.5 mg CL-20/L, and 7.5% for a concentration of 0.05 mg CL-20/L.

2.3.8 Statistical analysis. The data were evaluated by two-way and repeated measures analysis of variance (ANOVA). Differences between exposure groups and their respective controls were considered significant when $p \leq 0.05$ using the Dunnett's test. Statistical analysis was performed using JMPIN (Version 4, SAS® Institute, Cary, NC, USA).

2.4 Results

2.4.1 Subacute study. No clinical or overt toxic symptoms were observed during the 5-d exposure period to CL-20. The change in average weight gain (expressed as the % of respective control) for each exposure group is shown in Fig. 2.3. Data indicates that a dose-dependent decrease in body weight gain occurred during the first 5 days of this study ($p \leq 0.05$). On the second study day, the CL-20 quail exposure groups $\geq$ 964 mg/kg BW/d gained less weight than the control group ($p \leq 0.05$). However, this effect was not detected by the end of the study (day 14, or 10 d of no CL-20 exposure). Initial body weights prior to exposure were not significantly different between groups (data not shown).

Liver weights were significantly elevated in the highest dose group (5304 mg/kg BW/d), whereas other organ weights (brain and spleen) were not significantly different between CL-20 treated and control groups (Fig. 2.4). Increases in plasma sodium and creatinine levels were found in birds treated with 5304 mg CL-20/kg BW/d, compared with controls (Table 2). Hematological parameters (heterophil to lymphocyte ratios, and hematocrit) in the subacute study were not statistically different between exposure groups when compared to controls (data not shown).

2.4.2 Subchronic study. No clinical or overt toxic symptoms were observed during the 42-d exposure period in this study. Aggressive behavior was noted in one individual who was subsequently isolated from the control group. Another bird from a different control group had to be euthanized due to injuries inflicted by an aggressive control bird (that was hence removed from the triplicate). These observations were not related to exposure. Based on feed consumption (feed consumed per number of individuals in each dose group) and the mean body weight, the calculated daily doses to quail consuming the CL-20 diet were 0, 0.96, 10, and 94 mg CL-20/kg BW, for the measured 0, 11, 114, and 1085 mg CL-20/kg feed exposure groups, respectively. Throughout this study, no differences in feed consumption or body weight were

observed between exposure groups (data not shown). Clinical chemistry analysis of quail blood taken from the subchronic study (Table 2.1) revealed that the aspartate aminotransferase (AST) level was increased by 1.25 times in the birds of the highest measured feed dose level (1085 mg CL-20/kg) relative to controls ($p \leq 0.05$).

Embryo weight was found to decrease significantly and in a dose-dependent manner (Fig. 2.5). Based on the measured exposure concentration, the unbounded lowest observed adverse effect level (LOAEL) for this effect was 11 mg/kg feed. The unintentional evaporation of the preserving medium from some of the storage vials caused a dehydration of the embryos making these samples unsuitable for further analysis. Embryos in vials that still contained formalin were analyzed. In the 114 mg/kg feed exposure group, two of the 6 embryos had multiple (both cranial and facial) deformities. Whereas in the highest dose group (1085 mg/kg feed), three of the 9 embryos experienced beak curvatures, possible mid brain enlargement, and classical one sided development with micro-opthalamia. These CL-20 exposure-related deformities were not observed in the control group; however, two of the nine control embryos showed very slight ocular asymmetric deviations. No decrease in the rate of development was detected, all embryos being at stages 30-31 (Hamburger and Hamilton, 1951). In addition, CL-20 exposure tended to decrease the number of eggs laid per female ($0.48 < p < 0.99$ for the different treatment groups, using the Dunnett's two-sided t-test for multiple comparisons) compared to controls (Fig. 2.6).

2.4.3 CL-20 recovery from tissue. CL-20 was not detected in the plasma or selected organs (brain, spleen, heart and liver) of quail treated with CL-20 (data not shown), despite the excellent recovery of the chemical (99-105 %) using different spiked tissues (Fig. 2.7 and 2.8). This method was specific for CL-20 detection only.

2.5 Discussion

Few acute and subchronic effects were found from oral CL-20 exposures to quail in this study. However, our 42-d subchronic study indicates that CL-20 exposure led to significant decreases in embryo weight without a corresponding effect on developmental stage. This would suggest an effect on energy metabolism rather than a decrease in the rate of embryonic development, *per se*. Although we have evidence that CL20 exposure caused abnormal developmental effects, these results should be considered preliminary until a more rigorous teratological evaluation is done using increased number of replicates. Bushan *et al.* (2004a, 2004b) reported the formation of nitrite (NO_2^-), nitrous oxide (N_2O), ammonia, formic acid, ammonium or glyoxal from the enzymatic biodegradation of CL-20 by the flavin containing enzymes, nitroreductase and salicylate 1-monooxygenase. Quail have been found to possess both Phase I and II biotransformation enzymes (Gregus *et al.* 1983). It is possible that quail may possess similar enzymes capable of degrading CL-20 and that the presence of the resulting degradation products may be related to the observed CL-20 toxicity in our present studies. A preliminary survey of the avian toxicity literature failed to identify studies characterizing the toxic effects of the latter CL-20 degradation products to quail. To further characterize the reported effect of embryo malformation by CL-20 and gain more information about the mechanism of CL-20 toxicity (direct and indirect effects of CL-20 on the embryo), *in ovo* approaches are suggested.

Earlier studies have shown that exposure to the monocyclic nitramine explosive RDX causes central nervous system (CNS) disturbances in birds (northern bobwhite), terrestrial salamanders and mammals (rats and miniature swine) (Schneider *et al.* 1976; Levine *et al.* 1981; Gogal *et al.* 2003; Johnson *et al.* 2004). Rats injected intraperitoneally with 500 mg RDX/kg BW had seizures prior to death. Miniature swine treated intravenously with RDX showed convulsions from 12 to 24 h after exposure. A recent study reported that red-backed salamanders (*Plethodon cinereus*)

exposed to 5,000 mg RDX/kg soil in laboratory microcosms for 28 days showed signs of neuromuscular effects, including lethargy, convulsions, rolling and gaping motions, hyperactivity, as well as a marked weight loss (Johnson *et al.* 2004). In contrast to these earlier studies using RDX, none of the CL-20 treated birds in our present studies exhibited signs of neurotoxicity.

Earlier studies by Gogal *et al.* (2003) showed that RDX in a water vehicle was lethal to the northern bobwhite at a single dose (≥ 187 mg RDX/kg BW) after 72 h exposure. In our subacute study, CL-20 caused no mortality in adult Japanese quail gavaged with up to 5304 mg CL-20/kg BW. These data suggest that CL-20 is not as lethal to galliform species as RDX. Physico-chemical differences between RDX (aqueous solubility at 20°C = 42.58 mg/L, log K_{ow} = 0.90) and CL-20 (aqueous solubility at 20°C = 3.16 mg/L, log K_{ow} = 1.92) (Monteil-Rivera *et al.* 2004) may favor the absorption or bioavailability of RDX relative to CL-20, and may help to explain the differences in acute toxicity.

Increased liver to BW ratios were observed in the highest dose group (5304 mg CL-20/kg BW/d), 10 d post CL-20 exposure, whereas the spleen- and heart- to body weight ratios were not significantly altered compared to controls. This effect contrasts with the earlier results showing decreased liver to BW ratios observed for northern bobwhite exposed to RDX in feed (Gogal *et al.* 2003). Birds exposed to RDX for 14 days caused significant increases in the hematological parameters such as heterophil counts, and the heterophil/lymphocyte ratio, as well as an increase in the packed cell volume (PCV) (Gogal *et al.* 2003). In contrast, our study using CL-20 did not show an effect of exposure on the heterophil/lymphocyte ratio or on PCV, suggesting that CL-20 is not an immunotoxicant, or a mitogen at least during this period of exposure. It is possible that immunological effects may have occurred but were reversed during our exposure schedule that allowed 10-d of no exposure with CL-20.

In conclusion, although CL-20 has certain structural similarities to the monocyclic nitramine RDX (Fig. 1), this polycyclic nitramine does not appear to demonstrate the same profile of toxicities as RDX in quail. Our present study has provided basic toxicity data, and suggests that further research should be carried out to study the developmental effects of CL-20 in birds.

2.5.1 Acknowledgements. We thank Drs. Fanny Monteil-Rivera and Mark S. Johnson for their review of an earlier version of this article. In addition, we thank Manon Sarrazin, Sabine Dodard and Louise Paquet from Biotechnology Research Institute (National Research Council of Canada) for their technical assistance, the US Strategic Environmental Research and Development Program (SERDP Project CP1256) for financial support, and Thiokol Propulsion Inc. for providing the ε-CL-20.

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2.7 Figure Captions

2.1 Molecular structures of the cyclic nitramine explosives CL-20, HMX and RDX

2.2 Schedule for the two CL-20 exposure studies: subacute study (A) and subchronic study (B).

2.3 Changes in body weight of juvenile Japanese quail gavaged with CL-20 for 5 days (subacute study). Exposure schedule is shown in Fig. 2. Each value (expressed as % of control which was set at 100%) is the average ± SEM (standard error)(n = 6 to 10 birds per group). * above bars denotes value is statistically different from the control ($p \leq 0.05$). The average body weights of the control group (g ± SEM) (n = 6 birds) for study days 2, 3, 4, 5, 10, and 14 were: 98 ± 8, 106 ± 6, 116 ± 7, 126 ± 8, 172 ± 8, and 202 ± 9, respectively.

2.4 Somatic index of selected organs of juvenile Japanese quail gavaged with CL-20 for 5 days followed by 10 days of vehicle only (no CL-20). Data are expressed as mean ± SD (n = 44 birds). Study design is shown in Fig. 2. * above bars denotes value is statistically different from the control ($p \leq 0.05$).

2.5 Effects of 42 days dietary exposure of CL-20 on Japanese quail embryo weights. Data are mean embryo weight ± SD. (n = number of embryos evaluated). * above bars denotes values statistically different from the control ($p \leq 0.05$).

2.6 Effects of 42 d dietary exposure of CL-20 on the mean number of eggs produced per hen. These exposure effects were not significant compared to controls ($p > 0.05$).

