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UNIVERSITY OF ALBERTA

SOME OSCILLATION RESULTS FOR DIFFERENTIAL SYSTEMS

by

Lakshma Reddy Ganta

A THESIS SUBMITTED TO

THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

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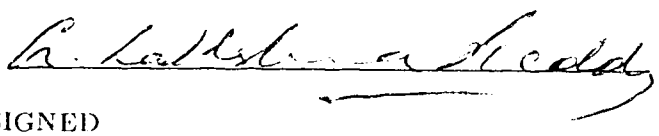

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Date April 26, 1990

To the loving memories of my parents, the late Sri. Yadava Reddy Ganta and the late Smt. Ramanamma Ganta.

ABSTRACT

In this thesis we consider second-order vector-matrix differential systems of the form

$$(P(t)y')' + Q(t)y = 0$$

$$(P(t)Y')' + Q(t)Y = 0$$

where $P(t)$ and $Q(t)$ are real continuous $n \times n$ symmetric matrix-valued functions with $P(t)$ being positive definite, $y(t)$ an n-dimensional vector and $Y(t)$ an $n \times n$ matrix function. By making use of the behaviour of the eigenvalues of the coefficient matrices, a number of oscillation results are proved. In addition, some comparison results for the system are also obtained.

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TABLE OF CONTENTS

CHAPTER I

INTRODUCTION

The oscillation theory for the second order scalar differential equation

$$(p(t)x')' + q(t)x = 0 \tag{1}$$

where $p(t) > 0$, $q(t)$ are continuous real valued functions on the interval $[0, \infty)$, has been studied by many authors. The first fundamental result was the comparison theorem of Sturm. The Sturm comparison theorem in its restricted form was first discovered by Sturm [51] in 1836. Starting with Picone [50] in 1909, many researchers have since then improved Sturm's theorem to include a wide class of coefficients. For the formally self adjoint second order scalar differential equation

$$x'' + q(t)x = 0, \tag{2}$$

Kneser [30] proved in 1893 that if the limit

$$\lim_{t \to \infty} t^2 q(t) \tag{3}$$

exists and is equal to α then equation (2) is oscillatory if $\alpha > \frac{1}{4}$ and nonoscillatory if $\alpha < \frac{1}{4}$. In 1918 Fite [24] proved that $q(t) > 0$ together with

$$\int_0^\infty q(s)ds = +\infty \tag{4}$$

forms a sufficient condition for the oscillation of (2). Hille [27] generalized Kneser's results to obtain the following:

(i) equation (2) is oscillatory if

$$\liminf_{t\to\infty} \; t\int_t^\infty q(s)ds > \frac{1}{4}, \tag{5}$$

(ii) equation (2) is nonoscillatory if

$$\limsup_{t\to\infty} \; t\int_t^\infty p(s)ds < \frac{1}{4}. \tag{6}$$

It was in 1949 that Wintner [57] removed the condition $q(t) > 0$ in Fite's result. He proved that

$$\lim_{T\to\infty} \; \frac{1}{T}\int_0^T \int_0^t q(s)dsdt = +\infty \tag{7}$$

implies the oscillation of (2). Later Hartman [26] proved that in the case of nonexistence of the limit in (7), the following condition

$$-\infty < \liminf_{T\to\infty} \; \frac{1}{T}\int_0^T \int_0^t q(s)dsdt < \limsup_{T\to\infty} \; \frac{1}{T}\int_0^T \int_0^t q(s)dsdt \tag{8}$$

is also sufficient for the oscillation of (2). These results were further generalized by Olech, Opial and Wazewski [49] and by Willett [56]. Several researchers [26, 52, 55, 57] have obtained a number of results concerning the oscillation and nonoscillation of equation (1). A well known and more practical result is that of Leighton [39], who proved that equation (1) is oscillatory if

$$\int_0^\infty \frac{ds}{p(s)} = \int_0^\infty q(s)ds = +\infty. \tag{9}$$

Many researchers were and still are interested in knowing the extent to which the oscillation and nonoscillation criteria of the scalar equation (1) have analogues for the vector-matrix differential system

$$(P(t)Y')' + Q(t)Y = 0 \tag{10}$$

where $P(t)$, $Q(t)$ are real continuous n by n symmetric matrix-valued functions on $[0,\infty)$ with $P(t)$ positive definite for all $t \geq 0$. As in the scalar case, particular attention was paid to the case when $P(t) = I$, namely, the formally self adjoint differential system

$$Y'' + Q(t)Y = 0. \tag{11}$$

Etgen [18] has shown that (11) is oscillatory if

$$\lim_{t\to\infty} \int_0^t Tr\ Q(s)ds = \infty. \tag{12}$$

This result was extended by Noussair and Swanson [48], which in turn was further generalized by Allegretto and Erbe [1]. Etgen and Pawlowski [20] have shown that equation (11) is oscillatory if there exists a positive linear functional g such that

$$\lim_{t\to\infty} g\Big[\int_0^t Q(s)ds\Big] = +\infty. \tag{13}$$

This result was generalized to the self adjoint equation (9). Etgen and Lewis [19] have shown that equation (9) is oscillatory if there exists a positive linear functional g such that the scalar equation

$$(g[P(t)]x')' + g[Q(t)]x = 0 \tag{14}$$

is oscillatory. It has been conjectured [28] that equation (11) is oscillatory at infinity whenever

$$\lim_{t\to\infty} \lambda_1\Big(\int_0^t Q(s)ds\Big) = +\infty$$

where for any symmetric matrix A, $\lambda_1(A)$ denotes the largest eigenvalue of A. In the case of $Q(t)$ positive semi-definite, the conjecture follows from condition (13). Similarly, the conjecture was first established under various additional conditions by Mingarelli [44, 45], Kwong *et al.* [38], Atkinson *et al.* [2], Kaper and Kwong [36], Butler and Erbe [6, 7], before it was finally settled by Byers, Harris and Kwong [10] and also by Kaper and Kwong [37]. It is clear that the eigenvalues of the coefficient matrices $P(t)$ and $Q(t)$ play a central role in obtaining oscillation results for equation (10). By drawing more from the intermediate eigenvalues, using a kind of "trade off", one can obtain a number of results for both equations (10) and (11).

In Chapter 2 we introduce notation, give some definitions and enumerate some basic results which are used in the subsequent chapters. In Chapter 3 we obtain oscillation criteria for the formally self adjoint system (11). Chapter 4 deals with oscillation criteria for the self adjoint equation (10), which can be regarded as a generalization of Leighton's result. In Chapter 5 we consider the problem of multiplying the coefficient matrices by matrices which preserve the oscillation property of the original systems.

CHAPTER II

PRELIMINARIES

2.1. Notation

The standard notations of differential and integral calculus are used throughout. We recall the following definitions [49] which we shall use in the coming chapters.

For any subset E of the real line R, $mes(E)$ denotes the Lebesgue measure of the subset E.

DEFINITION 2.1.1: Let $F(t)$ denote a continuous real valued function and ℓ be such that $-\infty \leq \ell \leq +\infty$. Then

$$\lim_{t \to \infty} \text{approx inf} \;\; F(t) = \ell$$

if

$$mes\{t : F(t) \leq \ell_1\} < +\infty \quad \text{for all} \quad \ell_1 < \ell$$

and

$$mes\{t : F(t) \leq \ell_2\} = +\infty \quad \text{for all } \ell_2 > \ell.$$

DEFINITION 2.1.2: Let $F(t)$ denote a continuous real valued function and L be a scalar such that $-\infty \leq L \leq +\infty$. Then

$$\lim_{t \to \infty} \text{approx sup} \;\; F(t) = L$$

if

$$mes\{t : F(t) \geq L_1\} = +\infty \quad \text{for all } L_1 < L$$

and

$$mes\{t : F(t) \geq L_2\} < +\infty \quad \text{for all } L_2 > L.$$

DEFINITION 2.1.3: For a continuous real valued function $F(t)$ and a scalar m we write

$$\lim_{t\to\infty} \text{approx } F(t) = m$$

if

$$\lim_{t\to\infty} \text{approx inf } F(t) = \lim \text{approx} \sup_{t\to\infty} F(t) = m.$$

From these definitions, we have, for a continuous real valued function $F(t)$,

$$\liminf_{t\to\infty} F(t) \leq \lim_{t\to\infty} \text{approx inf } F(t) \leq \lim_{t\to\infty} \text{approx sup } F(t) \leq \limsup_{t\to\infty} F(t).$$

2.2. Matrix theory

Unless stated otherwise capital letters will be used to denote real square matrices of order $n \times n$. The transpose of a matrix A is denoted by the matrix A^*. The symbol I will denote the identity matrix. The dimension of I will be clear from the context. The symbol O will denote the zero element. Again, it would be evident from the context whether it is the zero matrix, zero vector or a scalar. For n-tuples in R^n and scalars, lower case letters and Greek letters

are used respectively, and will be mentioned at the beginning of their usage from time to time if there is ambiguity.

Standard notations of matrix theory are also used throughout. Let A be an $n \times n$ matrix. Then A^{-1} will denote the inverse of A if A is nonsingular, i.e., $\det A \neq 0$. A matrix valued function $A(t) = (a_{ij}(t))$ is said to be continuous if each of its entries $a_{ij}(t)$ is continuous. Similarly $A(t)$ is said to have a property from calculus if and only if its entries $a_{ij}(t)$ all have that property. For obvious reasons, symmetric matrices will play an important role. We denote the set of all symmetric matrices of order $n \times n$ by S. With the usual addition of matrices and multiplication of a matrix with a scalar, S is a linear vector space over the reals. For any real symmetric matrix A, its eigenvalues, all of which are necessarily real, will be denoted by $\lambda_k(A)$, $1 \leq k \leq n$, and are assumed to be ordered so that

$$\lambda_1(A) \geq \lambda_2(A) \geq \cdots \geq \lambda_n(A).$$

As usual $Tr\ A$ will denote the trace of the matrix A. We have

$$Tr\ A = \sum_{i=1}^{n} \lambda_i(A).$$

A symmetric matrix $A \in S$ is *positive semi-definite* (*positive definite*) if for all $x \in R^n$, $x \neq 0$, the usual inner product (x, Ax) is non-negative (positive). To say A is positive definite (positive semi-definite) the notation $A > 0$ ($A \geq 0$) will be used throughout. For A and B in S, $A > B$ means $A - B > 0$.

A linear function $\phi : S \to R$ satisfying

$$\phi(A + B) = \phi(A) + \phi(B) \quad \text{and}$$

$$\phi(\lambda A) = \lambda\phi(A)$$

where $\alpha \in R$, $A, B \in S$, is called *positive* and *normalized* provided

$$\phi(A) > 0 \quad \text{if} \quad A > 0 \text{ and}$$

$$\phi(I) = 1.$$

A non-linear functional $q : S \to R$ is called *superadditive* and (*positively*) *superhomogeneous* if

$$q(A + B) \geq q(A) + q(B) \quad \text{and}$$

$$q(\alpha A) \geq \alpha q(A) \quad \text{for} \quad \alpha \geq 0$$

where $A, B \in S$, and is called *positive* and *normalized* if

$$q(A) > 0 \quad \text{if} \quad A > 0 \text{ and}$$

$$q(I) = 1.$$

Correspondingly, a functional $p : S \to R$ is called *subadditive* and (*positively*) *subhomogeneous* if

$$p(A + B) \leq p(A) + p(B) \quad \text{and}$$

$$p(\alpha A) \leq \alpha p(A) \quad \text{for } \alpha \geq 0.$$

Note that the conditions on q imply that q is concave and hence is continuous on S. A similar remark is valid for p.

As a positive linear functional ϕ is continuous, we have for integrable $A(t)$,

$$\phi\Big(\int_a^t A(s)ds\Big) = \int_a^t \phi[A(s)]ds, \quad a, t \in R^+$$

and

$$\phi[B'(t)] = (\phi[B(t)])', \quad t \in R^+$$

whenever $B(t)$ is differentiable.

2.3. Results on symmetric matrices

The purpose of this section is to collect some basic results based on the topics discussed in the previous section. These results will be used repeatedly in the subsequent chapters. Discussion of undefined terms and detailed accounts of the topics discussed here and in the previous section can be found in the references [5, 41, 42]. In the following discussion, all matrices will be assumed symmetric.

Let

$$A = \begin{bmatrix} a_{11} & a_{12} & \dots & a_{1n} \\ a_{21} & a_{22} & \dots & a_{2n} \\ \vdots & \vdots & \vdots & \vdots \\ a_{n1} & a_{n2} & \dots & a_{nn} \end{bmatrix}$$

be a symmetric matrix. The principal sub-matrices of A are

$$A_1 = [a_{11}], \quad A_2 = \begin{bmatrix} a_{11} & a_{12} \\ a_{21} & a_{22} \end{bmatrix} \dots A_k = \begin{bmatrix} a_{11} & \dots & a_{1k} \\ \vdots & & \\ a_{k1} & \dots & a_{kk} \end{bmatrix}$$

These matrices are all symmetric and we have

$$\lambda_{j+1}(A_{k+1}) \leq \lambda_j(A_k) \leq \lambda_j(A_{k+1})$$

where $\lambda_j(A_k)$ denotes the j^{th} characteristic root of the principal submatrix

$$A_k = \begin{bmatrix} a_{11} & \dots & a_{1k} \\ \vdots & \vdots & \vdots \\ a_{k1} & \dots & a_{kk} \end{bmatrix}.$$

LEMMA 2.3.1. *If $A \geq 0$ then all eigenvalues of A are non-negative.*

LEMMA 2.3.2. *If $A \geq B$, then $Tr\ A \geq Tr\ B$.*

LEMMA 2.3.3. *If $A \geq B$ and $C \geq D$, then $A + C \geq B + D$.*

LEMMA 2.3.4. *(i) If $0 \leq A$ and $A \not\leq I$, then $Tr\ A > 1$.*

(ii) If $A \not\leq B$ and $B \geq C$, then $A \not\leq C$.

LEMMA 2.3.5. *If $A > B$ and $C = C^*$, then $CAC > CBC$.*

LEMMA 2.3.6. *(i) If $A(t) \geq 0$ for $t \in (a, b)$, then*

$$\int_a^b A(t)dt \geq 0.$$

(ii) If $A(0) \geq 0$ and $A'(t) \geq 0$, then $A(t) \geq 0$.

(iii) $\int_a^b Tr\ \{A(t)^{-1}A'(t)A(t)^{-1}\}dt = Tr\ \{A(a)^{-1}\} - Tr\ \{A(b)^{-1}\}$.

LEMMA 2.3.7. *If A and B are positive semi-definite and $A^2 \geq B^2$, then $A \geq B$.*

LEMMA 2.3.8. *Let C and A be positive definite. If $ACA \geq BCB$, then $A \geq B$.*

For $1 \leq k \leq n$, and a symmetric matrix A, we define

$$\sigma_k(A) = \lambda_1(A) + \cdots + \lambda_k(A)$$

and

$$\rho_k(A) = \lambda_n(A) + \lambda_{n-1}(A) + \cdots + \lambda_{n-k+1}(A)$$

where the n eigenvalues of A are ordered in descending order of magnitude.

LEMMA 2.3.9. *If A and B are symmetric, then*

(i) $\sigma_k(A+B) \leq \sigma_k(A) + \sigma_k(B)$;

(ii) $\rho_k(A) + \rho_k(B) \leq \rho_k(A+B)$;

(iii) $|\sigma_k(A)|^2 \leq k\sigma_k(A^2)$;

(iv) $|\rho_k(A)|^2 = |\sigma_k(-A)|^2 \leq k\sigma_k(A^2)$;

(v) $Tr\ (A) \leq (n-k+1)\sigma_k(A) \leq n\sigma_k(A)$.

2.4. Definitions

There is a vast literature on the subject of the existence and location of zeros of solutions of differential equations. In the absence of formal expressions for the solution of a differential equation, attention is directed to the problem of obtaining essential characteristics of the solutions of the equation, by directly analyzing the equation itself. One such qualitative study is the subject of oscillation theory.

Consider the second-order scalar differential equation

$$(p(t)x')' + q(t)x = 0, \tag{2.4.1}$$

where $p(t) > 0$, $q(t)$ are continuous, real valued functions on the interval $R^+ = [0, \infty)$ and $'$ denotes differentiation with respect to t. The oscillation theory for the equation (2.4.1) has been studied by many authors [25, 51, 55, 57] and continues to flourish.

DEFINITION 2.4.1: Equation (2.4.1) is said to be *oscillatory* on the interval $R^+ = [0, \infty)$ if one (hence all) solution $x(t) \not\equiv 0$ of (2.4.1) has arbitrarily large zeros. The equation is said to be ***nonoscillatory*** if it is not oscillatory. Oscillatory on the interval $R^+ = [0, \infty)$ and oscillatory at ∞ mean the same.

The beginning of such a qualitative study can be traced back to the classic paper of Sturm [51], wherein his celebrated comparison theorem was proved.

We are mainly concerned with the self adjoint differential system

$$(P(t)Y')' + Q(t)Y = 0 \tag{2.4.2}$$

where $P(t), Q(t), Y(t)$ are all $n \times n$ real continuous matrix functions. We also assume that $P(t)$, $Q(t)$ are symmetric and that $P(t)$ is positive definite for $t \in [0, \infty)$. Also, particular attention will be paid to the special case when $P(t) = I$, that is, to the equation

$$Y'' + Q(t)Y = 0. \tag{2.4.3}$$

We also have the vector systems associated with equations (2.4.2) and (2.4.3)

$$\left(P(t)y'\right)' + Q(t)y = 0 \tag{2.4.4}$$

and

$$y'' + Q(t)y = 0 \tag{2.4.5}$$

where $y = \text{col}(y_1, y_2, \ldots, y_n)$ is an n-vector.

DEFINITION 2.4.2: Two distinct points t_0, $t_1 \in J = [a, b]$ are said to be (*mutually*) *conjugate* relative to equation (2.4.4) if there exists a nontrivial solution $y(t)$ of (2.4.4) such that $y(t_0) = y(t_1) = 0$.

DEFINITION 2.4.3: Equation (2.4.4) is said to be *disconjugate* on an interval J if every nontrivial solution of equation (2.4.4) vanishes at most once in J.

DEFINITION 2.4.4: Equation (2.4.4) is said to be *oscillatory* on $J = [a, \infty)$, $a \geq 0$, if for each $t_0 > a$ there exists a $t_1 > t_0$ such that equation (2.4.4) is not disconjugate on $[t_1, \infty)$.

DEFINITION 2.4.5: The system (2.4.5) is said to be *right-disfocal* on J if, for every pair of points a, $b \in J$, $a < b$, the only solution y satisfying the condition $y(a) = y'(b) = 0$ is the trivial solution.

DEFINITION 2.4.6: The system (2.4.5) is said to be *left-disfocal* on J if, for every pair of points a, $b \in J$, $a < b$, the only solution y satisfying the condition $y'(a) = y(b) = 0$ is the trivial solution.

For the first order system

$$y' + Q(t)y = 0 \tag{2.4.6}$$

we have the following concepts [47].

DEFINITION 2.4.7: A nontrivial solution $y = \text{col}(y_1, y_2, \ldots, y_n)$ of (2.4.6) is said to be *oscillatory* on an interval J if $y_k(t_k) = 0$ for some $t_k \in J$, $k = 1, 2, \ldots, n$.

DEFINITION 2.4.8: The first order system (2.4.6) is said to be *oscillatory* if it has at least one oscillatory solution vector.

DEFINITION 2.4.9: The first order system (2.4.6) is said to be *suborthogonal* on J if, for any nontrivial solution vector y and for any pair of points a, $b \in J$, the inner product $(y(a), y(b)) = \sum_{k=1}^{n} y_k(a) y_k(b) > 0$.

DEFINITION 2.4.10: A solution $Y(t)$ of the matrix differential equation (2.4.2) is called *nontrivial* if $\det Y(t) \neq 0$ for at least one $t \in [0, \infty)$.

DEFINITION 2.4.11: A solution $Y(t)$ of the matrix differential equation (2.4.2) is said to be *non-singular* at $a \in [0, \infty)$ if $\det Y(a) \neq 0$.

DEFINITION 2.4.12: A nontrivial solution $Y(t)$ of the matrix differential equation (2.4.2) is said to be *oscillatory* at ∞ if $\det Y(t)$ has infinitely many zeros in $[0, \infty)$. If it is not oscillatory then it is called *non-oscillatory*.

If one hopes to develop an analogue of the classical theory of oscillation for the scalar case, namely equation (2.4.1), for the linear system (2.4.2), one needs to consider only certain class of solutions of the equation of type (2.4.2). The following example [48] clarifies the situation. We know that the linear scalar equation

$$x'' + x = 0 \tag{2.4.7}$$

has only solutions of the form

$$x(t) = c_1 \sin t + c_2 \cos t \tag{2.4.8}$$

where c_1, c_2 are arbitrary constants. As all these solutions are oscillatory, equation (2.4.7) is oscillatory. Now consider the matrix analogue of the above equation, namely the matrix differential equation

$$Y'' + Y(t) = 0. \tag{2.4.9}$$

As in the case of equation (2.4.7), one expects to see that equation (2.4.9) is oscillatory (shortly we will give the precise definition). But the following matrix

$$Y(t) = \begin{pmatrix} \cos t & -\sin t \\ \sin t & \cos t \end{pmatrix}$$

is a solution of the matrix differential equation (2.4.9). As $\det Y(t) = 1$, we see that $Y(t)$ is a non-oscillatory solution of (2.4.9). Hence one has to consider a subclass of the class of solutions of equations of type (2.4.2). This subclass is that of all *prepared solutions.*

First, note that if $Y = Y(t)$ is a solution of the equation (2.4.2), then

$$Y^*(t)\big[P(t)Y'(t)\big] - \big[P(t)Y'(t)\big]^* Y(t) \equiv C \tag{2.4.10}$$

where C is an $n \times n$ constant matrix.

DEFINITION 2.4.13: A non-trivial solution $Y(t)$ of the matrix differential equation (2.4.2) will be called *prepared* (or *conjoined*, or *self-conjugate*) if

$$Y(t)^*\big[P(t)Y'(t)\big] - \big[P(t)Y'(t)\big]^* Y \equiv 0. \tag{2.4.11}$$

Note that a non-trivial solution $Y(t)$ of (2.4.2) is prepared if and only if $[P(t)Y'(t)]^*Y(t)$ is symmetric.

As a consequence of (2.4.10) and (2.4.11), $Y(t)$ is a prepared solution of (2.4.2) if and only if there exists $a \in [0, \infty)$ such that

$$Y^*(a)[P(a)Y'(a)] - [P(a)Y'(a)]^*Y(a) = 0.$$

Thus, prepared solutions can be obtained by starting out with appropriate initial values.

DEFINITION 2.4.14: The matrix differential equation (2.4.2) is said to be *oscillatory* if the determinant of every nontrivial prepared solution vanishes on $[a, \infty)$ for each $a > 0$.

Oscillation of the matrix differential equation (2.4.2) is equivalent to the oscillation of the vector differential equation (2.4.4) since any solution of (2.4.4) is of the form $y(t) = Y(t)\alpha$ for some constant vector α and some nontrivial prepared solution $Y(t)$ of (2.4.2). For this reason, very often, one studies equation (2.4.2) to study the oscillatory properties of equation (2.4.4).

Two basic techniques used in studying oscillation and non-oscillation of the scalar equation (2.4.1) have analogous approaches available for the differential systems. These techniques are generally known as the nonlinear Riccati integral equation approach and the variational approach [26].

2.5. Riccati and Variational Methods

In this section we briefly recall the essentials of the aforementioned approaches in connection with the study of oscillation and nonoscillation of differential systems.

Riccati Method: Let $Y = Y(t)$ be any solution (not necessarily prepared) of the matrix differential equation

$$\big(P(t)Y'(t)\big)' + Q(t)Y(t) = 0. \tag{2.5.1}$$

Taking $R(t) = -P(t)Y'(t)Y^{-1}(t)$ and using the fact that $Y(t)$ is a solution of (2.5.1), we get the Riccati equation

$$R'(t) = Q(t) + R(t)P^{-1}(t)R(t). \tag{2.5.2}$$

Equation (2.5.2) gives the Riccati integral equation

$$R(t) = R(a) + \int_0^t Q(s)ds + \int_a^t R(s)P^{-1}(s)R(s)ds. \tag{2.5.3}$$

If $R(t)$ is any solution of (2.5.2), from (2.5.2) it follows that $R(t)^*$ is also a solution of (2.5.2). From this observation and by the uniqueness theorem it follows that $R(t)$ is symmetric for all t if $R(t)$ is symmetric for some value of t.

If $Y = Y(t)$ is a nontrivial prepared solution of the matrix differential equation (2.5.1) with $\det Y(t) \neq 0$ for $t > a$, then $R(t) = -P(t)Y'(t)Y^{-1}(t)$ is a symmetric solution of (2.5.2) on the infinite interval $[a, \infty)$. Thus the matrix differential equation (2.5.1) is oscillatory if and only if the Riccati integral equation (2.5.3) has no continuous solution in some neighborhood of infinity.

Variational Method: As in the case of scalar equations, variational principles can be employed to obtain oscillation criteria for the matrix differential system [26].

For any subinterval $[a, b]$ of $[0, \infty)$, define

$$A_1(a, b) = \{y : [a, b] \to R^n,\ y(a) = y(b) = 0,\ y \in AC[a, b] \text{ and } y' \in L^2(a, b)\},$$

as the admissible class of vector functions.

We recall the following oscillation criterion for the matrix differential equation

$$Y'' + Q(t)Y = 0. \tag{2.5.4}$$

THEOREM 2.5.1 [26]. *The matrix differential equation (2.5.4) is oscillatory if and only if there is a sequence of intervals* $[a_n, b_n]$, *with* $\lim_{n\to\infty} a_n = \infty$, *and a sequence of functions* $Q_n \in A_1(a_n, b_n)$, *such that*

$$\int_{a_n}^{b_n} \{|Q_n'(t)|^2 - Q_n^*(t)Q(t)Q_n(t)\}dt < 0.$$

CHAPTER III

3.1. Introduction

In this chapter we are concerned with the second order $n \times n$ matrix differential equation

$$Y'' + Q(t)Y = 0, \quad t \in [a, \infty) \tag{3.1.1}$$

where $Y(t)$, $Q(t)$ are $n \times n$ real continuous matrix functions and $Q(t)$ is symmetric. We know that the corresponding scalar equation

$$y'' + q(t)y = 0, \quad t \in [a, \infty) \tag{3.1.2}$$

is nonoscillatory on $[a, \infty)$ if and only if the Riccati integral equation

$$r(t) = r(t_0) + \int_{t_0}^{t} q(s)ds + \int_{t_0}^{t} r^2(s)ds \tag{3.1.3}$$

has a continuous solution on $[t_0, \infty)$ for some t_0. If $y = y(t)$ with $y(t) \neq 0$ for $t \geq t_0$ is a solution of the scalar equation (3.1.2) then $r(t) = -y'/y$ is a solution of the associated Riccati integral equation (3.1.3). Similarly, we have seen that if $Y = Y(t)$ is a nontrivial prepared solution of the second order matrix differential equation (3.1.1), then $R(t) = -Y'Y^{-1}$ is a symmetric solution of the corresponding matrix Riccati equation

$$R(t) = R(t_0) + Q_1(t) + F(t) \tag{3.1.4}$$

where

$$Q_1(t) = \int_{t_0}^{t} Q(s)ds \tag{3.1.5}$$

$$F(t) = \int_{t_0}^{t} R^2(s). \tag{3.1.6}$$

For the scalar equation (3.1.2) Hartman [25] has shown that if equation (3.1.2) is nonoscillatory on $[a,\infty)$, then a necessary and sufficient condition that

$$\int^{\infty} \left(\frac{y'}{y}\right)^2 dt < \infty$$

holds for a solution $y \neq 0$ is that

$$\liminf_{T\to\infty} \frac{1}{T}\int_a^T \int_a^t q(s)dsdt > -\infty.$$

Hence when studying the oscillation behaviour of the scalar equation (3.1.2), one has to consider the two distinct cases namely, (i) the above limit does hold, and (ii) the above limit does not hold.