2.7 Recovery of CL-20 using spiked tissue samples (△, Brain; ◇, Spleen; ■, Heart) and the modified USEPA Method 8330A. Inset shows CL-20 recovery at spiked concentrations less than 0.8 μg CL-20/g tissue dry weight. R^2 value was determined by linear regression using least-squares method.

2.8 Recovery of CL-20 from spiked liver tissue using modified USEPA Method 8330A.

Table 2.1. Selected plasma biochemical parameters of adult Japanese quail exposed to CL-20 by gavage for 5 d followed by 10 days exposure with no CL-20

Parameters	CL-20 Exposure Groups (mg CL-20 per kg body weight per d) [a]					
	0	307	964	2439	3475	5304
PO_4 (mmol/L) [b]	3.22 ± 0.21 [c]	2.89 ± 0.26	2.91 ± 0.26	2.53 ± 0.21	2.73 ± 0.24	2.96 ± 0.39
TP (g/L)	19.2 ± 3.6	22.0 ± 2.6	22.3 ± 2.9	18.4 ± 2.9	20.8 ± 2.8	21.63 ± 3.25
Glu (mmol/L)	16.3 ± 1.0	17.0 ± 0.7	16.7 ± 1.0	16.4 ± 1.2	17.1 ± 1.3	16.36 ± 1.45
Cre (µmol/L)	20.0 ± 2.6	18.6 ± 3.2	20.3 ± 4.0	18.2 ± 5.4	21.8 ± 4.4	25.00 ± 3.16*
Na (mmol/L)	143.9 ± 0.8	145.5 ± 1.0	144.0 ± 0.8	144.6 ± 2.7	149.5 ± 1.6	151.7 ± 2.70*
ALP (IU/L)	592 ± 327	711 ± 253	1056 ± 301	485 ± 396	328 ± 275	509 ± 230
ALT (IU/L)	4.25 ± 1.50	4.20 ± 1.30	5.00 ± 0.82	4.80 ± 0.84	4.50 ± 1.05	4.50 ± 0.76
Uric (µmol/L)	201.3 ± 53.3	239.2 ± 78.1	269.0 ± 134.8	195.0 ± 37.6	216.0 ± 101.8	300.6 ± 68.5
AMY (IU/L)	336 ± 71	460 ± 190	377 ± 197	178 ± 60	436 ± 136	377 ± 193
AST (IU/L)	101.3 ± 11.7	87.3 ± 10.0	94.0 ± 10.1	107.2 ± 21.9	95.5 ± 13.5	101.9 ± 11.8
Chol (mmol/L)	5.32 ± 0.74	4.06 ± 0.91	5.17 ± 1.52	5.63 ± 2.50	4.67 ± 0.98	5.38 ± 1.44
Dbili (µmol/L)	1.25 ± 0.24	0.95 ± 0.21	0.78 ± 0.31	1.40 ± 0.50	0.83 ± 0.27	1.16 ± 0.49
Tbili- (µmol/L)	2.8 ± 0.5	2.6 ± 1.6	4.0 ± 1.2	2.2 ± 1.3	2.3 ± 1.1	3.5 ± 0.9
GGT (IU/L)	6.5 ± 4.5	8.8 ± 2.3	8.0 ± 1.4	8.4 ± 0.9	7.5 ± 2.2	10.0 ± 2.2
LDH (IU/L)	59.8 ± 13.0	48.8 ± 8.4	47.0 ± 9.5	49.2 ± 12.8	41.0 ± 12.7	56.4 ± 28.8
TG (mmol/L)	1.84 ± 0.20	1.42 ± 0.32	2.05 ± 0.91	2.04 ± 0.52	1.49 ± 0.36	2.06 ± 0.78
Mg (mmol/L)	0.15 ± 0.50	0.20 ± 0.01	0.28 ± 0.05	0.11 ± 0.14	0.10 ± 0.04	0.13 ± 0.7

[a] Measured concentrations of CL-20 intake by gavaged quails.

[b] Abbreviations used: Phosphate (PO_4), Total Proteins (TP), Glucose (Glu), Creatinine (Cre), Alkaline phosphatase (ALP), Alanine aminotransferase (ALT), Amylase (AMY), Aspartate aminotransferase (AST), Cholestrol (Chol), Direct Bilirubin (Dbili), Total Bilirubin (Tbili), γ-glutamyltransferase (GGT), Lactate dehydrogenase (LDH), Triglycerides (TG), Magnesium (Mg)

[c] Data are expressed as mean ± SD (standard deviation) (n = 4-8 birds per exposure group).

* Exposure group is significantly different from control using Dunnett's test ($p \leq 0.05$).

Table 2.2 Selected plasma biochemical parameters of adult Japanese quail fed CL-20 in the diet for 42 d

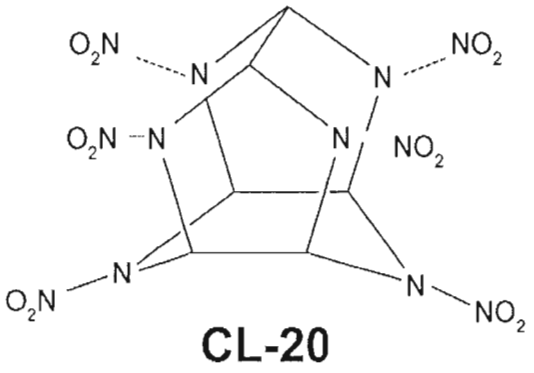
Parameters	CL-20 Exposure Groups (mg CL-20/kg feed) [a]			
	0	**11**	**114**	**1085**
PO_4 (mmol/L) [b]	2.87 ± 0.63 [c]	2.56 ± 0.39	3.03 ± 0.84	2.11 ± 0.71
TP (g/L)	31.6 ± 4.0	30.1 ± 2.3	33.5 ± 1.7	28.3 ± 3.2
Glu (mmol/L)	15.1 ± 0.99	15.4 ± 1.29	14.5 ± 0.8	14.4 ± 0.9
Cre (μmol/L)	30.3 ± 9.6	32.4 ± 4.7	24.4 ± 6.1	27.0 ± 9.6
Na (mmol/L)	144.4 ± 3.6	142.3 ± 1.3	139.8 ± 3.5	142.3 ± 1.3
ALT (IU/L)	5.16 ± 1.60	4.83 ± 2.22	6.00 ± 1.63	6.50 ± 1.51
Uric (μmol/L)	213.4 ± 58.7	303.0 ± 147.0	136.8 ± 71.2	232.3 ± 131.5
AMY (IU/L)	507 ± 179	368 ± 222	643 ± 304	472 ± 154
AST (IU/L)	133.3 ± 11.6	144.8 ± 23.5	154.6 ± 27.4	167.2 ± 23.2 *
Chol (mmol/L)	6.27 ± 2.06	4.84 ± 1.76	7.39 ± 3.85	4.63 ± 1.34
Tbili- (μmol/L)	7.65 ± 1.97	6.94 ± 1.45	8.12 ± 1.39	6.98 ± 0.96
GGT (IU/L)	5.88 ± 1.64	6.57 ± 1.13	6.13 ± 2.41	6.43 ± 1.90
LDH (IU/L)	54.8 ± 59.0	31.3 ± 27.0	34.8 ± 51.0	74.2 ± 39.0
TG (mmol/L)	11.7 ± 2.4	9.8 ± 2.7	10.8 ± 2.2	9.2 ± 2.7
Mg (mmol/L)	1.63 ± 0.33	1.36 ± 0.23	1.48 ± 0.29	1.34 ± 0.25
K (mmol/L)	2.9 ± 0.5	3.2 ± 0.3	2.7 ± 0.3	2.9 ± 0.4
Cl (mmol/L)	110.1 ± 4.2	107.8 ± 3.9	107.1 ± 3.5	110.3 ± 3.2

[a] Measured concentration in feed using USEPA Method 8330A.

[b] Abbreviations used: Phosphate (PO_4), Total Proteins (TP), Glucose (Glu), Creatinine (Cre), Alanine aminotransferase (ALT), Amylase (AMY), Aspartate aminotransferase (AST), Cholestrol (Chol), Total Bilirubin (Tbili), γ-glutamyltransferase (GGT), Lactate dehydrogenase (LDH), Triglycerides (TG).

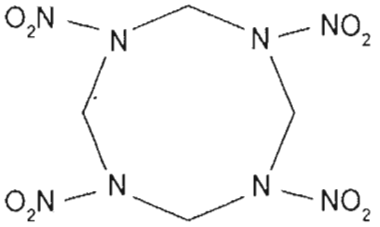
[c] Data are expressed as mean ± SD (standard deviation) (n= 7-9 birds per group).

* denotes exposure group is significantly different from control using Dunnett's test ($p \leq 0.05$).