3.2. Recent results

From the above considerations it follows that one has to consider the extended real-valued function L which is defined on the class of $n\times n$ continuous real symmetric matrices defined on $[a,\infty)$ by

$$L(Q) \equiv \liminf_{T\to\infty} \frac{1}{T}\int_a^T \int_a^t Tr\ Q(s)dsdt.$$

The value of $L(Q)$ will play a central role in the discussion to follow. The following lemma is due to Mingarelli [44] [see also Butler, Erbe and Mingarelli [9]], and is the systems analogue of the scalar result of Hartman [25] mentioned earlier in this chapter.

LEMMA 3.2.1 [9]. *Assume that the second order matrix differential equation (3.1.1) is nonoscillatory on* $[a,\infty)$. *Then a necessary and sufficient condition that*

$$\lim_{T\to\infty} \int_t^T R^2(s)ds$$

exists for any solution $R = -Y'Y^{-1}$ *of*

$$R(t) = R(t_0) + \int_{t_0}^t Q(s)ds + \int_{t_0}^t R^2(s)ds$$

where $Y(t)$ *is a prepared solution of equation (3.1.1) is that*

$$L(Q) = \liminf_{T\to\infty} \frac{1}{T} \int_a^T \int_a^t Tr\ Q(s)dsdt > -\infty.$$

□

As in the scalar case, by considering the two distinct cases $L(Q) = -\infty$, $L(Q) > -\infty$ separately, Butler, Erbe and Mingarelli [9] obtained a number of oscillation criteria for the equation (3.1.1), some of which are generalizations of the scalar results mentioned earlier.

THEOREM 3.2.2 [9]. *Assume* $L(Q) > -\infty$. *Then equation (3.1.1) is oscillatory if any of the following conditions hold.*

(A) $\limsup_{T\to\infty} \frac{1}{T} \int_a^T \lambda_1\Big(\int_a^t Q(s)ds\Big)dt = +\infty;$

(B) $\limsup_{T\to\infty} \frac{1}{T} \int_a^T \Big[\lambda_1\Big(\int_a^t Q(s)ds\Big)\Big]^2 dt = +\infty;$

(C) $\lim\,\text{approx}\sup_{T\to\infty} \lambda_1\Big(\int_a^T Q(s)ds\Big) = +\infty;$

(D) $\lim\operatorname{approx}\inf_{T\to\infty} \lambda_1\Big(\int_a^T Q(s)ds\Big) = -\infty.$

□

THEOREM 3.2.3 [9]. *Assume $L(Q) = -\infty$. Then (3.1.1) is oscillatory if*

$$\lim\operatorname{approx}\sup_{T\to\infty} \lambda_n\Big(\int_a^T Q(s)ds\Big) > -\infty. \qquad \square$$

As mentioned earlier, Wintner [57] showed that the scalar equation (3.1.2) is oscillatory if

$$\lim_{T\to\infty} \frac{1}{T}\int_a^T \int_a^t q(s)dsdt = +\infty.$$

A partial generalization of this result to systems is

THEOREM 3.2.4 [9]. *Assume that*

$$\lambda_1\Big(\int_a^t Q(s)ds\Big) > 0$$

for sufficiently large t, and that

$$\lim_{t\to\infty} \left|\frac{\lambda_1\Big(\int_a^t Q(s)ds\Big)}{\lambda_n\Big(\int_a^t Q(s)ds\Big)}\right| > 0.$$

Then (3.1.1) is oscillatory if

$$\lim_{T\to\infty} \frac{1}{T}\int_a^T \lambda_1\Big(\int_a^t Q(s)ds\Big)dt = +\infty.$$

□

Oscillation results for the equation $(3.1.1)_n$ which involve the quotient of $\lambda_1\Big(\int_a^t Q(s)ds\Big)$ and $\lambda_n\Big(\int_a^t Q(s)ds\Big)$ with some other conditions, were also given by Butler and Erbe [6].

3.3. Oscillation theorems

Consider the second order matrix differential equation

$$Y'' + Q(t)Y = 0, \quad t \in [a, \infty). \tag{3.3.1}$$

Once again, by considering two different cases depending on the value of

$$L(Q) = \liminf_{T\to\infty} \frac{1}{T}\int_a^T \int_a^t Tr\ Q(s)dsdt,$$

and by using the behavior of the intermediate eigenvalues one can obtain the following results.

THEOREM 3.3.1. *The matrix differential equation (3.3.1) is oscillatory if*

(i) $L(Q) > -\infty$, *(3.3.2)*

(ii) $\liminf\limits_{t\to\infty}$ *approx* $\sigma_k\Big(\int_a^t Q(s)ds\Big) = -\infty$, *(3.3.3)*

for some $1 \le k \le n$, *where* $\sigma_k(A) = \sum_{i=1}^{k} \lambda_i(A)$.

THEOREM 3.3.2. *The matrix differential equation (3.3.1) is oscillatory if*

(i) $L(Q) = -\infty$,

(ii) $\limsup\limits_{t_0\to\infty}$ *approx* $\rho_k\Big(\int_a^t Q(s)ds\Big) > -\infty$, *(3.3.4)*

for some $1 \leq k \leq n$, *where* $\rho_k(A) = \sum_{i=1}^{k} \lambda_{n-k+i}(A)$.

Before we give the proofs of these theorems, we note that by taking $Q(t) = \operatorname{diag}(q_1(t), \ldots, q_n(t))$, with suitable $q_i(t)$, one can construct examples to illustrate that any one of the (ii) conditions may hold for some k_0, $1 < k_0 \leq n$, but may fail for all $1 \leq k < k_0$. Thus each of the above results gives a sequence of n tests for the oscillation of equation (3.3.1).

PROOF OF THEOREM 3.3.1: Assume that equation (3.3.1) is nonoscillatory on $[a, \infty)$. Let $Y = Y(t)$ be a nontrivial prepared solution of (3.3.1) with $\det Y(t) \neq 0$ for $t \geq a$. Then $Z = -Y'Y^{-1}$ is a symmetric solution of the Riccati equation

$$Z(t) = Z(a) + \int_a^t Q(s)ds + \int_a^t Z^2(s)ds \tag{3.3.5}$$

for $t \geq a$. From the assumption made at the beginning of this proof and condition (3.3.2), we conclude, by using Lemma [3.2.1], that

$$\lim_{T \to \infty} \int_t^T Tr\ Z^2(s)ds \tag{3.3.6}$$

exists as a finite number. From (3.3.3), it follows that for $M > 0$,

$$mes\Big\{t : \sigma_k\Big(\int_a^t Q(s)ds\Big) < -M\Big\} = +\infty.$$

From (3.3.6) and (3.3.5) we get

$$Z(t) + \int_t^\infty Z^2(s)ds + C = \int_{t_0}^t Q(s)ds$$

where $C = -Z(a) - \int_a^\infty Z^2(s)ds$. Since $Z^2(s) \geq 0$, we have

$$\sigma_k(Z(t)) \leq \sigma_k\Big(Z(t) + \int_t^\infty Z^2(s)ds\Big)$$

$$= \sigma_k\Big(\int_a^t Q(s)ds - C\Big).$$

The subadditive nature of σ_k gives

$$\sigma_k(Z(t)) \leq \sigma_k\Big(\int_a^t Q(s)ds\Big) + \sigma_k(-C).$$

If $M > 1 + |\sigma_k(-C)|$, then

$$\sigma_k\Big(\int_a^t Q(s)ds\Big) < -M$$

which implies $\sigma_k(Z(t)) < -1$. Hence

$$mes\{t : \sigma_k(Z(t)) < -1\} = +\infty.$$

This in turn implies

$$\int_a^\infty \big(\sigma_k Z(t)\big)^2 dt = +\infty.$$

Now using the fact that for a symmetric matrix A we have $|\sigma_k(A)|^2 \leq k\sigma_k(A^2)$, we arrive at

$$\int_a^\infty k\sigma_k(Z^2(t))dt = +\infty$$

and whence

$$\int_a^\infty Tr\ Z^2(t)dt = +\infty,$$

which is a contradiction to (3.3.6). Hence equation (3.3.1) is oscillatory.

PROOF OF THEOREM 3.3.2: Assume $L(Q) = -\infty$ and (3.3.4) holds. If equation (3.3.1) is nonoscillatory, then we may assume that there exists a solution $Y = Y(t)$ of (3.3.1), with $\det Y(t) \neq 0$ on $[a, \infty)$. Then $Z = -Y'Y^{-1}$ is a symmetric solution of the Riccati equation

$$Z(t) = Z(a) + \int_a^t Z^2(s)ds + \int_a^t Q(s)ds. \tag{3.3.5}$$

Since

$$\sigma_k\Big(-\int_a^t Q(s)ds\Big) = -\rho_k\Big(\int_a^t Q(s)ds\Big),$$

we have

$$\begin{aligned}\sigma_k\Big(Z(t) - \int_a^t Q(s)ds\Big) &\leq \sigma_k(Z(t)) + \sigma_k\Big(-\int_a^t Q(s)ds\Big) \\ &= \sigma_k(Z(t)) - \rho_k\Big(\int_a^t Q(s)ds\Big).\end{aligned}$$

Upon noting that for symmetric matrices A we have $Tr(A) \leq (n-k+1)\sigma_k(A) \leq n\sigma_k(A)$, we have

$$\begin{aligned}\frac{1}{n}\,Tr\int_a^t Z^2(s)ds &= \frac{1}{n}\,Tr\Big(Z(t) - \int_a^t Q(s)ds - Z(a)\Big) \\ &\leq \sigma_k\Big(Z(t) - Z(a) - \int_a^t Q(s)ds\Big) \\ &\leq \sigma_k(Z(t)) - \rho_k(Z(a)) - \rho_k\Big(\int_a^t Q(s)ds\Big).\end{aligned}$$

That is,

$$\rho_k(Z(a)) + \frac{1}{n}\,Tr\Big(\int_a^t Z^2(s)ds\Big) \leq \sigma_k(Z(t)) - \rho_k\Big(\int_a^t Q(s)ds\Big).$$

As

$$\frac{1}{n}\ Tr\ \int_a^t Z^2(s)ds \geq \frac{1}{n}\int_a^t \sigma_k(Z^2(s))ds,$$

we obtain

$$\rho_k(Z(a)) + \frac{1}{n}\ \int_a^t \sigma_k(Z^2(s))ds \leq \sigma_k(Z(t)) - \rho_k\Big(\int_a^t Q(s)ds\Big). \tag{3.3.7}$$

If

$$m = \lim_{t\to\infty} \operatorname{approx\,sup}\, \rho_k\Big(\int_a^t Q(s)ds\Big),$$

then for any $\epsilon > 0$, it follows that

$$mes\Big\{t : \rho_k\Big(\int_a^t Q(s)ds\Big) \geq m - \epsilon\Big\} = +\infty.$$

We observe that if

$$\rho_k\Big(\int_a^t Q(s)ds\Big) \geq m - \epsilon,$$

then

$$\rho_k(Z(a)) + \frac{1}{n}\int_a^t \sigma_k(Z^2(s))ds \leq \sigma_k(Z(t)) - m + \epsilon$$

whence

$$mes\big\{t : \rho_k(Z(a)) + \frac{1}{n}\int_a^t \sigma_k(Z^2(s))ds \leq \sigma_k(Z(t)) - m + \epsilon\big\} = +\infty.$$

Since $L(Q) = -\infty$, it follows that

$$\int_{t_0}^t Tr\ Z^2(s)ds \to +\infty$$

as $t \to \infty$ and therefore

$$\sigma_k\Big(\int_a^t Z^2(s)ds\Big) \to +\infty$$

as $t \to +\infty$. We note that if the set E is defined by

$$E = \left\{ t \in [a+1, \infty) : \frac{1}{2n} \int_a^t \sigma_k(Z^2(s))ds \leq \sigma_k(Z(t)) \right\},$$

then $mes(E) = +\infty$. By defining $P(t)$ as

$$P(t) = \int_a^t \sigma_k(Z^2(s))ds,$$

we have

$$P'(t) + \sigma_k(Z^Z(t)) \geq \frac{1}{k}(\sigma_k(Z(t))^2$$

and for $t \in E$ we have

$$\sigma_k(Z(t)) \geq \frac{1}{2n} \int_a^t \sigma_k(Z^2(s))ds$$

which implies that

$$(\sigma_k(Z(t)))^2 \geq \frac{1}{4n^2} P^2(t).$$

That is,

$$P'(t) \geq \frac{1}{4n^2 k} P^2(t).$$

Integrating over the set E gives

$$\frac{1}{P(a+1)} \geq \int_E \frac{p'(t)}{p^2(t)} dt \geq \frac{1}{4n^2 k} \cdot \mu(E) = +\infty,$$

a contradiction. Hence equation (3.3.1) is oscillatory. □

A simple modification of the above proof leads to the following result.

THEOREM 3.3.3. *The differential system (3.3.1) is oscillatory if*

(i) $L(Q) = -\infty$;

(ii) $\limsup_{t\to\infty}^{approx} Tr\Big(\int_a^t Q(s)ds\Big) > -\infty.$ *(3.3.8)*

3.4. More oscillation criteria.

For the scalar equation

$$y'' + q(t)y = 0, \tag{3.4.1}$$

Olech, Opial and Wazewski [49] proved that

$$\lim_{T\to\infty}^{approx} \int_a^T q(s)ds = +\infty$$

or

$$\liminf_{T\to\infty}^{approx} \int_a^T q(s)ds < \limsup_{T\to\infty}^{approx} \int_a^T q(s)ds$$

implies oscillation of (3.4.1). As noted before, Wintner [57] established that equation (3.4.1) is oscillatory in the case

$$\lim_{T\to\infty} \frac{1}{T}\int_a^T \int_a^t q(s)dsdt = +\infty.$$

In case the above limit fails to exist, Hartman [26] showed that

$$-\infty < \liminf_{T\to\infty} \frac{1}{T}\int_a^T \int_a^t q(s)dsdt < \limsup_{T\to\infty} \frac{1}{T}\int_a^T \int_a^t q(s)dsdt$$

guarantees the oscillation of the equation (3.4.1). We now concern ourselves with the matrix equation

$$Y'' + Q(t)Y = 0. \tag{3.4.2}$$

Recently Erbe [15] generalized the above mentioned oscillation criterion for the scalar equation to derive an oscillation criterion for the second order differential system (3.4.2). For this he exploited the behaviour of the largest eigenvalue of the integral of the coefficient matrix $Q(t)$ together with the value of $L(Q)$. More precisely, Erbe proved

THEOREM 3.4.1 [15]. *The matrix differential equation (3.4.2) is oscillatory if*

(i) $L(Q) > -\infty$;

(ii) there exists $\delta > 0$ *such that for all large* t_0, *we have*

$$\eta(t_0) - \mu(t_0) \geq \delta$$

where

$$\eta(t_0) \equiv \limsup_{T\to\infty} \frac{1}{T}\int_{t_0}^{T} \lambda_1\Big(\int_{t_0}^{t} Q(s)dsdt\Big)$$

$$\mu(t_0) \equiv \liminf_{T\to\infty} \frac{1}{T}\int_{t_0}^{T} \lambda_1\Big(\int_{t_0}^{t} Q(s)dsdt\Big).$$

We are interested in determining the extent to which the criteria given by Olech, Opial and Wazewski mentioned earlier have analogues for the matrix equation. In this direction, we have

THEOREM 3.4.2. *The matrix differential equation (3.4.2) is oscillatory if*

(i) $L(Q) > -\infty$;

(ii) there exists $\delta > 0$ *and* k, $1 \leq k \leq n$, *such that for all large* t_0,

$$\eta_k(t_0) - \mu_k(t_0) \geq \delta \tag{3.4.3}$$

where

$$\eta_k(t_0) = \limsup_{T\to\infty} \frac{1}{T}\int_{t_0}^{T} \sigma_k\Big(\int_{t_0}^{t} Q(s)ds\Big)dt.$$

$$\mu_k(t_0) = \liminf_{T\to\infty} \frac{1}{T}\int_{t_0}^{T} \sigma_k\Big(\int_{t_0}^{t} Q(s)ds\Big)dt.$$

PROOF: Assume that the equation (3.4.2) is nonoscillatory. Let $Y = Y(t)$ be a nontrivial prepared solution of (3.4.2) with $\det Y(t) \neq 0$ for $t \geq t_0$. Then $Z = -Y'Y^{-1}$ is a solution of the Riccati equation

$$Z(t) = Z(t_0) + \int_{t_0}^{t} Q(s)ds + \int_{t_0}^{t} Z^2(s)ds$$

for $t \geq t_0$. First we suppose that both $\eta_k(t_0)$ and $\mu_k(t_0)$ are finite. By defining

$$Q_1(t;t_0) \equiv \int_{t_0}^{t} Q(s)ds \tag{3.4.4}$$

$$A(t;t_0) \equiv Z(t_0) + \int_{t_0}^{t} Z^2(s)ds \tag{3.4.5}$$

and using Lemma [3.2.1], we see that $\lim_{t\to\infty} A(t;t_0)$ exists and hence $\lim_{t\to\infty} Tr\ A(t;t_0)$ exists. Take $\epsilon > 0$ such that $\epsilon < \frac{\delta}{3}$. Using the fact that

$$\lim_{T\to\infty} \int_{t}^{T} Tr\ Z^2(s)ds \tag{3.4.6}$$

exists as a finite number, we may suppose that t_0 is sufficiently large so that

$$\int_{s}^{t} Tr\ Z^2(u)du < \epsilon \tag{3.4.7}$$

for $t_0 \leq s < t < +\infty$ and also $\sigma_k(Z^2(t_0)) < \frac{\epsilon^2}{k}$. The Riccati equation takes the form

$$Z(t) = A(t;t_0) + Q_1(t;t_0). \tag{3.4.8}$$

Using the subadditivity of σ_k we have

$$\sigma_k(Z(t)) \leq \sigma_k(A(t;t_0)) + \sigma_k(Q_1(t;t_0)). \tag{3.4.9}$$

Now, using $\rho_k(A)+\sigma_k(B) \leq \sigma_k(A+B) \leq \sigma_k(A)+\sigma_k(B)$ for symmetric matrices, we obtain

$$\sigma_k(Z(t)) \geq \rho_k(A(t;t_0)) + \sigma_k(Q_1(t;t_0)). \tag{3.4.10}$$

From (3.4.5) we get

$$\sigma_k(A(t;t_0) \leq \sigma_k(Z(t_0)) + \sigma_k\Big(\int_{t_0}^{t} Z^2(s)ds\Big).$$

Now using $|\sigma_k(A)|^2 \leq k\sigma_k(A^2)$ we get

$$|\sigma_k(Z(t_0))|^2 \leq k\sigma_k(Z^2(t_0)) < \epsilon^2.$$

We also have

$$\sigma_k\Big(\int_{t_0}^{t} Z^2(s)ds\Big) \leq Tr \int_{t_0}^{t} Z^2(s)ds < \epsilon.$$

Hence from (3.4.9), it follows that

$$\sigma_k(Z(t, \leq 2\epsilon + \sigma_k(Q_1(t;t_0)) \tag{3.4.11}$$

for $t \geq t_0$. Now using the superadditivity of ρ_k, we have that

$$\rho_k(A(t_0;t)) \geq \rho_k(Z(t_0)) + \rho_k\Big(\int_{t_0}^{t} Z^2(s)ds\Big) > -\epsilon,$$

so that from (3.4.10), we obtain

$$\sigma_k(Z(t)) > -\epsilon + \sigma_k(Q_1(t;t_0)) \tag{3.4.12}$$

for $t \geq t_0$. We let $T_n \to \infty$ and $\tau_n \to \infty$ so that

$$\lim_{T_n \to \infty} \frac{1}{T_n} \int_{t_0}^{T_n} \sigma_k(Q_1(t;t_0))dt \equiv \eta_k(t_0) \tag{3.4.13}$$

and

$$\lim_{\tau_n \to \infty} \frac{1}{\tau_n} \int_{t_0}^{\tau_n} \sigma_k(Q_1(t;t_0)dt \equiv \mu_k(t_0). \tag{3.4.14}$$

From (3.4.11) we see that

$$\frac{1}{\tau_n} \int_{t_0}^{\tau_n} \sigma_k(Z(t))dt < \frac{2\epsilon(\tau_n - t_0)}{\tau_n} + \frac{1}{\tau_n} \int_{t_0}^{\tau_n} \sigma_k(Q_1(t;t_0))dt \tag{3.4.15}$$

and from (3.4.12) we obtain

$$\frac{1}{T_n} \int_{t_0}^{T_n} \sigma_k(Z(t))dt > \frac{-\epsilon(T_n - t_0)}{T_n} + \frac{1}{T_n} \int_{t_0}^{T_n} \sigma_k(Q_1(t;t_0)dt. \tag{3.4.16}$$

Now by the Schwartz inequality

$$\begin{aligned} \left|\frac{1}{T} \int_{t_0}^{T} \sigma_k(Z(t))dt\right|^2 &\leq \frac{1}{T} \int_{t_0}^{T} (\sigma_k(Z(t))^2 dt \\ &\leq \frac{k}{T} \int_{t_0}^{T} \sigma_k(Z^2(t))dt \\ &\leq \frac{k}{T} \int_{t_0}^{T} Tr\ Z^2(t)dt \to 0 \end{aligned}$$

as $T \to \infty$. Hence taking limits in (3.4.15) and (3.4.16) as $\tau_n \to \infty$, $T_n \to \infty$ we get

$$-\epsilon + \eta_k(t_0) \leq 0 \leq 2\epsilon + \mu(t_0)$$

which in turn gives

$$\eta_k(t_0) - \mu_k(t_0) < \delta,$$

a contradiction to (3.4.3), thus proving the theorem when both $\eta_k(t_0)$ and $\mu_k(t_0)$ are finite. Since $\sigma_k(A) \leq k\lambda_1(A)$ for all symmetric matrices A, in the case

$$\eta_k(t_0) = \limsup_{T\to\infty} \frac{1}{T}\int_{t_0}^{T} \sigma_k\Big(\int_{t_0}^{t} Q(s)ds\Big)dt = +\infty,$$

it follows that

$$\limsup_{T\to\infty} \int_{t_0}^{T} \lambda_1\Big(\int_{t_0}^{t} Q(s)ds\Big)dt = +\infty.$$

In this case the theorem follows by Theorem [3.2.2]. Finally, we assume that $\eta_k(t_0) < +\infty$ and $\mu_k(t_0) = -\infty$. That is,

$$\lim_{\tau_n} \frac{1}{\tau_n}\int_{t_0}^{\tau_n} \sigma_k(Q_1(t;t_0))dt = -\infty.$$

Hence, we get

$$-\epsilon + \eta_k(t_0) \leq 0 \leq 2\epsilon + \mu_k(t_0) = -\infty$$

a contradiction. This completes the proof in its entirety. □

THEOREM 3.4.3. *Assume $L(Q) > -\infty$. Then the matrix differential equation (3.4.2) is oscillatory if there exists $\delta > 0$ such that for all large t_0 we have*

$$\ell(t_0) + \delta \leq m(t_0)$$

where

$$\ell(t_0) = \lim_{t\to\infty} \operatorname{approx\,inf}\ \lambda_1(Q_1(t;t_0));$$

$$m(t_0) = \lim_{t\to\infty} \operatorname{approx\,sup}\ \lambda_1\Big(\int_{t_0}^{t} Q(s)ds\Big).$$

□

We can now state and prove a more general result :

THEOREM 3.4.4. *The matrix differential equation (3.4.2) is oscillatory if*

(i) $L(Q) > -\infty$;

(ii) there exists $\delta > 0$ *such that for all large* t_0, *we have*

$$m_k(t_0) - \ell_k(t_0) \geq \delta \tag{3.4.17}$$

for some $1 \leq k \leq n$, *where*

$$\ell_k(t_0) \equiv \lim_{t\to\infty} \text{approx inf } \sigma_k\Big(\int_{t_0}^{t} Q(s)ds\Big);$$

$$m_k(t_0) \equiv \lim_{t\to\infty} \text{approx sup } \sigma_k\Big(\int_{t_0}^{t} Q(s)ds\Big);$$

$$\sigma_k(A) = \sum_{i=1}^{k} \lambda_i(A).$$

PROOF: Assume that equation (3.4.2) is nonoscillatory. As before let $Y = Y(t)$ be a nontrivial prepared solution of (3.4.2) with $\det Y(t) \neq 0$ for $t \geq t_0$. Then $Z = -Y'Y^{-1}$ is a symmetric solution of the Riccati equation

$$Z(t) = Z(t_0) + \int_{t_0}^{t} Q(s)ds + \int_{t_0}^{t} Z^2(s)ds$$

for $t \geq t_0$. By defining $Q_1(t;t_0)$ and $A(t;t_0)$ as before and using Lemma [3.2.1] it is easy to see that $\lim_{t\to\infty} Tr\ A(t;t_0)$ exists. Taking $\epsilon > 0$ such that $\epsilon < \frac{\delta}{3}$,

as in the proof of the previous theorem, we may suppose that t_0 is sufficiently large so that

$$\int_{t_0}^{\infty} \sigma_k(Z^2(s))ds < \epsilon \qquad (3.4.18)$$
$$|\sigma_k(Z(t_0))| < \epsilon$$

and

$$|\rho_k(Z(t_0))| < \epsilon.$$

Now using the Riccati equation and the subadditivity of σ_k we get

$$\begin{aligned}
\sigma_k(Z(t)) &\leq \sigma_k(A(t;t_0)) + \sigma_k(Q_1(t;t_0)) \\
&\leq \sigma_k(Z(t_0)) + \sigma_k\Big(\int_{t_0}^{t} Z^2(s)ds\Big) + \sigma_k(Q_1(t;t_0)) \\
&< \epsilon + \int_{t_0}^{t} \sigma_k(Z^2(s))ds + \sigma_k(Q_1(t;t_0)) \\
&< 2\epsilon + \sigma_k(Q_1(t;t_0)). \qquad (3.4.19)
\end{aligned}$$

Similarly, using that fact that $\rho_k(A)+\sigma_k(B) \leq \sigma_k(A+B)$ for symmetric matrices A and B, we get

$$\begin{aligned}
\sigma_k(Z(t)) &\geq \rho_k(Z(t_0)) + \sigma_k\Big(\int_{t_0}^{t} Z^2(s)ds + Q_1(t;t_0)\Big) \\
&\geq \rho_k(Z(t_0)) + \rho_k\Big(\int_{t_0}^{t} Z^2(s)ds\Big) + \sigma_k(Q_1(t;t_0)) \\
&> -\epsilon + \sigma_k(Q_1(t;t_0)). \qquad (3.4.20)
\end{aligned}$$

For a symmetric matrix A, we know that

$$|\rho_k(A)|^2 = |\sigma_k(-A)|^2 \leq k\sigma_k(A^2).$$

Therefore it follows that $\sigma_k(Z(t))$ cannot be $\leq -\eta$ on a set of infinite measure and $\sigma_k(Z(t))$ cannot be $\geq \eta$ on a set of infinite measure for any $\eta > 0$. Hence we get

$$\begin{aligned} \lim_{t\to\infty}\text{approx}\ \ \sigma_k(Z(t)) &= 0 \\ &= \lim_{t\to\infty}\text{approx inf}\ \ \sigma_k(Z(t)) \\ &= \lim_{t\to\infty}\text{approx sup}\ \ \sigma_k(Z(t)). \end{aligned} \tag{3.4.21}$$

Therefore, from (3.4.19) we have

$$\begin{aligned} 0 &\leq \lim_{t\to\infty}\text{approx inf}(\sigma_k(A(t;t_0)) + \sigma_k(Q_1(t;t_0)) \\ &\leq 2\epsilon + \lim_{t\to\infty}\text{approx inf}\ \ \sigma_k(Q_1(t;t_0)) \\ &= 2\epsilon + \ell_k(t_0). \end{aligned} \tag{3.4.22}$$

Similarly, it follows from (3.4.20) that

$$\begin{aligned} 0 &\geq \lim_{t\to\infty}\text{approx sup}\left(\rho_k(Z(t_0)) + \sigma_k\Big(\int_{t_0}^{t} Z^2(s)ds + Q_1(t;t_0)\Big)\right. \\ &\geq -\epsilon + \lim_{t\to\infty}\text{approx sup}\ \ \sigma_k(Q_1(t;t_0)) \\ &= -\epsilon + m_k(t_0). \end{aligned} \tag{3.4.23}$$

Combining (3.4.22) and (3.4.23) we get

$$m_k(t_0) - \ell_k(t_0) < \delta,$$

a contradiction. Hence (3.4.2) is oscillatory.