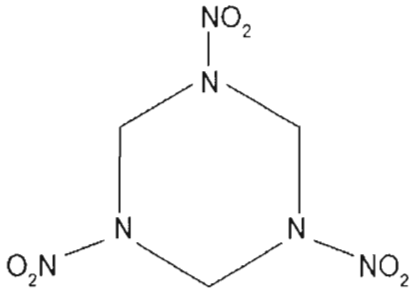
CL-20

(Hexanitrohexaazaisowurtzitane)



HMX

(octahydro- 1, 3, 5, 7- tetranitro- 1, 3, 5, 7-tetrazocine)



RDX

(hexahydro- 1, 3, 5- trinitro- 1, 3, 5 -triazine)

Figure 2.1

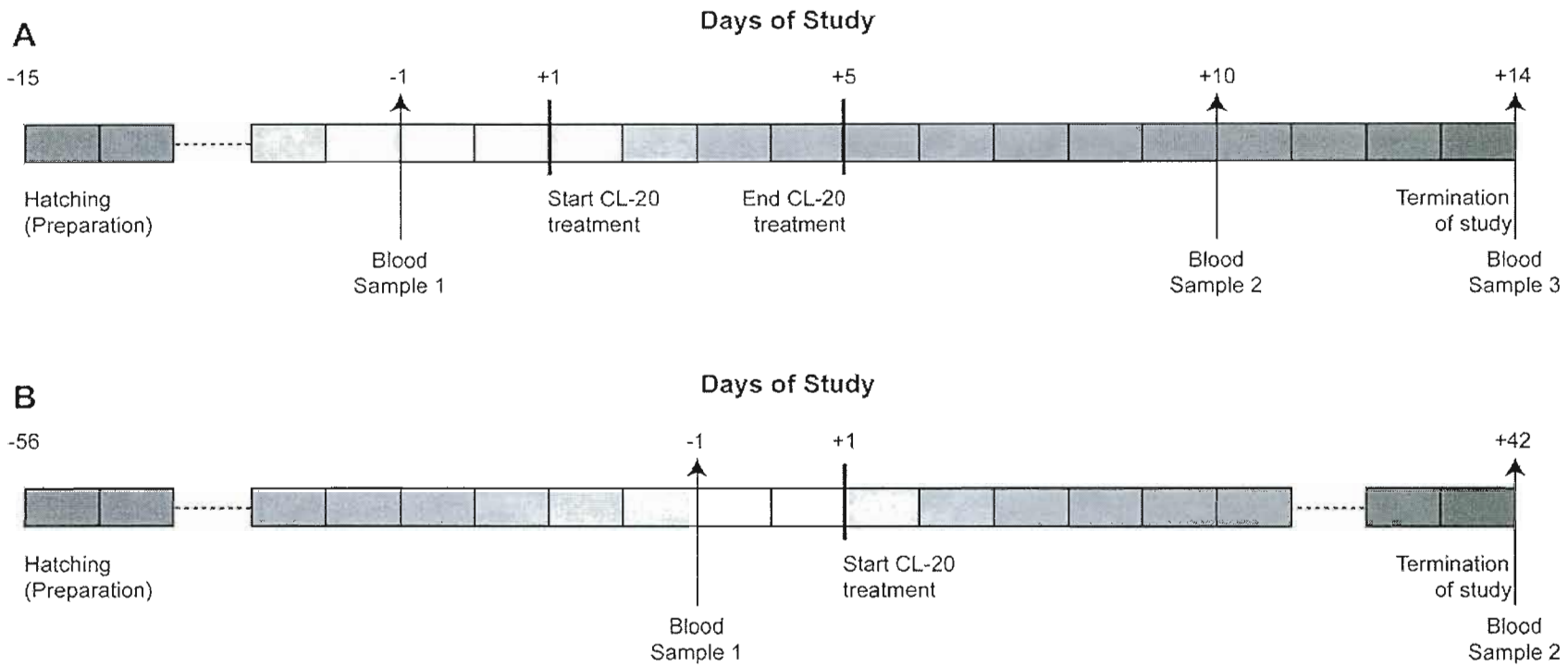

A
Days of Study
-15
-1
+1
+5
+10
+14
Hatching
(Preparation)
Blood
Sample 1
Start CL-20
treatment
End CL-20
treatment
Blood
Sample 2
Termination
of study
Blood
Sample 3
B
Days of Study
-56
-1
+1
+42
Hatching
(Preparation)
Blood
Sample 1
Start CL-20
treatment
Termination
of study
Blood
Sample 2

Figure 2.2

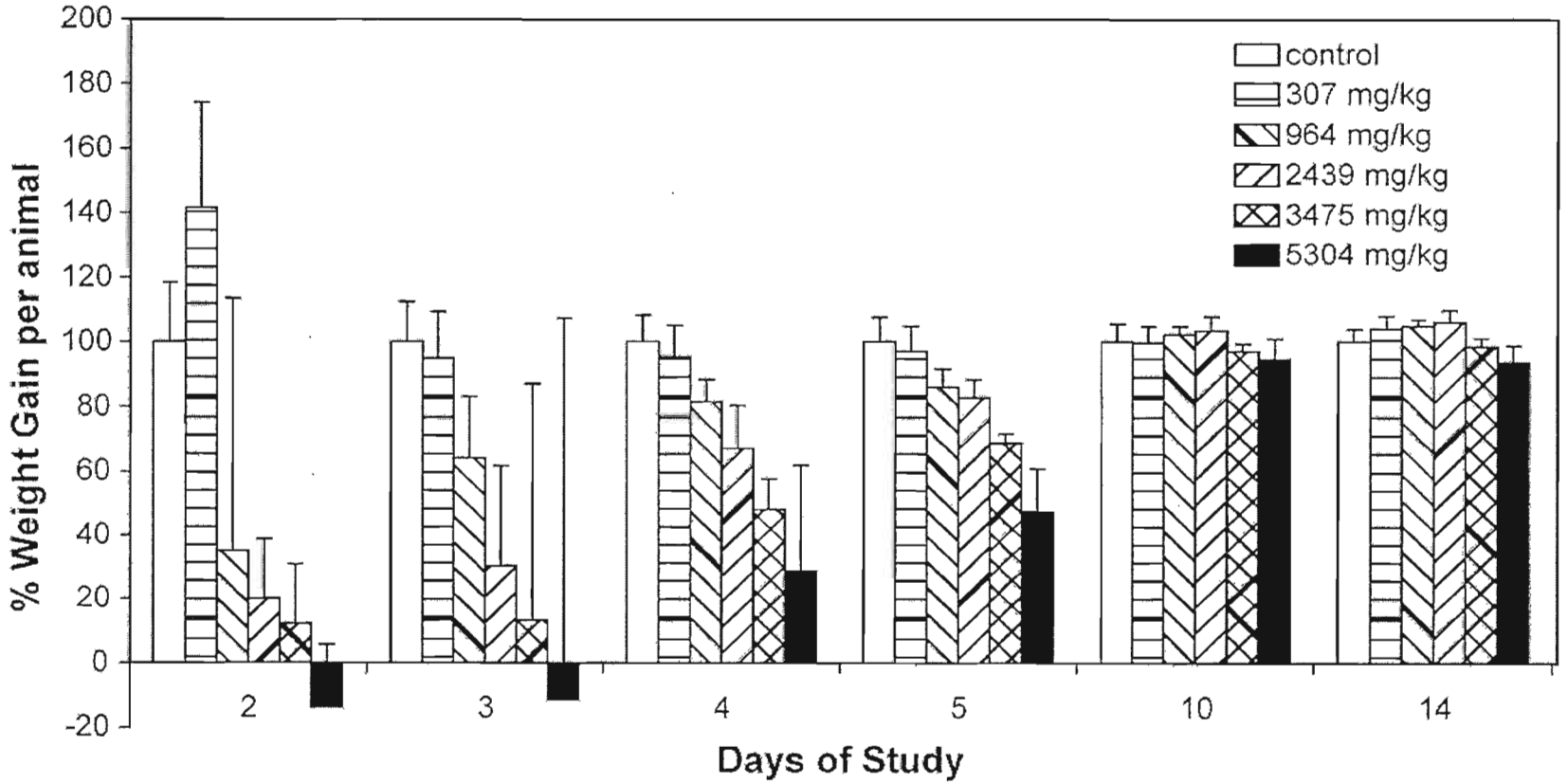

200
180
160
140
120
100
80
60
40
20
0
-20
% Weight Gain per animal
2
3
4
5
10
14
Days of Study
control
307 mg/kg
964 mg/kg
2439 mg/kg
3475 mg/kg
5304 mg/kg

Figure 2.3

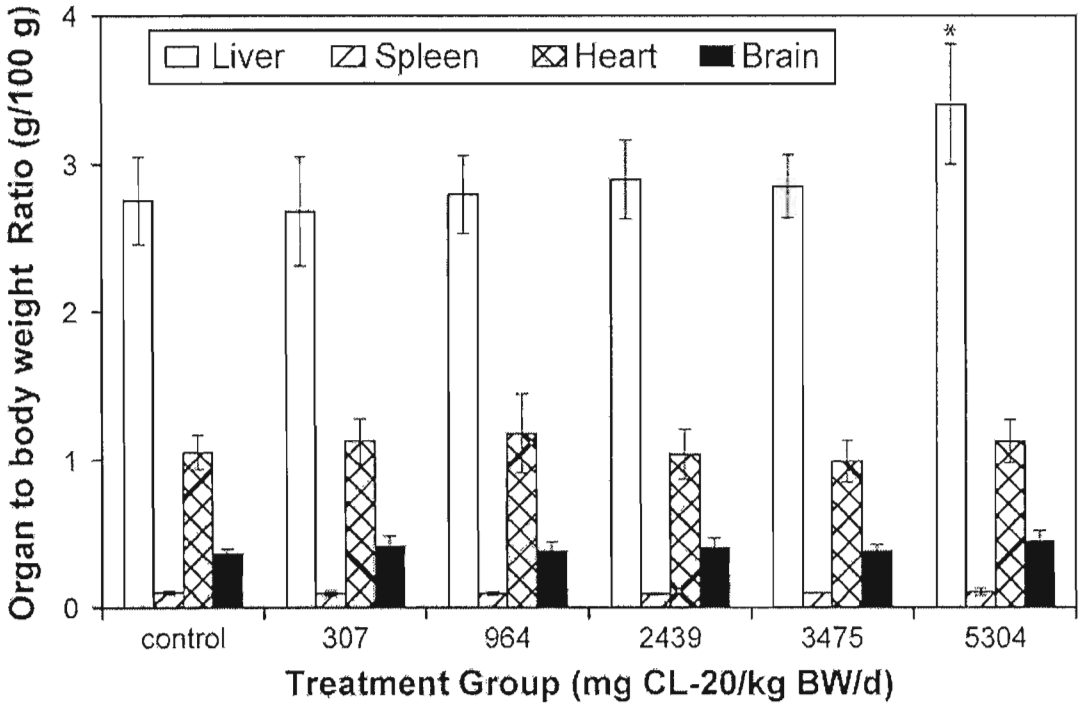

Liver
Spleen
Heart
Brain
Organ to body weight Ratio (g/100 g)
0
1
2
3
4
*
control
307
964
2439
3475
5304
Treatment Group (mg CL-20/kg BW/d)