CHAPTER IV

LEIGHTON-TYPE RESULTS FOR SYSTEMS

4.1. Introduction

For the second-order scalar differential equation

$$(p(t)x')' + q(t)x = 0 \tag{4.1.1}$$

where $p(t) > 0$, $q(t) \in C[a, +\infty)$, many oscillation criteria have been developed [26, 52, 56, 58]. A well known result due to Leighton [39], generalizes the earlier oscillation criterion of Fite-Wintner which states that the second order scalar differential equation

$$x'' + q(t)x = 0 \tag{4.1.2}$$

is oscillatory in case the coefficient $q(t)$ satisfies the condition

$$\int_a^\infty q(s)ds = +\infty. \tag{4.1.3}$$

LEIGHTON'S THEOREM. *The second-order scalar differential equation (4.1.1) is oscillatory if*

$$\int_a^\infty p^{-1}(s)ds = \int_a^\infty q(s)ds = +\infty. \tag{4.1.4}$$

□

It is of interest to look for analogues of these results for systems.

4.2. An Example

Consider the corresponding self-adjoint matrix differential equation

$$(P(t)Y')' + Q(t)Y = 0 \tag{4.2.1}$$

where P, Q, Y are $n \times n$ real continuous matrix valued functions with $P(t), Q(t)$ symmetric and $P(t)$ positive definite for $t \in [a, +\infty)$. We also consider the formally self-adjoint matrix differential equation

$$Y'' + Q(t)Y = 0 \tag{4.2.2}$$

where $Q(t)$ is as above.

We shall be interested in an analogue of Leighton's theorem for equation (4.2.1). Consider the following simple example [7]): Let $P(t) = Q(t) = \text{diag}(e^{2t}, e^{-2t})$ in (4.2.1). That is, consider the 2-dimensional system

$$\left(\begin{bmatrix} e^{2t} & 0 \\ 0 & e^{-2t} \end{bmatrix} \begin{bmatrix} y_1 \\ y_2 \end{bmatrix}' \right)' + \begin{bmatrix} e^{2t} & 0 \\ 0 & e^{-2t} \end{bmatrix} \begin{bmatrix} y_1 \\ y_2 \end{bmatrix} = 0.$$

Then a nontrivial prepared nonoscillatory solution is $Y(t) = \text{diag}(e^{-t}, e^{t})$. However, one can see that

(i) $\lambda_1\left(\int_0^t P^{-1}(s)ds\right)$ and $\lambda_1\left(\int_0^t Q(s)ds\right)$ both tend to $+\infty$ as $t \to \infty$;

(ii) $\int_0^t \det(P^{-1}(s))ds$ and $\int_0^t \det(Q(s))ds$ both tend to $+\infty$ as $t \to +\infty$;

(iii) $\int_0^t Tr(P^{-1}(s))ds$ and $\int_0^t Tr\ (Q(s))ds$ both tend to $+\infty$ as $t \to +\infty$.

In the light of the above example, it is not obvious what the analogous theorem for system (4.2.1) should be.

4.3. A Conjecture

Using positive linear functionals Etgen and Pawlowski [20] have shown that equation (4.2.2) is oscillatory at ∞ if there exists a positive linear functional ϕ on the set of all $n \times n$ real symmetric matrices such that

$$\lim_{t\to\infty} \phi\Big(\int_0^t Q(s)ds\Big) = \infty. \tag{4.3.1}$$

As the trace of a matrix is a positive linear functional, it immediately follows that equation (4.2.2) is oscillatory at ∞ whenever

$$\lim_{t\to\infty} Tr\Big(\int_0^t Q(s)ds\Big) = +\infty. \tag{4.3.2}$$

There are several results dealing with the oscillation of the equation (4.2.1), and they are largely based on the use of positive linear functionals [1, 7, 19, 20, 21, 28, 54]. The basic result obtained is that equation (4.2.1) is oscillatory on $[0,\infty)$ in case there exists a positive linear functional ϕ such that the scalar equation

$$(\phi(P(t))y')' + \phi(Q(t))y = 0$$

is oscillatory, where it is assumed, without loss of generality, that ϕ is normalized so that $\phi(I) = 1$.

Although condition (4.3.2) implies the oscillation of equation (4.2.2), Mingarelli [45], observed that the condition

$$\liminf_{t\to\infty} Tr\Big(\int_0^t Q(s)ds\Big) = -\infty \tag{4.3.3}$$

may or may not imply oscillation of the equation (4.2.2).

It has been conjectured (see Hinton and Lewis [28]) that the equation (4.2.2) is oscillatory at ∞ if

$$\lim_{t\to\infty} \lambda\{Q_1(t)\} = +\infty$$

where $Q_1(t) = \int_0^t Q(s)ds$.

In the case of a non-negative definite coefficient matrix $Q(t)$, using the fact that for such $Q(t)$,

$$\lambda_1\Big(\int_s^t Q(s)ds\Big) \leq Tr\Big(\int_0^t Q(s)ds\Big)$$

Mingarelli proved that equation (4.2.2) is oscillatory. He further showed that the conjecture is also true in the case when $Q(t)$ is a constant matrix, namely,

THEOREM 4.3.1. *Let Q be a symmetric constant matrix. Then*

(A) Equation (4.2.2) is oscillatory at ∞ if Q possesses at least one positive eigenvalue.

(B) If $\lambda_1(Q) \leq 0$, the equation (4.2.2) is non-oscillatory, and in fact, disconjugate on $[0,\infty)$.

□

Mingarelli [45] also proved that the conjecture is true under the additional assumption that

$$\liminf_{t\to\infty} \frac{1}{t} Tr\Big(\int_0^t Q(s)ds\Big) > -\infty.$$

He [44] later replaced the above condition by

$$\liminf_{t\to\infty} \frac{1}{t}\int_0^t Tr\Big(\int_0^s Q(\sigma)d\sigma\Big)ds > -\infty.$$

These results were further generalized by Akiyama, Kaper, Kwong and Mingarelli [38]. Butler and Erbe [6] showed that the conjecture is true even if (4.3.4) does not hold, provided a weaker condition than (4.3.4) holds. In fact, they proved:

THEOREM 4.3.2 [6]. *Let $g(t)$ be a positive, absolutely continuous, real valued function which is nondecreasing on $[a,\infty)$ and assume*

(i) $\liminf\limits_{t\to\infty}\ \dfrac{1}{g(t)}Tr\Big(\displaystyle\int_a^t Q(s)ds\Big) = \ell > -\infty;$

(ii) $\liminf\limits_{t\to\infty}\ \dfrac{1}{g(t)}\displaystyle\int_a^t \Big(\lambda_1\int_a^s Q(\sigma)d\sigma\Big)^2 ds = +\infty$

and that $\lim\limits_{t\to\infty}\ \lambda_1\Big(\displaystyle\int_a^t Q(s)ds\Big) = +\infty.$

Then (4.2.2) is oscillatory.

□

It was also shown by Butler and Erbe that the condition

$$\lim_{t\to\infty}\ \lambda_1\Big(\int_a^t Q(s)ds\Big) = +\infty$$

can also be weakened somewhat provided a certain relation holds between the largest and smallest eigenvalues of $\int_a^t Q(s)ds$ as $t\to\infty$.

THEOREM 4.3.3 [7]. *Equation (4.2.2) is oscillatory if one of the following set of hypotheses holds:*

(1) (a) $\liminf\limits_{t\to\infty}\ \lambda_1\Big(\displaystyle\int_a^t Q(s)ds\Big) = \infty$ *and*

(b) $\limsup\limits_{t\to\infty}\ \left|\dfrac{\lambda_1(Q_1(t,a))}{\lambda_n(Q_1(t,a))}\right| > 0;$

or

(2) (a) $\limsup\limits_{t\to\infty}$ *approx* $\lambda_1(Q_1(t,a)) = \infty$ *and*

(b) $\liminf\limits_{t\to\infty}$ *approx* $\left|\dfrac{\lambda_1(Q_1(t,a))}{\lambda_n(Q_1(t,a))}\right| > 0.$

□

Using the behaviour of the other eigenvalues, Atkinson, Kaper and Kwong [2] proved

THEOREM 4.3.4 [2]. *Equation (4.2.2) is oscillatory at infinity if*

$$\lim_{t\to\infty} \lambda_i\Big\{\int_a^t Q(s)ds\Big\} = \infty, \quad i = 1, 2, \ldots, n-1.$$

□

Kaper and Kwong in [36] first gave an affirmative answer to the conjecture when the dimension n equals 2. Finally, Kaper and Kwong [37] showed that the equation (4.2.2) is oscillatory if the largest eigenvalue of the matrix $\int_a^t Q(s)ds$ is sufficiently large on a sufficiently large set of t-values, thus confirming and strengthening the conjecture.

THEOREM 4.3.5 [37]. *There exists a finite number T_n, whose value depends on the dimension n, such that equation (4.2.2) is oscillatory at infinity whenever*

$$\liminf_{\alpha\to\infty}\{\alpha \ mes \ S_\alpha(a,\infty)\} \geq T,$$

where $S_\alpha(a,\infty) = \Big\{t \in (a,\infty) : \lambda_1\Big(\int_a^t Q(s)ds\Big) \geq \alpha\Big\}$ *for any positive number* α.

Using the method of Kaper and Kwong [37], we can obtain a similar result for the equation (4.2.1), in case $P(t)$ ***is diagonal such that*** $P(t) \leq I$.

Taking $R(t) = -P(t)Y'(t)Y^{-1}(t)$ and using the fact that $Y(t)$ is a solution of (4.2.1) we get the Riccati equation

$$R(t) = R(a) + \int_a^t Q(s)ds + \int_a^t R(s)P^{-1}(s)R(s)ds$$

which can be written as

$$R(t) = F(t) + \int_a^t R(s)P^{-1}(s)R(s)ds \tag{4.3.4}$$

where

$$F(t) = R(a) + Q_1(t) \tag{4.3.5}$$

and $Q_1(t) = \int_a^t Q(s)ds$. For $\alpha > 0$, define S_α as

$$S_\alpha(a,b) = \{t \in (a,b) : \lambda_1\{F(t)\} \geq \alpha\}.$$

LEMMA 4.3.6. *There exists T_n such that*

$$\int_a^b R(s)P^{-1}(s)R(s)ds \geq \frac{1}{10}I \tag{4.3.6}$$

whenever mes $S_1(a,b) \geq T_n$.

PROOF: Lemma 4.3.6 will be proved by using induction on the dimension n. When $n = 1$, we are dealing with the scalar case. In this case $\lambda_1\{F(t)\} = F(t)$ and we also have

$$\int_a^b R(s)P^{-1}(s)R(s)ds = \int_a^b P^{-1}R^2(s)ds.$$

As $P^{-1}(t) > 0$, $R^2(t) \geq 0$, it follows from (4.3.4), using $F(t) \geq 1$, that $R(t) \geq 1$. Hence

$$\begin{aligned}\int_a^b R(s)P^{-1}(s)R(s)ds &= \int_a^b P^{-1}(s)R^2(s)ds \\ &\geq P^{-1}(t_*)\int_a^b R^2(s)ds \\ &\geq P^{-1}(t_*)mes\ S_1(a,b)\end{aligned}$$

where $t_* \in [a,b]$ such that $P^{-1}(t_*) = \min_{t\in[a,b]} P^{-1}(t)$. We can take $T_1 = \frac{1}{10}$ and the lemma is true.

Now assume that Lemma 4.3.6 is true for dimensions less than or equal to n. For $k \leq n$ we can establish a number of results, some of which show that the constant $\frac{1}{10}$ can be dropped if the set S_α is large enough and others establish the relation between the size $S_\alpha(a,b)$ and nonexistence of continuous solutions to Riccati equations. In fact, using change of variables and translation we obtain

LEMMA 4.3.7. *If* $mes\ S_\alpha(a,b) \geq \frac{10}{\alpha}T_n$ *then*

$$\int_a^b R(s)P^{-1}(s)R(s)ds \geq \alpha I.$$

The connection between $S_\alpha(a,b)$ and the nonexistence of a continuous solution to the Riccati equation is established in the following

LEMMA 4.3.8. *The Riccati equation (4.3.4) does not have a continuous solution on* $[a, b)$ *if mes* $S_1(a, b) \geq 20T_n$.

If possible, assume the contrary. Taking $a_1 = a$ and $b_1 < b$ such that *mes* $S_1(a_1, b_1) = 10\ T_n$ we get

$$\int_{a_1}^{b_1} R(s)P^{-1}(s)R(s)ds \geq I.$$

Now let $a_2 = b_1$ and choose b_2 such that *mes* $S_1(a_3, b_3) = \frac{10\ T_n}{2}$. Consider

$$R(t) = F_2(t) + \int_{a_2}^{t} R(s)P^{-1}(s)R(s)ds, \quad t \geq a_2$$

where $F_2(t) = F(t) + \int_{a_1}^{b_1} R(s)P^{-1}(s)R(s)ds$. Note that [5, 42]

$$\lambda_1\{F_2(t)\} \geq \lambda_1\{F(t)\} + \lambda_n\Big\{ \int_{a_1}^{b_1} R(s)P^{-1}(s)R(s)ds \Big\}$$

$$\geq 2 \quad \text{for all } t \in S_1(a_2, b_2).$$

Hence by the previous lemma we get

$$\int_{a_2}^{b_2} R(s)P^{-1}(s)R(s)ds \geq 2I.$$

Now let $a_3 = b_2$ and choose b_3 such that *mes* $S_1(a_3, b_3) = \frac{10T_n}{4}$. Consider

$$R(t) = F_3(t) + \int_{a_3}^{t} R(s)P^{-1}(s)R(s)ds, \quad t \geq a_3,$$

where

$$F_3(t) = F(t) + \int_{a_1}^{b_1} R(s)P^{-1}(s)R(s)ds + \int_{a_2}^{b_2} RP^{-1}(s)R(s)ds.$$

Note that $\lambda_1\{F_3(t)\} \geq 4$ for all $t \in S_1(a_3, b_3)$. Hence we obtain

$$\int_{a_3}^{b_3} R(s)P^{-1}(s)ds \geq 4I.$$

Note that $b_n \to \bar{b} \leq b$ where $\bar{b}$ is such that $mes\ S_1(a, \bar{b}) = \frac{\pi^2}{6} 10\ T_n$ and $\int_a^t R(s)P^{-1}(s)R(s)ds$ exceeds any multiple of I as $t \to \bar{b}$. Hence lemma (4.3.8) follows.

LEMMA 4.3.9. *If* $mes\ S_\alpha(a, b) \geq \frac{20\ T_n}{\alpha}$, *then the Riccati equation (4.3.4) does not have a continuous solution on* $[a, b)$.

Assume the contrary. That is, we assume that

$$R(t) = F(t) + \int_a^t R(s)P^{-1}(s)R(s)ds$$

exists as a continuous solution on $[a, b)$. Using $\bar{R}(t) = \frac{1}{\alpha} R\left(\frac{t}{\alpha}\right)$ we get

$$\bar{R}(t) = \bar{F}(t) + \int_{\alpha a}^t \bar{R}(s)\bar{P}^{-1}(s)\bar{R}(s)ds$$

exists as a continuous solution on $[\alpha a, \alpha b)$, where $\bar{F}(t) = \frac{1}{\alpha} F\left(\frac{t}{\alpha}\right)$ and $\bar{P}(t) = P\left(\frac{t}{\alpha}\right)$. Letting $\bar{S}(\alpha a, \alpha b) = \{t \in (\alpha a, \alpha b) : \lambda_1\{\bar{F}(t)\} \geq 1\}$ note that $mes\ \bar{S}(\alpha a, \alpha b)$ $\alpha\ mes\ S_\alpha(a, b) \geq 20\ T_n$. Hence $\bar{R}(t)$ is not a continuous solution, a contradiction.

LEMMA 4.3.10. *If* $mes\ S_1(a, b) \geq \frac{20\ T_n}{\delta^2}$, $\delta > 0$, *then*

$$R(t) = \delta\left[F(t) + \int_a^t R(s)P^{-1}(s)R(s)ds\right]$$

does not have a continuous solution on $[a, b)$.

To prove this, let $\bar{R}(t) = \frac{1}{\delta} R\left(\frac{t}{\delta^2}\right)$ and note that $\bar{R}(t)$ satisfies

$$\bar{R}(t) = \bar{F}(t) + \int_{\delta^2 a}^{t} \bar{R}(s)\bar{P}^{-1}(s)\bar{R}(s)ds, \quad [\delta^2 a, \delta^2 b)$$

where $\bar{P}(s) = P\left(\frac{s}{\delta^2}\right)$ and $\bar{F}(s) = F\left(\frac{s}{\delta^2}\right)$, if $R(t)$ satisfy (4.3.4). Defining

$$\bar{S}_1(\delta^2 a, \delta^2 b) = \{t \in (\delta^2 a, \delta^2 b) : \lambda_1\{\bar{F}(t)\} \geq 1\}$$

note that $mes\ \bar{S}_1(\delta^2 a, \delta^2 b) = \delta^2 mes\ S_1(a, b)$. As long as $R(t)$ is a continuous solution on $[a, b)$, $\bar{R}(t)$ is a continuous solution on $[\delta^2 a, \delta^2 b)$. Hence we get the conclusion.

Now we continue the proof of Lemma 4.3.6. Assume that Lemma 4.3.6 is not true for $n + 1$, where we take $T_{n+1} = 2000T_n + 40n^2(n + 1)$. Hence there exists a right neighborhood $[a, b)$ of a where $mes\ S_1(a, b) \geq T_{n+1}$, but the solution of (4.3.4) does not satisfy the ordering relation (4.3.6). Without loss of generality we may assume that

$$\int_a^b R(s)P^{-1}(s)R(s)ds = \ \mathrm{diag}(\delta_1, \delta_2, \ldots, \delta_{n+1})$$

where $\delta_1 \geq \delta_2 \geq \cdots \geq \delta_{n+1}$. Then $\delta_{n+1} < \frac{1}{10}$. Using the following notation,

$$R(t) = [r_{ij}]_{(n+1)\times(n+1)}, \ G(t) = \int_a^t R(s)P^{-1}(s)R(s)ds = [\rho_{ij}(t)]_{(n\times 1)(x+1)},$$

and using the fact that $P'(t)$ is diagonal and $R^T = R$, we get

$$\sigma_{n+1} = \sum_{j=1}^{n+1} \int_1^b r_{n+1,j}^2(t)p_{jj}(t)dt < \frac{1}{10}.$$

Hence we ,

$$\int_a^b r_{n+1,j}^2(t)p_{jj}^{-1}(t)ds < \frac{1}{10} \quad \text{for} \quad j = 1, 2, \ldots, n+1. \tag{4.3.7}$$

Using these inequalities which put an upper bound on the measure of the set of points t in (a, b) where $\sqrt{r_{n+1,j}^2(t)p_{jj}^{-1}(t)}$ exceeds any given value we obtain

$$mes\Big\{t \in (a,b) : \sqrt{r_{n+1,j}^2(t)p_{jj}^{-1}(t)} \geq \frac{1}{20n}\Big\} < 40n^2. \tag{4.3.8}$$

If (4.3.8) is not true then we get

$$\int_a^b r_{n+1,j}^2(t)p_{jj}^{-1}(t)dt \geq \frac{1}{400n^2} \cdot 40n^2 \geq \frac{1}{10}$$

which contradicts (4.3.7). Thus, if we eliminate from $S_1(a, b)$ all those points t where

$$\sqrt{r_{n+1,j}^2(t)p_{jj}^{-1}(t)} \geq \frac{1}{20n}$$

for some j and call the remaining set $S_1^{\#}(a, b)$,

$$S_1^{\#}(a,b) = \Big\{t \in (a,b) : \lambda_1\{F(t)\} \geq 1 \quad \text{and} \quad \sqrt{r_{n+1,j}^2(t)P_{jj}^{-1}(t)} < \frac{1}{20n}, \quad j = 1, 2, \ldots, n+1\Big\},$$

then, as $mes\ S_1(a, b) \geq T_{n+1}$, using (4.3.8) we get

$$mes\ S_1^{\#}(a,b) > T_{n+1} - 40n^2(n+1) = 2000T_n.$$

We introduce the following partitions of $R(t)$, $G(t)$ and $P(t)$:

$$R(t) = \begin{bmatrix} R_{n\times n}^{\#}(t) & r_{n\times 1}^{\#}(t) \\ \left(r^{\#^T}(t)\right)_{1\times n} & \alpha(t) \end{bmatrix}, \qquad P(t) = \begin{bmatrix} P_{n\times n}^{\#}(t) & 0_{n\times 1} \\ 0_{1\times n} & p_{n+1,n+1}^{-1}(t) \end{bmatrix}$$

$$G(t) = \begin{bmatrix} G_{n\times n}^{\#}(t) & g_{n\times 1}^{\#}(t) \\ \left(g^{\#^T}(t)\right)_{1\times n} & \beta(t) \end{bmatrix}$$

and noting that

$$G(t) = \int_a^t \begin{bmatrix} R^{\#}_{n\times n} & r^{\#}_{n+1} \\ r^{\#^T}_{1\times n} & \alpha(s) \end{bmatrix} \begin{bmatrix} P^{\#^{-1}}_{n\times n} & 0 \\ 0 & p_{n+1,n+1} \end{bmatrix} \begin{bmatrix} R^{\#}_{n\times n}(s) & r^{\#}_{n\times 1}(s) \\ r^{\#^T}_{1\times n}(s) & \alpha(s) \end{bmatrix} ds,$$

we have, in $n \times n$ dimensions

$$G^{\#}(t) = \int_a^t R^{\#} P^{\#^{-1}} R^{\#}(s) ds + \int_a^t r^{\#} p^{-1}_{n+1,n+1} r^{\#^T} ds.$$

By taking

$$A(t) = \begin{bmatrix} R^{\#} - \frac{1}{10}\int_a^t R^{\#} P^{\#^{-1}} R^{\#} ds + \frac{9}{10} I_n & 0_{n\times 1} \\ 0_{1\times n} & \frac{19}{20} \end{bmatrix}$$

$$U(t) = \begin{bmatrix} \frac{1}{10}\int_a^t r^{\#}_{n\times 1} p^{-1}_{n+1,n+1} r^{\#^T}_{1\times n} ds & 0_{n\times 1} \\ 0_{1\times n} & 0_{1\times 1} \end{bmatrix}$$

$$V(t) = \begin{bmatrix} \frac{9}{10} G^{\#} & g^{\#} \\ g^{\#^T} & \frac{8}{10} + \beta(t) \end{bmatrix}, \quad W(t) = \begin{bmatrix} \frac{9}{10} I_n & -r^{\#}(t) \\ -r^{\#^T} & \frac{3}{20} - \alpha(t) \end{bmatrix}$$

we see that

$$F(t) = R(t) - G(t) = A(t) - U(t) - V(t) - W(t).$$

From the definition $U(t) \geq 0$ for all $t \in [a,b]$. Now from the definition of $S_1^{\#}(a,b)$ we have, on $S_1^{\#}(a,b)$ that

$$\sqrt{r^2_{n+1,j}(t) p^{-1}_{jj}(t)} < \frac{1}{20n}, \quad j = 1,2,\ldots,n$$

$$|\alpha(t)| = \left| r_{n+1,n+1}(t) \right| < \sqrt{r^2_{n+1,n+1}(t) p^{-1}_{n+1,n+1}(t)} < \frac{1}{20n} < \frac{1}{20}$$

$$\beta(t) = \int_a^t \sum_{i=1}^{n+1} r^2_{n+1,i} P^{-1}_{ii} = \rho_{n+1,n+1}(t) \leq \rho(b) = \sigma_{n+1} < \frac{1}{10}.$$

For any $(n+1)$-dimensional vector $x = (x_1, x_2, \ldots, x_{n+1})^T = (x^{\#}, x_{n+1})^T$ we have

$$\begin{aligned}(x, V(t)x) &= \frac{9}{10}\langle x^{\#}, G^{\#}(t)x^{\#}\rangle + 2\langle x^{\#}, g^{\#}(t)x_{n+1}\rangle \\ &\quad + \Big(\frac{8}{10} + \beta(t)\Big)x_{n+1}^2 \\ &> \frac{1}{9}\langle x^{\#}, G^{\#}(t)x^{\#}\rangle + 2\langle x^{\#}, g^{\#}(t)x_{n+1}\rangle \\ &\quad + 9\beta(t)x_{n+1}^2 + 8\Big(\frac{1}{10} - \beta(t)\Big)x_{n+1}^2 \\ &= \langle y, G(t)y\rangle + 8\Big(\frac{1}{10} - \beta(t)\Big)x_{n+1}^2\end{aligned}$$

where $y = \Big(\frac{1}{3}x^{\#}, 3x_{n+1}\Big)^T$. Also we have

$$\begin{aligned}(x, w(t)x) &= \frac{9}{10}\|x^{\#}\|^2 - 2\langle x^{\#}, r^{\#}x_{n+1}\rangle + \Big(\frac{3}{20} - \alpha(t)\Big)x_{n+1}^2 \\ &\geq \frac{1}{10}\|x\|^2 - 2\sum_{j=1}^{n}|r_{j,n+1}|\ \|x\|^2 + \Big(\frac{1}{20} - \alpha(t)\Big)x_{n+1}^2\end{aligned}$$

using the inequalities which hold on $S_1^{\#}(a,b)$ we get $V(t) \geq 0$ and $w(t) \geq 0$ for all $t \in S_1^{\#}(a,b)$.