Figure 2.4

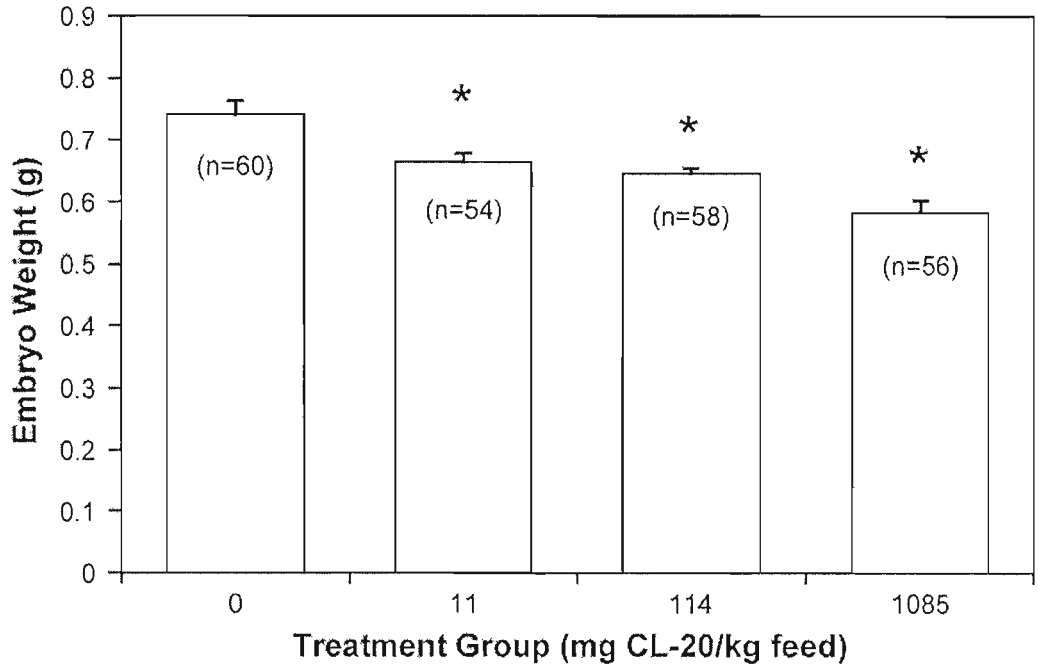

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Embryo Weight (g)
(n=60)
(n=54)
(n=58)
(n=56)
*
*
*
0
11
114
1085
Treatment Group (mg CL-20/kg feed)

Figure 2.5

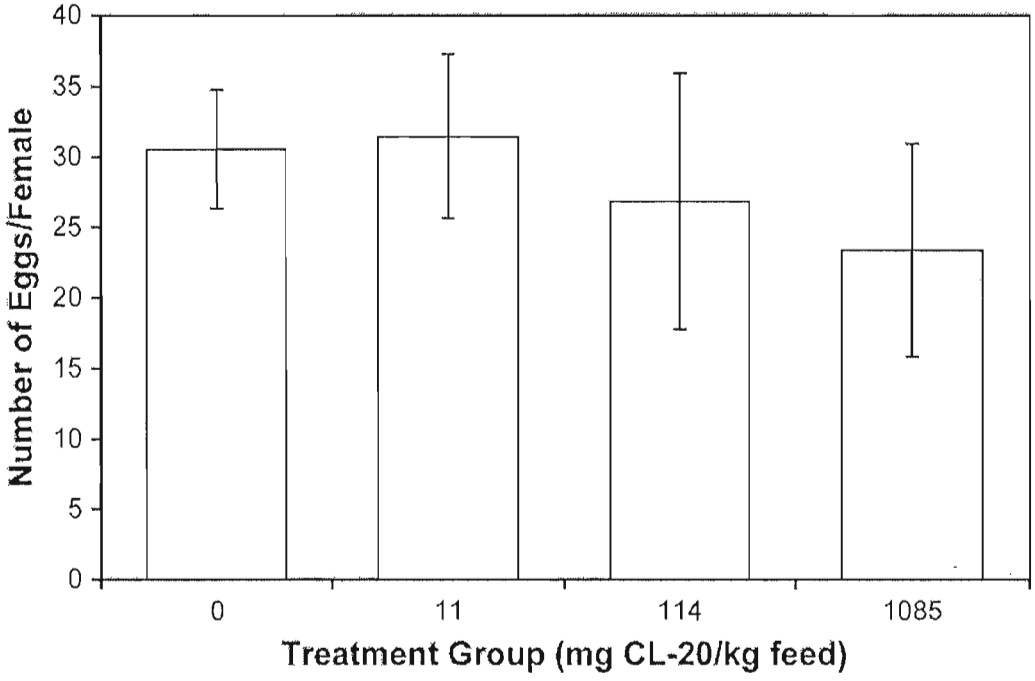

Number of Eggs/Female
40
35
30
25
20
15
10
5
0
0
11
114
1085
Treatment Group (mg CL-20/kg feed)

Figure 2.6

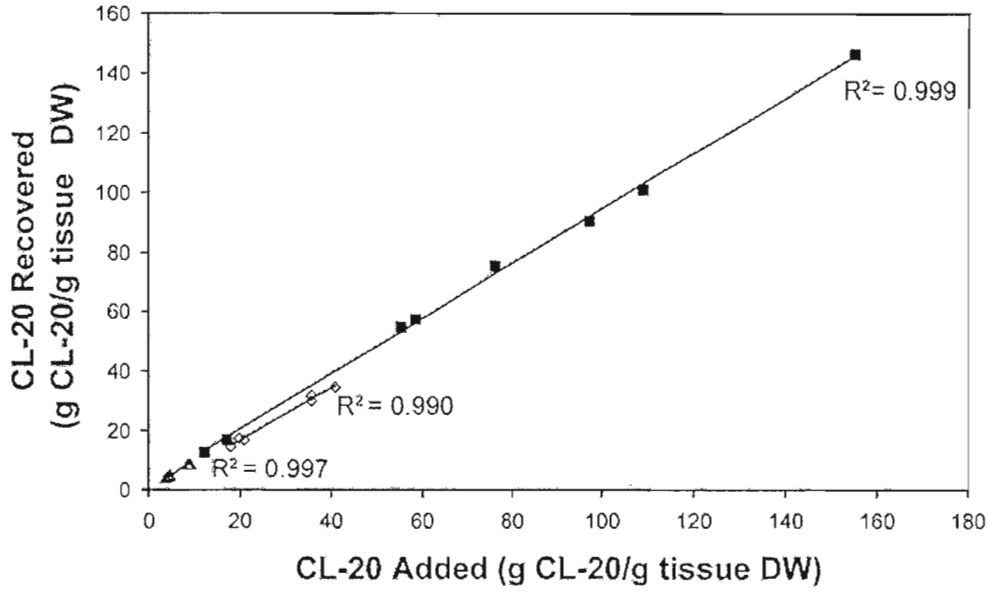

CL-20 Recovered (g CL-20/g tissue DW)
CL-20 Added (g CL-20/g tissue DW)
R²= 0.999
R² = 0.990
R² = 0.997
0
20
40
60
80
100
120
140
160
180

Figure 2.7

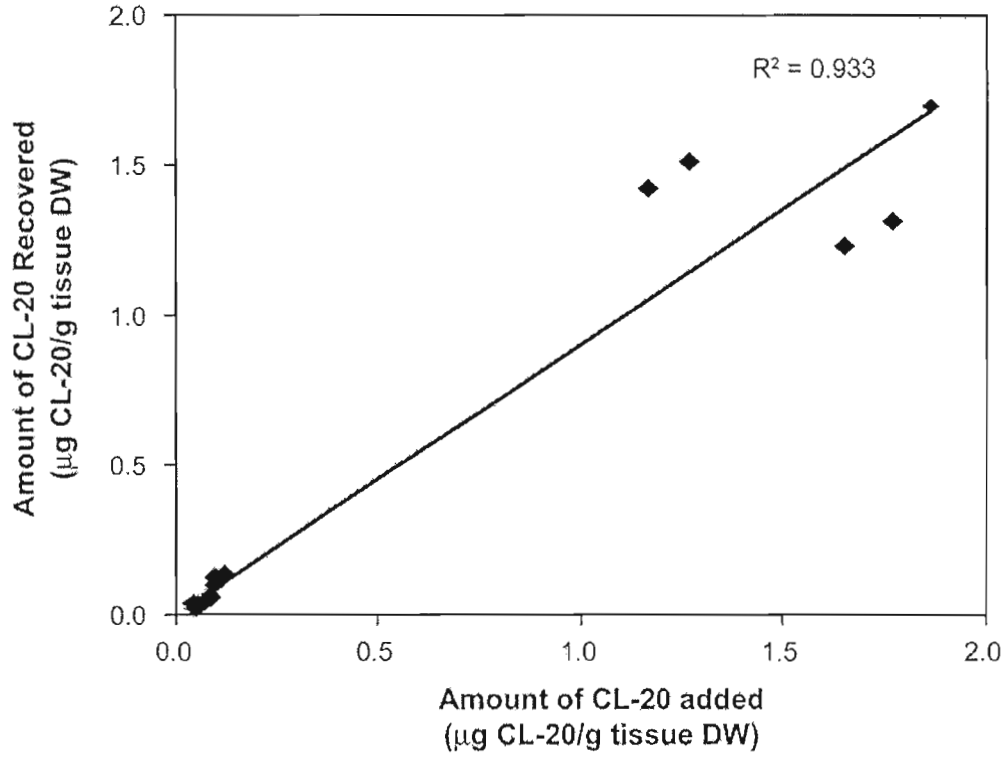

2.0
1.5
1.0
0.5
0.0
0.0
0.5
1.0
1.5
2.0
R² = 0.933
Amount of CL-20 Recovered
(µg CL-20/g tissue DW)
Amount of CL-20 added
(µg CL-20/g tissue DW)

Figure 2.8

CHAPTER III

Purification of a cytosolic GST from Japanese quail (*Coturnix coturnix japonica*) capable of biotransforming CL-20

Ghalib Bardai [a,b], Geoffrey I. Sunahara [a,*], Philip A. Spear [b], Stephan Grosse[a], and Jalal Hawari[a]

[a]Biotechnology Research Institute, National Research Council of Canada, 6100 Royalmount Avenue, Montreal, Quebec, Canada, H4P 2R2

[b]Centre de Recherche TOXEN, Département des Sciences Biologiques, Université du Québec à Montréal, CP. 8888, succursale Centre-ville, Montréal, Québec, Canada, H3C 3P8

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* Corresponding Author:

Geoffrey I. Sunahara, Ph.D.
Biotechnology Research Institute
National Research Council of Canada
6100 Royalmount Ave.
Montreal, QC, Canada, H4P 2R2
Tel: (514)-496-8030
Fax: (514)-496-6265
geoffrey.sunahara@cnrc-nrc.gc.ca

3.1 Abstract

CL-20, a polycyclic nitramine, is an emerging environmental contaminant. Our earlier studies showed that CL-20 exposure to adult quail caused an increase in liver weight and liver aspartate aminotransferase activities and yet CL-20 was not detectable in quail brain, heart, spleen, or liver. We considered the possibility that adult quail liver may possess an enzymatic mechanism to biotransform CL-20. Liver homogenates were prepared from adult female Japanese quail (*Coturnix coturnix japonica*) prior to the CL-20 biotransformation assays and the GST purification procedures. Data indicates that the disappearance of CL-20 using whole cytosol was inhibited *in vitro* by either ethacrynic acid or the GSH analogue, s-octylglutathione, indicating a GST enzyme. Using size exclusion, and affinity chromatography, we partially purified and characterized a cytosolic glutathione S-transferase (GST) from quail liver capable of biotransforming CL-20. The molecular masses of the purified proteins were ~ 28 and 27 kDA, using SDS-PAGE silver staining. Partial N-terminal sequence analysis showed both alpha and mu classes of GST, having 100% homology to chicken and quail GST described in the literature. This partially purified enzyme preparation biotransformed CL-20 at a rate of 0.27 ± 0.05 nmol/min/mg protein, with the requirement of glutathione (GSH) as an obligatory co-factor. Nitrite (NO_2^-) production was 1.2 ± 0.09 nmol/min/mg protein. Data suggest that the purified protein biotransformed CL-20 as evidenced by the concomitant formation of NO_2^- with CL-20 removal. These data suggest that GST purified from female quail liver is capable of biotransforming CL-20 *in-vitro* and may explain the absence of CL-20 in liver *in-vivo*.

Key Words: Japanese quail; CL-20; GST; ethacrynic acid

3.2 Introduction

Hexanitrohexaazaisowurtzitane (HNIW) or CL-20 (Fig. 1) is an emerging energetic chemical whose widespread use might lead to environmental contamination of soil. To better understand the environmental impact of CL-20, we determined its toxicity to selected terrestrial, aquatic and avian receptors [1,2,3]. Recently, we investigated the toxic effects of CL-20 on the gallinaceous test species, the Japanese quail (*Coturnix coturnix japonica*) and demonstrated that CL-20 does not show the same biological toxicity as RDX [3,4]. Therefore, the biotransformation of CL-20 must be understood in order to determine, and predict its biological fate and effects.

Our earlier *in vivo* studies showed that subacute and subchronic CL-20 exposure to adult quail caused an increase in liver weight and enzyme activities, and showed developmental effects on embryos [3]. Analysis of quail plasma and the major organs (liver, brain, heart and spleen) showed that CL-20 was not present in these tissues suggesting that CL-20 have have been biotransformed *in vivo*. As a first approach, we considered the role of avian xenobiotic metabolizing pathways capable of biotransformations caused by oxidative, reductive, hydrolytic, conjugation, acetylation, and methylation reactions [5]. CL-20, an electrophilic molecule (Fig. 1) is a possible substrate for nucleophilic reactions [6,7]. Nucleophilic conjugation reactions with thiols on electrophilic substrates, formed via the glutathione S-transferases (GST) enzyme and glutathione (GSH) are well documented [8,9]. We hypothesize that the quail liver may contain GST type enzyme(s) capable of biotransforming CL-20 with glutathione as a required substrate.

In the present study, we report the purification of an avian GST capable of biotransforming CL-20 under both aerobic and anaerobic conditions. To the best of our knowledge, this is the first report describing the purification and characterization of an avian enzyme capable of biotransforming CL-20.

3.3 Materials and Methods

3.3.1 Chemicals and reagents. CL-20 (CAS 135285-90-4) in ε form (purity > 95%) was obtained from ATK Thiokol Propulsion (Brigham City, Utah, USA). Reduced glutathione (GSH), glutathione *S*-transferase (GST) from rat liver, 1-chloro-2,4-dinitrobenzene (CDNB), diphenyliodonium chloride (DPI), allopurinol, ethacrynic acid (EA) and s-octylglutathione (s-GSH) were purchased from Sigma Chemicals (Oakville, Ontario, Canada). Carbon monoxide (CO) was purchased from Aldrich Chemical Company (Milwaukee, WI). Sephadex G-25, Sepharose 12 and GST-Affinity columns, were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). All other chemicals and reagents were of the highest grades of purity available and were obtained from Sigma Chemicals. Deionized water was obtained using a Zenopure Mega-90 water purification system. All glassware was washed with phosphate-free detergent, rinsed with acetone, and acid-washed before a final rinse with deionized water.

3.3.2 Enzyme purification. All procedures were performed at 4°C, using an FPLC™ system (Amersham Pharmacia Biotech, Uppsala, Sweden) unless otherwise stated. Protein purification columns used in this study were pre-equilibrated in Buffer A (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) at 4°C. Adult female quail (9 weeks old) were obtained from a local farm (Ferme Bourgois, Mirabel, PQ, Canada). Animals were sacrificed by decapitation, livers were then removed, washed with isotonic saline, and minced (2 mm thickness). Seven livers in triplicate, (total wet weight about 120 g) were pooled and rapidly homogenized using a Polytron tissue grinder with 35 ml of phosphate buffered saline (Buffer A). The homogenate was centrifuged at 10,000*g* for 30 min, and the supernatant was further centrifuged at 100,000*g* for 60 min. The 100,000*g* supernatant (containing cytosol) was filtered through glass wool and used for further purification. A 70 ml aliquot of cytosol was then applied to a Sephadex G-25

desalting column (4 × 25 cm). The desalted cytosolic fraction was passed through a GSH affinity column (1 × 10 cm) at 1 ml/min, the affinity column was washed extensively with buffer A, and protein eluted with 50 mM Tris-HCl (pH 8.0) containing 10 mM GSH. Protein was monitored at 280 nm, and active fractions (those having high CDNB conjugating activity as described below) were collected. This procedure was repeated, active fractions were pooled. The buffer was then exchanged to Buffer A and the protein concentrated using Amicon (10,000 MW cut off) centrifugal filter units. Aliquots (300 µl) of the above concentrated fractions were applied onto a Sepharose 12 column (previously equilibrated with Buffer A) and eluted with the same buffer at a flow rate of 0.5 ml/min. Protein concentration was determined with a bicinchoninic acid protein assay kit from Pierce Chemical Company (Rockford, IL) using BSA as the standard.

3.3.3 SDS-PAGE and N-terminal sequencing. SDS–PAGE was performed on 12% gels each having a 5% stacking gel, as described by Laemmli [10] using a BioRad Mini Protean II electrophoresis system. The loading volume was 10 µl (containing 1 µg protein) per well. Molecular weights were determined from R_f values of standard marker proteins (BioRad Hercules, CA), and protein bands were visualized using a silver stain. Gel analysis was carried out using the Biorad Quantity One Analysis software. For N-terminal microsequencing, gels obtained from SDS-PAGE were soaked in transfer buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 10% methanol, pH 11) for 5 min to reduce the amount of Tris and glycine. During this time, a PVDF (polyvinylidene difluoride) membrane (BioRad Hercules, CA) was rinsed with 100% methanol and stored in transfer buffer. The gel, sandwiched between a sheet of PVDF membrane and several sheets of blotting paper, was assembled into a blotting apparatus (Mini Protein II, Biorad) and electroeluted for 15 min at 0.259 amperes in transfer buffer. The PVDF membrane was washed in deionized water and then stained with 0.1% Coomassie Blue R-250 in 50% methanol.

Destaining was carried out in a solution containing 50% methanol and 10% acetic acid. The membrane was finally rinsed in deionized water for 5-10 min and air dried. All steps were carried out at room temperature. Protein bands were then cut from the membrane, and N-terminal amino acid sequencing of isolated protein were obtained by automated Edman degradation performed on a model Procise LC 494 protein sequencer from Applied Biosystems (AB) (Foster City, CA) employing the general protocol of Hewick et al. [11]. Approximately one pmole of the protein was loaded on the sequencer and a standard program using liquid phase TFA was employed for sequencing. The phenylthiohydantoin amino acid (PTH-aa) derivatives were determined by comparison with standards (PTH-standards, AB) analyzed on line on a Model ABI 140D capillary separation system at the start of a sequence analysis.

3.3.4 Glutathione-S-Transferase (GST) Activity Assay. GST assays were performed at room temperature using a microtiter plate reader (Spectra MAX PLUS; Molecular Devices Corp, Sunnyvale, CA) which measures absorbance (340 nm) in a 96 well quartz microtiter plate. GST activity was measured with CDNB as the substrate and reduced GSH as the cofactor. CDNB (20 mM) was dissolved in anhydrous ethanol, and GSH (20 mM) was prepared fresh daily in sodium phosphate buffer (100 mM, pH 6.5). The reaction mixture (250 µl) contained: 100 mM sodium phosphate buffer (pH 6.5), 1 mM GSH, and 1 mM CDNB. The ethanolic concentration was less than 5% (unless otherwise stated). The increase in absorbance was recorded for 5 min to ensure that the reaction went to completion. Nonenzymatic base catalyzed conjugation of GSH with CDNB was subtracted from all assays, by including a blank (buffer only) consisting of all the assay components except the protein preparation containing GST activity. One unit of transferase activity was defined as the initial rate of one micromole of the product (S-2,4-dinitrobenzene-glutathione) formed per minute measured at 340 nm using an extinction coefficient of 9.6 mM^{-1} for the conjugate [12].

3.3.5 Kinetic studies. Initial-velocity studies of the purified enzyme were performed using the GST assay described above, except that varying concentrations of CDNB (from 0.02 to 5 mM), or GSH (from 0.07 to 5 mM) were used. Apparent enzyme kinetic constants (app. V_{max} and app. K_m) were calculated according to the Michelis-Menten equation. Specific activities were expressed as μmol/min/mg protein.