Taking $Z(t) = U(t)+V(t)+W(t)$ we have $Z(t) \geq 0$ on $S_1^{\#}(a,b)$ and $A(t) = F(t)+Z(t)$. Since $\lambda_1\{F(t)\} \geq 0$ on the set $S_1(a,b)$, it follows that $\lambda_1\{A(t)\} \geq 1$ on $S_1^{\#}(a,b)$. From the definition of $A(t)$, we see that any eigenvalue of $A(t)$ that is greater than 1 must also be an eigenvalue of the $n \times n$ submatrix in the top left corner, in the definition of $A(t)$. Hence for $t \in S_1^{\#}(a,b)$,

$$\lambda_1\Big\{R^{\#} - \frac{1}{10}\int_a^t R^{\#}P^{\#^{-1}}R^{\#}(s)ds + \frac{9}{10}I_n\Big\} \geq 1. \qquad (4.3.9)$$

Let $F^{\#}$ be defined in terms of $R^{\#}$ by

$$F^{\#}(t) = 10\Big[R^{\#} - \frac{1}{10}\int_a^t R^{\#}P^{\#^{-1}}R^{\#}(s)ds\Big]. \tag{4.3.10}$$

Then it follows from (4.3.9) that

$$\lambda_1\{F^{\#}(t)\} \geq 1, \quad t \in S^{\#}(a,b).$$

This leads to the contradiction. We can interpret the definition of $F^{\#}$ as a matrix Riccati equation for $R^{\#}$,

$$R^{\#}(t) = \frac{1}{10}\Big[F^{\#}(t) + \int_a^t R^{\#}P^{\#^{-1}}R^{\#}(s)ds\Big] \tag{4.3.11}$$

and then the existence of continuous solution $R(t)$ of (4.3.4) on $[a,b)$ implies the existence of a continuous solution $R^{\#}$ of (4.3.11) on $[a,b)$. On the other hand, because $mes\ S^{\#}(a,b) > 2000T_n$, (4.3.11) can not have a continuous solution on $[a,b)$. Hence the proof follows by induction. □

Now define

$$S_\alpha(a,\infty) = \{t \in (a,\infty) : \lambda\{Q_1(t)\} \geq \alpha\}$$

for any positive α. We then have

THEOREM 4.3.11. *There exists a finite number $T(n)$ such that*

$$(P(t)Y'(t))' + Q(t)Y(t) = 0 \tag{4.3.12}$$

is oscillatory at infinity whenever

$$\lim_{\alpha\to\infty}\inf\{\alpha\ mes\ S_\alpha(a,\infty)\} \geq T(n). \tag{4.3.13}$$

PROOF: Let $T(n) = 80T_n$, where T_n is the number guaranteed by Lemma 4.3.6. From (4.3.13) we get that $mes\ S_\alpha(a,\infty) \geq \left(\frac{40}{\alpha}\right)T_n$ for α sufficiently large. Furthermore we also have, when α is sufficiently large and $\lambda_1\{Q_1(t)\} \geq \alpha$ for some t, that $\lambda_1\{R(a) + Q_1(t)\} \geq \frac{\alpha}{2}$. Hence

$$mes\{t \in (a,\infty) : \lambda_1\{R(a) + Q_1(t)\} \geq \frac{\alpha}{2}\} \geq \frac{40}{\alpha} \cdot T_n.$$

But then by the results established as a consequence of Lemma (4.3.6), we get that the equation (4.3.12) does not have a nonsingular conjoined solution on $[a,\infty)$ and hence is oscillatory at infinity.

□

The above theorem expresses the fact that oscillation at infinity is possible whenever $\lambda_1\{Q(t)\}$ is sufficiently large on a sufficiently large set. The conjecture guarantees this phenomenon in the case of the formally self-adjoint equation

$$Y''(t) + Q(t)Y(t) = 0. \tag{4.3.14}$$

COROLLARY 4.3.12. *If there is a measurable subset J of $[a,\infty)$ of infinite measure, such that*

$$\lim_{t\to\infty} \lambda_1\{Q_1(t)\} = \infty$$

then (4.3.14) is oscillatory at infinity.

4.4. Different Proof of the Conjecture

In this section we are specifically concerned with the self adjoint matrix differential equation

$$(P(t)Y')' + Q(t)Y = 0 \tag{4.4.1}$$

where P, Q are real, symmetric, continuous matrix valued functions with $P(t)$ positive definite. In addition to the condition

$$\lim_{t\to\infty} \lambda_1\Big(\int_0^t Q(s)ds\Big) = \infty \tag{4.4.2}$$

which comes from the corresponding formally self-adjoint system

$$Y'' + Q(t)Y = 0, \tag{4.4.3}$$

various conditions on $P(t)$ (namely on the eigenvalues of $P(t)$) are required, as the following discussion will show, in order to conclude that the equation (4.4.1) is oscillatory. The following are some such results by Butler and Erbe:

THEOREM 4.4.1. *The equation (4.4.1) is oscillatory if*

(i) $\lim_{t\to\infty} \lambda_1\Big(\int_a^t Q(s)ds\Big) = \infty;$

(ii) $\lim_{t\to\infty} \int_a^t \lambda_n(P^{-1}(s))ds = \infty;$

and

(iii) $\liminf_{t\to\infty} \dfrac{\lambda_1\Big(\int_a^t Q(s)ds\Big)}{\lambda_n\Big(\int_a^t Q(s)ds\Big)} > 0.$

□

The following result extends a result due to Mingarelli [45]:

THEOREM 4.4.2 [7]. *Let $g(t)$ be a positive, absolutely continuous, nondecreasing function on $[a, \infty)$ and assume*

(i) $\liminf_{t\to\infty} \dfrac{1}{g(t)}\, Tr\Big(\int_a^t Q(s)ds\Big) > -\infty;$

(ii) $\lim_{t\to\infty} \dfrac{1}{g(t)} \int_a^t \lambda_n(P^{-1}(s))\Big[\lambda_1 \int_a^s Q(\sigma)d\sigma\Big]^2 ds = +\infty;$

and

$$\textit{(iii)}\ \lim_{t\to\infty}\ \lambda_1\Big(\int_a^t Q(s)ds\Big) = \lim_{t\to\infty}\ \int_a^t \lambda_n(P^{-1}(s))ds = +\infty;$$

then equation (4.4.1) is oscillatory.

□

Using principal submatrices [5] and functionally commutative matrices [Chapter 5] Butler and Erbe also proved

THEOREM 4.4.3 [7]. *Let $g(t)$ be as in the above theorem and assume $P(t)$ is functionally commutative on $[a,\infty)$. Assume further that there exists k, $1 \le k < n$ such that*

$$\textit{(i)}\ \liminf_{t\to\infty}\Big(\frac{1}{g(t)}\Big)Tr\Big(\int_a^t Q_k(s)ds\Big) > -\infty;$$

$$\textit{(ii)}\ \lim_{t\to\infty}\Big(\frac{1}{g(t)}\Big)\int_a^t \lambda_k\big[(P^{-1}(s))_k\big]\Big[\lambda\Big(\int_a^s Q_k(\sigma)\Big)\Big]^2 ds = +\infty;$$

and

$$\textit{(iii)}\ \lim_{t\to\infty}\ \lambda_1\Big(\int_a^t Q_k(s)ds\Big) = \lim_{t\to\infty}\ \int_a^t \lambda_k\big[(P^{-1}(s))_k\big]ds = +\infty.$$

Then equation (4.4.1) is oscillatory.

□

Recently Byers, Harris and Kwong [10] proved

THEOREM 4.4.4 [10]. *The equation (4.4.1) is oscillatory if*

$$\limsup_{\lambda\to\infty}\ \lambda\Big(\int_{S_\lambda} \lambda_n(P^{-1}(t))dt\Big) > n$$

where

$$S_\lambda = \Big\{t \in [0,\infty) : \lambda_1\Big(\int_0^t Q(s)ds\Big) \ge \lambda\Big\}.$$

□

The above Theorem also proves the conjecture.

Coles [12] gave some more oscillation criteria for (4.4.1) by using the notion of weighted means introduced by Byers, Harris and Kwong [10]. Let $f_1, f_2, \ldots, f_N$ be non-negative, locally integrable scalar-valued functions defined on $[0, \infty)$. For a matrix-valued function $H(t)$ and $t \geq a \geq a$, define

$$I_1(t, a; H) = \int_a^t f_1(s)H(s)ds$$

$$J_1(t, a; H) = \int_a^t f_1^2(s)H(s)ds$$

$$i_1(t, a) = \int_a^t f_1(s)ds.$$

For $2 \leq k \leq N$:

$$I_k(t, a; H) = \int_a^t f_k(s)I_{k-1}(s, a; H)ds$$

$$J_k(t, a; H) = \int_a^t f_k^2(s)f_{k-1}^{-1}(s)J_{k-1}(s, a; H)ds$$

$$i_k(t, a) = \int_a^t f_k(s)i_{k-1}(s)ds.$$

For $1 \leq k \leq N$:

$$I_k(t; H) = I_k(t, 0, H)$$

$$J_k(t; H) = J_k(t, 0; H)$$

$$i_k(t) = i_k(t, 0).$$

Let

$$S_\lambda = \left\{ t \geq 0; \lambda_1 \left\{ i_n^{-1}(t) I_N \left(t; \int_0^t Q(s)ds \right) \right\} \geq \lambda \right\}$$

$$S_\lambda(t) = S_\lambda \cap [t, \infty)$$

$$L(\lambda) = \limsup_{t \to \infty} \; i_N(t) \cdot \int_{S_\lambda(t)} f_N(s) \lambda_n \{ J_N^{-1}(s; P) \} ds.$$

Now we can state the results of Coles:

THEOREM 4.4.5. *Equation (4.4.1) is oscillatory if*

$$\limsup_{\lambda \to \infty} \; \lambda \int_{S_\lambda} f_N(s) \lambda_n \{ J_N^{-1}(s; P) \} ds > \frac{n}{i_N(\infty)}.$$

THEOREM 4.4.6. *Equation (4.4.1) is oscillatory if*

$$\limsup_{\lambda \to \infty} \; \lambda L(\lambda) > n.$$

4.5. Leighton-type results for systems

By relaxing the stringent conditions on the smallest eigenvalue of $P(t)$, by going to some other intermediate eigenvalue of $P(t)$, and by strengthening the conditions on the intermediate eigenvalues of $Q(t)$ one can obtain an oscillation result for equation (4.4.1). This result can be viewed as a proper generalization of Leighton's result mentioned earlier in this chapter. The method of proof is modeled along the techniques used in Byers, Harris and Kwong [10] and the result obtained includes Theorem 4.4.4 as a special case when $k = 1$.

THEOREM 4.5.1. *The equation (4.4.1) is oscillatory if there exists* k, $1 \leq k \leq n$ *such that*

$$\limsup_{\lambda \to \infty} \lambda \int_{S_\lambda^k} \lambda_{n-k+1}(P^{-1}(t))dt > n$$

where

$$S_\lambda^k = \Big\{ t \in (0,\infty); \quad \lambda_k\Big(\int_0^t Q(s)ds \Big) \geq \lambda \Big\}.$$

The proof of the above theorem will be based on the following notation and lemmas.

On the set S of all symmetric $n \times n$ matrices we define a relation $\underset{k}{\geq}$, $1 \leq k \leq n$ as follows:

DEFINITION 4.5.2: Two symmetric $n \times n$ matrices A and B are $A \underset{k}{\geq} B$ if and only if $0 \underset{k}{\leq} (A - B)$ iff $\lambda_{n-k+1}(A - B) \geq 0$.

Note that in the case $k = 1$, the notion $\underset{k}{\geq}$ is nothing but the notion of positive semi-definiteness for symmetric matrices.

LEMMA 4.5.3. *If* A *and* B *are positive semi-definite such that* $A > 0$ *and* $A^2 \underset{k}{\geq} B^2$, *then* $A \underset{k}{\geq} B$.

LEMMA 4.5.4. *If* C *and* A *are positive definite then for any symmetric matrix* B,

$$ACA \underset{k}{\geq} BCB \quad \textit{implies} \quad A \underset{k}{\geq} B.$$

Negating Lemma 4.5.4, we have

LEMMA 4.5.5. *If C and A are positive definite then for a symmetric matrix B,*

$$A \underset{k}{\geq} B \Longrightarrow ACA \underset{k}{\geq} BCB.$$

PROOF OF LEMMA 4.5.3. Taking $X = A - B$ note that

$$\begin{aligned} A^2 - B^2 &= A^2 - (A - X)^2 \\ &= AX + XA - X^2. \end{aligned}$$

Hence we get

$$AX + XA = (A^2 - B^2) + X^2.$$

As X^2 is positive and $(A^2 - B^2) \underset{k}{\geq} 0$ we get

$$AX + XA \underset{k}{\geq} 0.$$

That is, there exists a subspace V_{n-k+1} of dimension $n - k + 1$ such that, for u in V_{n-k+1} we have

$$u^*(AX + XA)u \geq 0.$$

Suppose that the lemma is false. This will imply that there exists an orthonormal set $u_1, u_2, \ldots, u_k$ such that

$$u_i^* X u_i = \lambda_{n-i+1}(X) < 0$$

for $1 \leq i \leq k$.

Now for these u_i, $1 \leq i \leq k$, we have

$$\begin{aligned} u_i^*(AX + XA)u_i &= 2u_i^* AXu_i \\ &= 2\lambda_{n-i+1}(X) u_i^* A u_i < 0. \end{aligned}$$

This contradicts the observation made earlier.