3.3.6 CL-20 biotransformation assay. Enzyme-catalyzed biotransformation assays and inhibition studies were performed under anaerobic or aerobic conditions, using 6-ml glass septum-sealed vials with constant agitation in the dark at 37°C for 2 h. Both anaerobic and aerobic conditions were tested to determine optimal incubation conditions, because no literature exists on the purification of a vertebrate enzyme capable of biotransforming CL-20. Anaerobic conditions were created by purging the reaction mixture with argon gas for 15 min. For the CL-20 biotransformation studies, each 1 ml test unit contained: CL-20 (28 μmol), enzyme preparation (0.75 mg protein) or cytosol (5 mg protein), reduced glutathione (100 μM) and Buffer A. Inhibition studies were assessed by incubating the cytosol or the pure enzyme preparation, with the following selected inhibitors: diphenyliodonium chloride (DPI), allopurinol, ethacrynic acid (EA), s-octylglutathione (s-GSH), and carbon monoxide (CO). Controls were prepared by omitting enzyme or GSH in the reaction mixture. To determine CL-20 concentrations, the reaction was stopped with the addition of 1 ml acetonitrile, vortexed and placed in the dark at 4°C for 1 hr to allow for protein precipitation. The solution was subsequently filtered through a 0.45 μm membrane. CL-20 was measured by high-pressure liquid chromatography (HPLC) (see below). Activity of the enzymatic biotransformation of CL-20 was expressed as micromoles CL-20 per minute per milligram of protein.

3.3.7 Analytical procedures. CL-20 concentration was quantified by UV as previously described [3]. Nitrite was quantified using a reverse polarity capillary electrophoretic method described by Okemgbo et al. [13]. An Agilent 3D CE system was fitted with a bare silica bubble capillary (Part no: G1600-61232, total length 64.5 cm, effective length 56 cm, internal diameter 50 μm). The separation buffer (pH 9.2) contained 25 mM sodium tetraborate and 25 mM hexamethonium bromide, in MilliQ water. Injections were performed hydrodynamically (25 sec, 50 mbar, injection volume 34 nl). The separation voltage was - 30 kV (cathode at inlet). Quantification was obtained by peak area using direct absorbance at 220 nm. Using commercial external standards (Alltech Assoc., Deerfield, IL, USA), the instrumental quantification limit was 0.2 ppm (N=10, peak area 4.5 mAU-s, RSD 3.9%). N_2O and glyoxal were analyzed as described previously [14,15].

3.3.8 Statistical analysis. Significant differences among mean activities were evaluated using Student's *t*-test. Differences with $P \leq 0.05$ were denoted as statistically significant. The kinetic constants (K_m and V_{max}) were determined by Eadie-Hofstee plots, and were compared to the K_m and V_{max} constants generated by the Michelis-Menten equation using Sigma Plot® (Point Richmond, CA).

3.4 Results and discussion

The analysis of the major quail organs including brain, heart, liver, and spleen in our previous studies showed no residual CL-20 present in these organs [3]. The absence of CL-20 in tissue samples suggested that either: a) the compound was not absorbed and did not enter general circulation, or b) the compound was absorbed and rapidly metabolized. The former hypothesis was ruled out based on visible changes in liver weight and enzyme activities (aspartate aminotransferase), and the developmental effects on embryos caused by CL-20 [3]. The liver was chosen as the organ of choice for purification because our previous data demonstrated that the liver was most

impacted (increased liver weights and selected enzymes). It is also well known that this organ possesses the ability to metabolize a broad range of compounds in birds [5].

To determine if the adult female quail liver possesses the enzymatic ability to biotransform CL-20, we tested different liver fractions including: crude extract (containing both membrane and soluble fractions), and supernatants from the 10,000*g*, and the 100,000*g* (cytosol) fractions for their ability to biotransform CL-20. We found that the rate of CL-20 biotransformed increased with each successive supernatant fraction (10,000*g* or 100,000*g*). Furthermore, we noted that under anaerobic conditions, the amount of CL-20 biotransformed was greater than that under aerobic conditions (Table 3.0). Although complex, under aerobic conditions, cells produce reactive oxygen species (ROS) (radicals), which would normally be quenched by GSH [16,17]. As expected, under anaerobic conditions, the rate of ROS formation decreases and the ratio of free GSH to oxidized (GSSG) increases. Therefore, under anaerobic conditions, and in the purified enzyme assays we would expect to see a greater rate of CL-20 biotransformation due to the fact that the available relative amount of GSH would be increased. The monitoring of GSH levels in the assay vials would help in confirming this speculation.

To identify the possible enzymes capable of CL-20 biotransformation in the cytosol, we used a strategy that involved the use of non specific enzyme inhibitors, and specific inhibitors (DPI) to enzymes known to biotransform CL-20. The effects of DPI (an inhibitor of flavoenzymes) [18], CO (cytochrome P450 inhibitor), allopurinol (a xanthine oxidase inhibitor) [14], EA (a GST inhibitor) [19,20], and s-GSH (a glutathione analogue), were all tested for their ability to inhibit CL-20 biotransformation *in vitro*. Only EA and s-GSH caused a significant decrease in the

biotransformation of CL-20 in quail liver cytosol (Table 3.0), and suggested the involvement of a soluble GST- like enzyme.

GST activity from quail liver was then isolated from crude cytosol using GSH affinity chromatography and gel filtration technique (Table 3.1). The GST type enzyme that eluted from the affinity column had a specific activity of 19.3 μmol/min/mg protein, which is comparable to that of the purified quail GST (24.8 μmol/min/mg), described by Dai et al. [21]. Table 1 shows that further purification using gel filtration increased the specific activity by 30% to 26.6 μmol/min/mg protein, with only a 3% loss in recovery.

SDS-PAGE analysis of the purified enzyme preparation (Fig. 2) revealed that the enzyme from crude liver homogenate was purified to homogeneity by the GSH affinity column. The proteins eluted from the GSH affinity column resolved two major bands, which were identified as, QL1 and QL2 in this article (Fig. 2, Lane 6). The subunit M_r of the QL1 and QL2 were estimated to be ~ 28 and ~27 kDa respectively, and were within the range of the molecular masses reported for avian GSTs (24-30 kDa) [21-24]. The two major bands resolved from the GSH affinity column were in good agreement with results obtained by Dai et al [21], Chang et al. [23], and Hsieh et al. [24] who showed the presence of a mu GST following affinity purification from quail and chicken liver.

Partial N-terminal amino acid sequence analysis of QL1 and QL2 from the GSH affinity column are listed in Table 3. An alignment of the N-terminal amino acid sequence of QL1 with the corresponding chicken and quail class (QL2) mu sequences confers 100% homology for the first 9 amino acids. Alignment of QL2 (class alpha) from this study with the corresponding chicken sequence, shows 100% homology for the first 12 amino acids, indicating that two different GST's were retained on the

affinity column. Initial-velocity kinetic analysis was performed on the partially purified enzyme preparation (Fig. 3). When GSH or CDNB was plotted (v) versus [S] as the varied-concentration substrate with different concentrations of GSH or CDNB as the fixed-concentration substrate, a classical hyperbolic curve was obtained. The apparent kinetic parameters (K_{mGSH}, K_{mCDNB}, V_{maxGSH}, $V_{maxCDNB}$) were obtained from Eadie-Hofstee (EH) plots, and were used to determine if the experimental data sets fitted the Michelis-Menten (MM) model (Fig. 3). The K_{mGSH}, K_{mCDNB}, V_{maxGSH} , and $V_{maxCDNB}$ obtained from the EH plots were; 0.38 mM, 0.34 mM, 221 μmol/min/mg protein, and 176 μmol/min/mg protein respectively. While the MM kinetic parameters obtained were; 0.45, 0.42, 241, and 167; respectively. These results strongly suggest that the purified protein preparation from quail liver contained at least two GST-type enzymes. Further studies were carried out to determine whether these enzymes can metabolize CL-20 *in vitro*.

Biotransformation of CL-20 only occurred when incubated in the presence of both purified enzyme and GSH, and was completely inhibited in the presence of ethacrynic acid (Fig. 4) and were consistent with the results using whole cytosol (Table 1). The incubation of CL-20 with GSH (no enzyme added) only had no effect on the biotransformation of CL-20. We also incubated CL-20 with other reducing agents such as BME (β-mercaptoethanol), DTT (dithiothreitol), or NADH, and found that in the presence of these stronger reducing agents, CL-20 was not biotransformed (data not shown).

The absolute requirement of both GSH and enzyme for the biotransformation of CL-20 offers further evidence that the enzyme responsible for the biotransformation of CL-20 may be a GST enzyme. GSTs catalyze the following reaction (GSH + R-X → GSR + RX) [7]. The primary function of the N-terminal domain of GST is to provide a hydrophilic site to activate the sulfhydral group on GSH (GSH → GS•) nucleophile

activation (G for binding glutathione), which is the most conserved region in all of the cytosolic enzymes, and an adjacent H- site which provides a hydrophobic environment for the binding of structurally diverse electrophilic xenobiotic substrates [6]. These data suggest that the formation of a thiol radical $GS^{\bullet}$, is required for the biotransformation of CL-20. The diuretic drug ethacrynic acid (EA), an α,β-unsaturated ketone, and the glutathione analogue s-octyl glutathione both inhibited the biotransformation of CL-20 (Table 1). Glutathione analogues bearing hydrophobic R groups are effective inhibitors of cytosolic GSTs via simultaneous occupation of the peptide and substrate binding site. EA is both a substrate and inhibitor of GST [20]. Cameron et al. [25] have shown through crystallographic studies using human alpha class A1-1 that EA appears to bind to the active site of GSH transferase. Therefore, we can conclude that both GSH and binding of CL-20 to the active site is required for biotransformation to occur.

Ogawa et al. [26,27] demonstrated that the biotransformation of the organic nitrate esters, nitroglycerin (NTG) and isosorbide dinitrate (ISDN) occurred in rabbit cytosol. These authors also demonstrated the denitration of NTG and ISDN was potentiated by the presence of GSH, and was inhibited by *S*-alkyl GSH, a GST analogue. This line of evidence suggests that GSTs may be involved in the initial denitration of CL-20.

Time course studies showed that the disappearance of CL-20 in the presence of GSH and enzyme, was accompanied by the release of nitrite (Fig. 5) suggesting the occurrence of an initial denitration step. Such denitration might have been initiated via a thiol radical. The accumulation of nitrate (NO_3^-) levels, was not significant, indicating that nitrite released from the biotransformation of CL-20 was not oxidized to nitrate. The rate of CL-20 biotransformation determined from the linear portion of the curve shown in Fig. 5 was 0.27 ± 0.02 nmol/min/mg protein, with the production of 1.2 ± 0.09 nmols of NO_2^- /min/mg protein. The stoichiometry indicates that

approximately 4 NO_2^- were produced for every CL-20 biotransformed. The decrease in enzyme activity (decreased CL-20 biotransformation) and nitrite production from 120 to 240 min could be explained by the fact that; a) increasing concentrations of nitrite or other CL-20 intermediates [14,18,28] may have caused a product inhibition of GST; or b) the remaining CL-20 concentration at 120 min. was below the K_{mCL-20} of the enzyme . These hypotheses would have to be confirmed by product-inhibition and enzyme CL-20 kinetic studies.

Although we have suggested that the biotransformation of CL-20 in the presence of GSH occurs enzymatically, GSTs can also act as ligandins, (bind a wide range of hydrophobic compounds but do display any enzymatic activity) [29,30]. Therefore, we could not rule out the possibility of CL-20 binding to GST in the presence of GSH only. However, two lines of evidence do not support this hypothesis: firstly the concomitant release of nitrite would suggest that binding only may not be occurring, and secondly if binding was occurring then it should have occurred with the commercial rat liver GST (Fig. 4). Future kinetic studies should be performed to determine if non-competitive inhibition exists towards the GST substrate, 1-chloro-2,4-dinitrobenzene (CDNB) in the presence of GST and CL-20, because ligandin binding is characterized by this type of kinetics [30].

In summary, the adult female quail liver contains a soluble GST-type enzyme capable of biotransforming CL-20 with the concomitant release of nitrite, and may explain the lack of toxicity observed in our previous study [3]. The enzyme preparation described here contained a mixture of both α and μ type GSTs that showed 100% homology to the first 12 amino acids on similar chicken GST. With the chicken genome sequenced, recombinant protein expression of these sequences would help in identifying the specific enzyme capable of CL-20 biotransformation. It would be interesting to determine the biotransformation products of this reaction, to determine

if the nitrite we observed in the present study was a direct result of GST denitration, or the result of an initial reaction leading to unstable intermediaries that results in the release of nitrite.

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3.6 Figure Captions

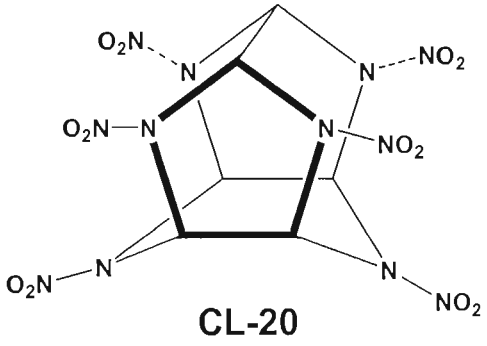
Fig. 3.1 Molecular structure of the polycyclic nitramine CL-20.

Fig. 3.2 Silver stain of SDS-PAGE purification of quail CL-20 degrading enzyme. Lanes 1 and 7, molecular weight markers (97 kDa Phosphorylase b, 66 kDa BSA, 45 kDa Ovalbumin, 31 kDa Carbonic anhydrase, 21.5 kDa Trypsin); Lane 2, Crude extract; Lane 3, 10,000*g* fraction; Lane 4, 100,000*g* fraction; Lane 5, total proteins eluted from the Glutathione affinity column; Lane 6, GST following gel filtration column.

Fig. 3.3 Initial velocity patterns of partially purified GST. Michelis-Menten graph was generated using least squares analysis. Inset: Secondary plots of Eadie-Hofstee. Table inset shows the apparent enzyme kinetic endpoints determined using either method. V_{max} and K_m are expressed as μmol/min/mg and mM, respectively.

Fig. 3.4 Biotransformation of CL-20 by: (1) Enzyme Preparation (EP) + CL-20, (2) CL-20 + GSH, (3) CL-20 + GSH + EP, (4) CL-20 + GSH + EP + EA, and (5) CL-20 only. Incubation conditions: 37°C, 2 h under aerobic conditions. Data are means ± SE (n = 3). * Significantly different ($P \leq 0.05$) from its appropriate control using the unpaired Student's *t*-test.

Fig. 3.5 Time course study of enzyme dependent biotransformation of CL-20 under aerobic conditions. Symbols indicate quantity of CL-20 (-△-) and nitrite (-■-). Incubation conditions: 37°C, 2 h under aerobic conditions. Data are the means of triplicates, and error bars represent SE.



CL-20

(Hexanitrohexaazaisowurtzitane)

Fig. 3.1

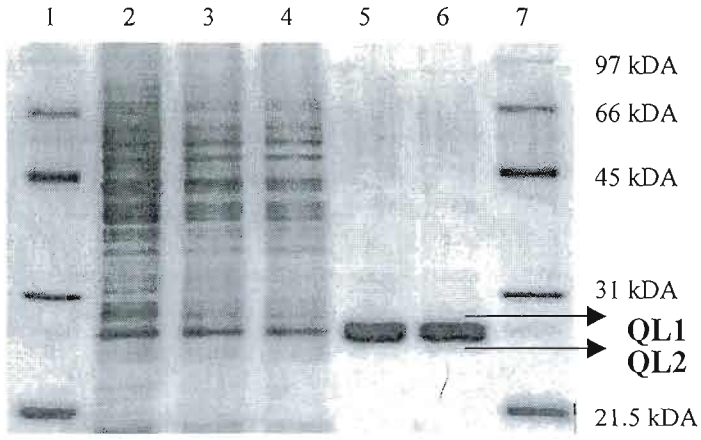


Fig. 3.2

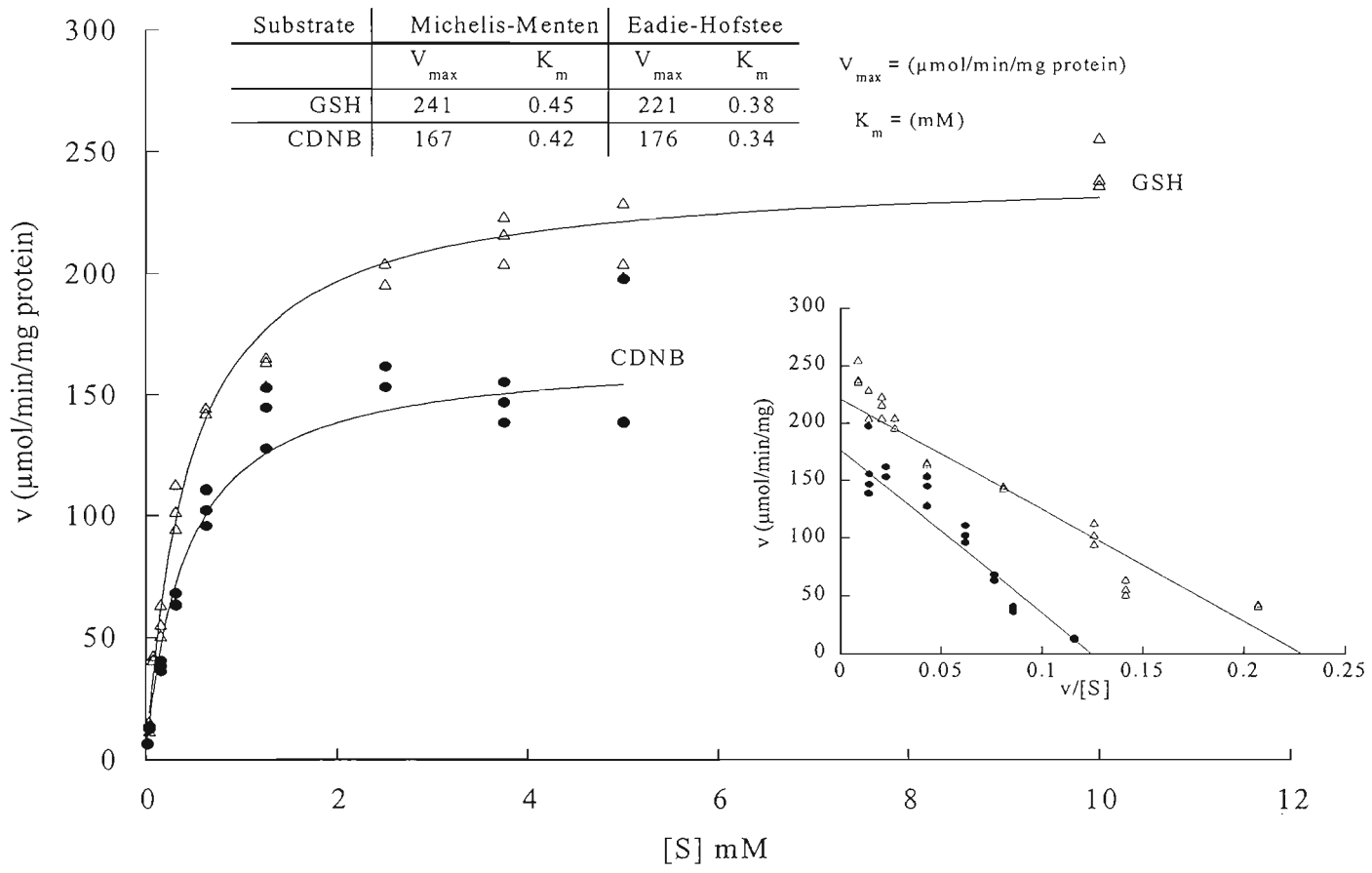

Substrate
Michelis-Menten
Eadie-Hofstee
V max
K m
V max
K m
GSH 241 0.45 221 0.38
CDNB 167 0.42 176 0.34
V max = (µmol/min/mg protein)
K m = (mM)
GSH
CDNB
v (µmol/min/mg protein)
[S] mM
v (µmol/min/mg)
v/[S]

Fig.3.3

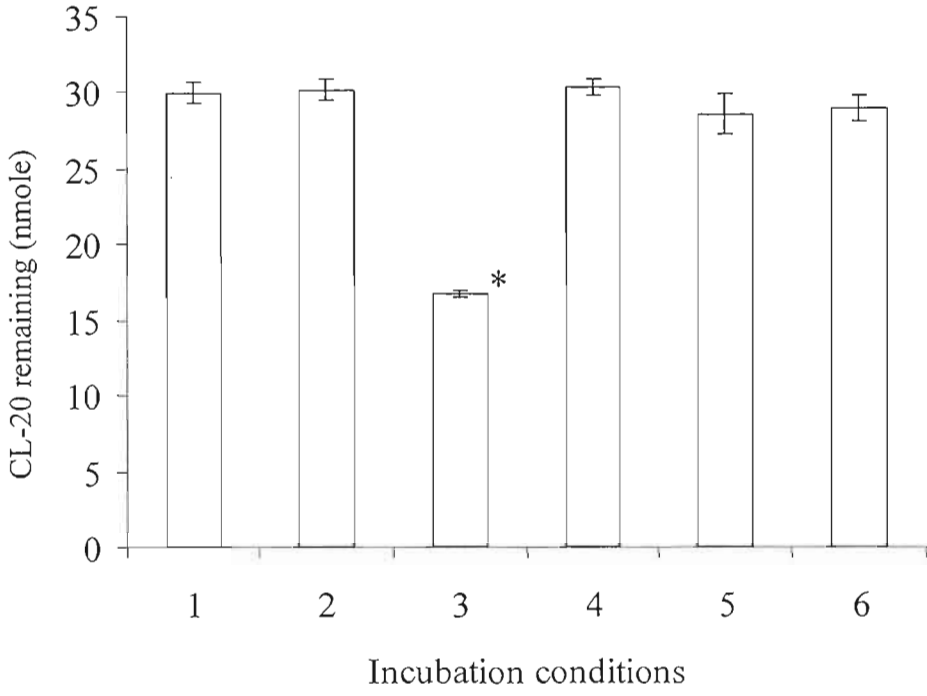

35
30
25
20
15
10
5
0
CL-20 remaining (nmole)
*
1
2
3
4
5
6
Incubation conditions

Fig. 3.4

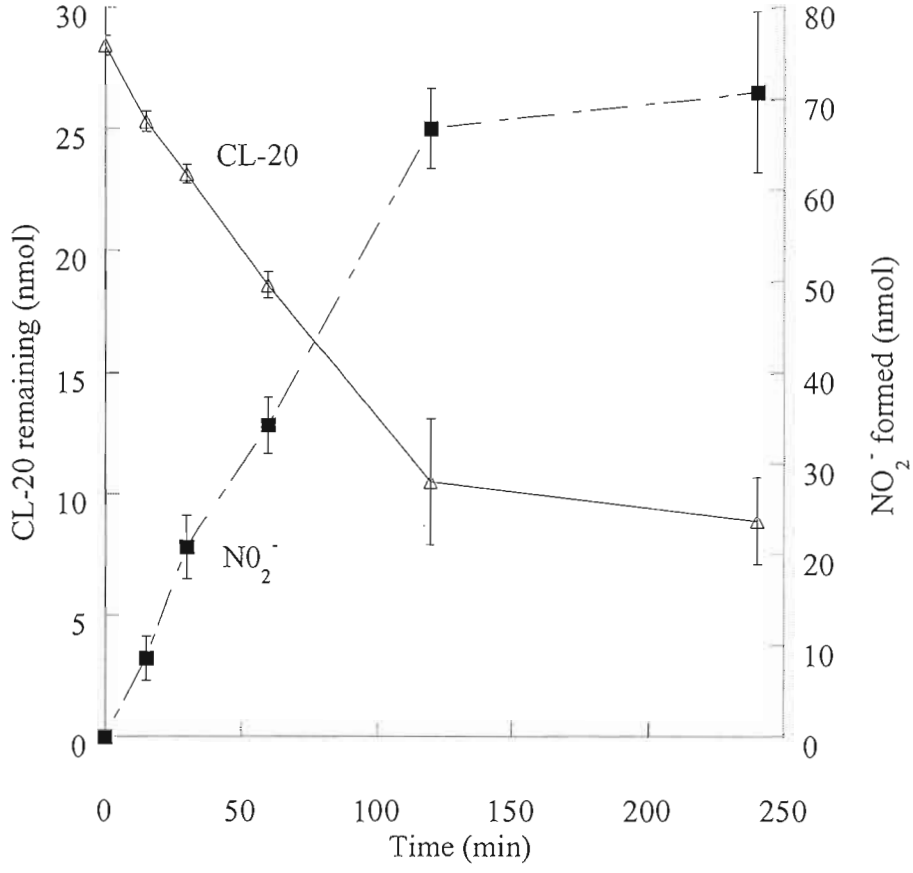

CL-20
NO_2^-
CL-20 remaining (nmol)
NO_2^- formed (nmol)
Time (min)
0
5
10
15
20
25
30
0
10
20
30
40
50
60
70
80
0
50
100
150
200
250

Fig. 3.5

Table 3.1 Effects of enzyme inhibitors and incubation conditions on CL-20 biotransformation activity in quail liver whole cytosol

Inhibitor (100 μM) or Incubation Condition	% CL-20 biotransformed *	% Activity
Anaerobic	37.1 ± 1.3^{a}	100
Aerobic	19.8 ± 3.2	53
Diphenyliodonium	34.4 ± 2.1^{ns}	93
Carbon monoxide	35.8 ± 1.9^{ns}	96
Ethacrynic acid	2.5 ± 1.6^{b}	7
Allopurinol	40.3 ± 1.3^{ns}	108
S-octylglutathione	7.5 ± 0.9^{b}	20

* Mean ± SE. (n=3). Hundred percent activity was equivalent to 0.01 nmol CL-20 transformed per mg protein per min.

[a] Significantly different from control (Aerobic incubation) at $P \leq 0.05$, as determined by Student's *t*-test.

[b] Significantly different from control (Anaerobic incubation) at $P \leq 0.05$, as determined by Student's *t*-test.

[ns] denotes no significant difference vs. control (Anaerobic incubation) ($P > 0.05$)

Table 3.2 Purification of Glutathione *S*-Transferase from quail liver

Purification Step	Volume (ml)	Total Protein (mg)	Total Activity (μmol/min)	Specific activity (μmol/min/mg protein)[a]	Recovery (%)	Purification (-fold)
1. Crude Homogenate	140	13300	3458	0.26		1.0
2. 10,000g supernatant	90	6750	2160	0.32	62	1.2
3. 100,000g supernatant	70	4480	2060	0.46	60	1.8
4. GST-Affinity Chromatography	1.6	33.6	649	19.3	19	74
5. Gel Filtration (Sepharose 12)	1.5	21	558	26.6	16	102

[a] Measured according to Habig et al.[10], using 1-chloro-2,4-dinitrobenzene as substrate

Table 3.3 N-Terminal Amino Acid Sequences of Glutathione *S*-Transferases

Transferase	Alignment of N-terminal amino acid sequences	GenBank primary accession number	Reference:
Quail GST QL1 (class-mu)	NH_2-VVTLGYWDI		This study
Chicken GST (class-mu)	NH_2-VVTLGYWDIRGLAHA	**S18464**	[21]
Mouse GST (class-mu 6)	MPVTLGYWDIRGLAHA	**CAA04060**	[31]
Quail GST QL2 (class mu)	NH_2-VVTLGYWDIRGLA A		[22]
Quail GST QL2 (class alpha)	NH_2- SGKPRLTYLNGR		This study
Chicken GST (class alpha)	NH_2- SGKPRLTYVNGRGRMESIR	**NP_990149**	[23]

CONCLUSION

Les travaux de recherche présentés dans le cadre de cette thèse ont montré que le CL-20 n'a pas d'effet toxique aigu sur les cailles adultes (males et femelles) mais qu'il a un effet nocif sur le développement des embryons. Au cours de l'étude sub-aiguë, les oiseaux exposés au CL-20 ont subi une perte de poids corporel inversement proportionnelle à la dose de CL-20 administrée. Par contre, cet effet est devenu non significatif à la fin de l'étude. La perte de poids corporel ne pouvant être attribuée à la toxicité du CL-20, elle pourrait être liée à une irritation croissante du tube gastro-intestinal. Une augmentation du poids du foie, des niveaux de sodium plasmique et de créatine a été observée chez les oiseaux ayant reçus les doses les plus élevées de CL-20. L'analyse des organes vitaux n'a pas décelé de résidu de CL-20, indiquant que le CL-20 a pu être métabolisé, non absorbé ou excrété rapidement. Nos résultats suggèrent que les effets toxiques du CL-20 se situent au niveau du foie (augmentation de son poids) et des reins (augmentation des niveaux du sodium plasmique et de la créatine). Ces résultats devront être confirmés par d'éventuelles études histopathologiques.

Aucun effet toxique n'a été observé chez les cailles adultes au cours de l'étude sub-chronique. Par contre, une augmentation du niveau d'aspartate aminotransférase (AST) a été mesurée chez les oiseaux exposés à la dose la plus élevée de CL-20. Ces résultats suggèrent donc également un effet toxique au niveau du foie. Une diminution du nombre d'œufs pondus par les femelles ainsi que des malformations embryonnaires ont été observées chez les oiseaux exposés au CL-20. Ces résultats suggèrent que le CL-20 a un effet sur le foie des femelles adultes.

La présente étude a finalement démontré que le foie des cailles femelles adultes contient une enzyme de type GST capable de biotransformer le CL-20, expliquant

ainsi les effets toxiques *in vivo*. Nous en concluons que le CL-20 a des effets chez les cailles femelles adultes pouvant influencer le développement embryonnaire. Ces résultats préliminaires devront être confirmés avec d'autres études ultérieures.

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