PROOF OF LEMMA 4.5.4. We have

$$ACA \underset{k}{\geq} BCB$$

if and only if

$$\lambda_{n-k+1}(ACA - BCB) \geq 0.$$

Let $C^{1/2}$ be a Hermitian square root of C. It follows that

$$\lambda_{n-k+1}(C^{1/2}ACAC^{1/2} - C^{1/2}BCBC^{1/2}) \geq 0.$$

That is,

$$\lambda_{n-k+1}((C^{1/2}AC^{1/2})^2 - (C^{1/2}BC^{1/2})^2) \geq 0.$$

From the definition of $\underset{k}{\geq}$ we have

$$(C^{1/2}AC^{1/2})^2 \underset{k}{\geq} (C^{1/2}BC^{1/2})^2.$$

Using Lemma 4.5.3, we get

$$C^{1/2}AC^{1/2} \underset{k}{\geq} C^{1/2}BC^{1/2}$$

which in turn gives (since $C^{1/2}$ is nonsingular)

$$A \underset{k}{\geq} B.$$

PROOF OF THE THEOREM 4.5.1. We use the Riccati Technique to obtain a contradiction.

If (4.4.1) is not oscillatory we obtain

$$R(t) = R(0) + \int_0^t Q(s)ds + \int_0^t R(s)P^{-1}(s)R(s)ds$$

$$= R(0) + Q_1(t) + \int_0^t R(s)P^{-1}(s)R(s)ds \tag{4.5.1}$$

where $R(t) = -P(t)Y'(t)Y^{-1}(t)$ is symmetric. Defining

$$F(t) = R(0) + Q_1(t)$$

$$A(t) = \int_0^t R(s)P^{-1}(s)R(s)ds,$$

we note that $A(0) = 0$, $A(t) \geq 0$, $A'(t) \geq 0$. From the definition of $S_\lambda^{(k)}$ and $F(t)$, for $\delta \in (0,1)$, by taking λ sufficiently large we obtain

$$F(t) \underset{k}{\nleq} \delta\lambda I \quad \text{for} \quad t \in S_\lambda^{(k)}.$$

It follows that

$$F(t) + A(t) \underset{k}{\nleq} \delta\lambda I + A(t). \tag{4.5.2}$$

Now

$$A'(t) = R(t)P^{-1}(t)R(t)$$

$$= (F(t) + A(t))P^{-1}(t)(F(t) + A(t))$$

$$\underset{k}{\nleq} (\delta\lambda I + A(t))(P^{-1}(t))(\delta\lambda I + A(t)).$$

We have

$$(\delta\lambda I + A(t))^{-1}A'(t)(\delta\lambda I + A(t))^{-1} \underset{k}{\leq} P^{-1}(t). \tag{4.5.3}$$

That is

$$0 \underset{k}{\leq} (P^{-1}(t) - W(t)) \tag{4.5.4}$$

where $W(t) = (\delta\lambda I + A(t))^{-1}A'(t)(\delta\lambda I + A(t))^{-1}$. Hence we get

$$\lambda_{n-k+1}(P^{-1}(t) - W(t)) < 0$$

which implies that

$$\sum_{i=1}^{k} \lambda_{n-i+1}(P^{-1}(t) - W(t)) < 0.$$

That is, $\rho_k(P^{-1}(t) - W(t)) < 0$ and hence

$$0 < \sigma_k(W - P^{-1}) \leq \sigma_k(W) + \sigma_k(-P^{-1})$$

...ying

$$\sigma_k(W) > -\sigma_k(-P^{-1}) = \rho_k(P^{-1}).$$

Therefore we get

$$\rho_k(P^{-1}) < \sigma_k(W) \leq Tr(W) \quad (\text{as } W \geq 0)$$
$$= Tr\Big(-\frac{d}{dt}(\delta\lambda I + A(t))^{-1}\Big).$$

Integrating through $S_\lambda^{(k)}$ from 0 to T we obtain

$$Tr(\delta\lambda I)^{-1} - Tr\big((\delta\lambda I + A(T))^{-1}\big) > \int_{S_\lambda^{(k)}} \rho_k(P^{-1}(s))ds$$

$$\geq \int_{S_\lambda^{(k)}} \lambda_{n-k+1}(P^{-1}(s))ds.$$

It follows that

$$Tr(\delta\lambda I)^{-1} > \int_{S_\lambda^{(k)}} \lambda_{n-k+1}(P^{-1}(s))ds.$$

That is

$$\frac{n}{\delta\lambda} > \int_{S_\lambda^{(k)}} \lambda_{n-k+1}(P^{-1}(s)ds.$$

Thus we get

$$\lambda \int_{S_\lambda^{(k)}} \lambda_{n-k+1}(P^{-1}(s))ds \leq \frac{n}{\delta} \quad \text{for large} \quad \lambda.$$

By letting $\lambda \to \infty$, we obtain a contradiction. □

We remark that examples may be visualized to realize that Theorem 4.5.1 can be applied even when Theorem 4.4.4 is not applicable.

CHAPTER V

COMPARISON THEOREMS

5.1. Introduction

Consider the second order linear differential scalar equation

$$x'' + kq(t)x = 0. \tag{5.1.1}$$

The function $q(t)$ is an oscillation coefficient if for all $k \in (0, K)$, the solutions to the above equation (5.1.1) are all oscillatory. This notion of oscillation coefficient was introduced in the papers of Utz [53] and Waltman [55].

Clearly the function $q(t) \geq 0$ is an oscillation coefficient if and only if equation (5.1.1) has oscillatory solutions for all $k > 0$.

For the linear second order differential scalar equation

$$(p(t)x')' + q(t)x = 0, \quad t \in [0, \infty) \tag{5.1.2}$$

where $p(t) > 0$ and $p(t)$, $q(t)$ are continuous, as anticipated by Hartman and Wintner [27], Fink and St. Mary [23] have shown that to each $q(t)$ one can associate a number k_0, $0 \leq k_0 \leq +\infty$ such that the equation

$$(p(t)y')' + kq(t)y = 0 \tag{5.1.3}$$

is oscillatory provided the number k satisfies $k > k_0$, and nonoscillatory if $0 \leq k \leq k_0$.

THEOREM 5.1.1 [23]. *If the self adjoint differential equation*

$$(p(t)x')' + kq(t)x = 0, \quad t \in [0, \infty)$$

has zeroes at a *and* b, $a < b$, *and* $j > k > 0$, *then*

$$(p(t)x')' + jq(t)x = 0$$

has at least one zero in $[a, b]$.

□

This is in the form of the Sturm comparison theorem, however $jq(t) \not\geq kq(t)$ unless $q(t) \geq 0$. In fact, $jq(t) \geq kq(t)$ on $\{t/q(t) \geq 0\}$ and $jq(t) < kq(t)$ on $\{t/q(t) < 0\}$. It is not true that $q_1 \geq q$ on $\{t/q(t) \geq 0\}$; $q_1 \leq q$ on $\{t/q(t) < 0\}$ implies

$$(p(t)x')' + q_1(t)x = 0 \tag{5.1.4}$$

oscillates faster than

$$(px')' + qx = 0. \tag{5.1.5}$$

By taking $q_1(t) = q(t)$ on $\{t/q(t) \geq 0\}$ and $q_1(t) = 2q(t)$ on $\{t/q(t) < 0\}$ and by using the Sturm comparison theorem, one observes that equation (5.1.4) oscillates slower than equation (5.1.5) and hence it can be concluded that the above theorem is not valid for a non-constant multiplier of a given function, in general. However, the result (Theorem 5.5.1) also follows easily from Sturm's First Comparison Theorem (cf. [26], p.334) by rewriting the equation and observing that $\frac{1}{j}p(t) < \frac{1}{k}p(t)$ if $j > k > 0$.

But Erbe [16] showed that the constant k can be replaced by a class of functions $k(t)$. That is, multiplying the coefficient $q(t)$ by a certain class of nonconstant functions also preserves the oscillation property.

THEOREM 5.1.2 [16]. *Let $x(t)$ be a nontrivial solution of*

$$(p(t)x')' + q(t)x = 0$$

satisfying $x(a) = x(b) = 0$. Let $k(t) \in C^{(1)}[a,b]$ satisfying $k(t) \geq 1$ on $[a,b]$ and assume pk' is nonincreasing on $[a,b]$. Then every solution of

$$(p(t)x')' + k(t)q(t)x = 0$$

has a zero on (a,b).

□

In [33] Kwong showed that Erbe's result is still true for a much wider class of functions.

THEOREM 5.1.3. [33]. *Let $k(t) \in C^{(1)}[0,\infty)$ such that*

(i) $k(t) \geq 1$

(ii) $2p(t)k'(t) - 3\int_0^t \frac{p(s)k'^2(s)}{k(s)}ds$ *is nonincreasing for large t. If the equation*

$$(px')' + qx = 0$$

is oscillatory, then so is

$$(px')' + k(t)q(t)x = 0.$$

5.2. Comparison problems for systems

Consider the second order $n \times n$ matrix differential equation

$$(P(t)Y')' + Q(t)Y(t) = 0, \quad t \in [a, \infty) \tag{5.2.1}$$

where P, Q, Y are $n \times n$ real continuous matrix functions with $P(t), Q(t)$ symmetric and $P(t)$ positive definite for $t \in [a, \infty)$. We also have the associated vector system

$$(P(t)y')' + Q(t)y = 0 \tag{5.2.2}$$

where $y = (y_1, y_2, \ldots, y_n)$ is an n-vector.

Our interest now is the following:

Assume that the equation (5.2.1) is oscillatory. What are the relations between P and P_1, Q and Q_1 which ensure that the equation

$$(P_1Y')' + Q_1Y = 0, \quad t \in [a, \infty)$$

is also oscillatory?

In one direction, as we have seen, by imposing a partial ordering on the set of Hermitian matrices, several authors have proved a number of results using a direct relation between P and P_1, Q and Q_1 in terms of the partial ordering. The other direction is the problem of determining for what matrices $A(t)$ for which the second order $n \times n$ matrix differential systems

$$(PY')' + AQY = 0$$

and

$$(PY')' + AQAY = 0$$

are oscillatory? It is this latter problem we shall concentrate on in this chapter.

5.3. Functionally commutative coefficients

First, we recall the definition of functional commutativity of a matrix.

DEFINITION 5.3.1: A continuous $n \times n$ matrix valued function $Q(t)$ is said to be functionally commutative on an interval J if

$$Q(s)Q(t) = Q(t)Q(s) \quad \text{for all} \quad s, t \in J.$$

We need the following characterization of functionally commutative matrices Freedman [22].

LEMMA 5.3.2. *Let $Q(t)$ be a diagonalizable $n \times n$ matrix which is functionally commutative on an interval J. Then $Q(t)$ has a constant set of eigenvectors and therefore there exists a constant matrix P such that $Q(t) = P^{-1}D(t)P$, where $D(t)$ is a diagonal matrix.*

LEMMA 5.3.3. *Let $A_1, \ldots, A_N$ be a given set of $n \times n$ matrices. Then the following statements are equivalent:*

(i) A_i is diagonalizable for each i, and A_i commute pairwise;

(ii) $A_1, \ldots, A_N$ can be simultaneously diagonalized.

The above lemma 5.3.3 may be found in Drazin, Dungey and Greuenberg [14]. For a differential equation with functionally commutative coefficients, we have

THEOREM 5.3.4. *Suppose that*

$$Y'' + Q(t)Y = 0, \quad t \in [a, \infty) \tag{5.3.1}$$

is oscillatory and assume that

(i) $Q(t)$ *is functionally commutative, symmetric and continuous;*

(ii) $A(t)$ *and* $Q(t)$ *commute;*

(iii) $A(t)$ *is symmetric, twice differentiable such that* $A(t) \geq I_n$ *and* $A''(t) \leq 0$.

Then

$$Y'' + A(t)Q(t)Y = 0 \tag{5.3.2}$$

is oscillatory.

PROOF: Since $Q(t)$ is symmetric and functionally commutative on the interval $J = [a, \infty)$, we can use the above lemma [5.3.2] and write

$$Q(t) = P^{-1}D(t)P \tag{5.3.3}$$

where $D(t)$ is diagonal and P is unitary. Using $X(t) = PY(t)$ in equation (5.3.1) we obtain

$$X''(t) + D(t)X(t) = 0. \tag{5.3.4}$$

The above equation (5.3.4) is oscillatory. Actually, $Y(t)$ is a prepared solution of (5.3.1) if and only if $X(t)$ is a prepared solution of (5.3.4), and in fact both equations exhibit the same oscillatory or nonoscillatory behavior. Thus, in particular the soltuion $X_0(t)$ satisfying

$$X_0(a) = 0, \quad X_0'(a) = I \tag{5.3.5}$$

is an oscillatory solution of (5.3.4). The solution of (5.3.4) satisfying the initial condition (5.3.5) is diagonal for all $t \geq a$, and so $X_0(t) = \text{diag}\{x_1(t), x_2(t), \ldots, x_n(t)\}$. For this solution, (5.3.4) can be written in the form

$$x_i'' + d_i(t)x_i(t) = 0, \quad i = 1, 2, \ldots, n$$

where $D(t) = \text{diag}\{d_1(t), d_2(t), \ldots, d_n(t)\}$.

Since $\det X_0(t) = \prod_{i=1}^{n} x_i(t)$, $X_0(t)$ oscillates if and only if one of the functions $x_i(t)$ oscillates. That is, the equation

$$x_{i_0}'' + q_{i_0}(t)x_{i_0}(t) = 0 \tag{5.3.6}$$

is oscillatory for some i_0, $1 \leq i_0 \leq n$. Therefore by Theorem 5.1.3, we get

$$x_{i_0}'' + \lambda_{i_0}(t)q_{i_0}x_{i_0}(t) = 0 \tag{5.3.7}$$

is oscillatory for some i_0 and for every $\lambda_{i_0}(t)$ such that $\lambda_{i_0}(t) \geq 1$ and $\lambda_{i_0}''(t) \leq 0$. Since $Q(t)$ and $A(t)$ are diagonalizable and commute, Lemma 5.3.3 shows that $Q(t)$ and $A(t)$ can be diagonalized by the same constant matrix P. That is

$$A(t)Q(t) = P^{-1}\Lambda DP \tag{5.3.8}$$

where $\Lambda = P^{-1}AP$ and $\Lambda(t) = \text{diag}\{\lambda_1(t), \ldots, \lambda_n(t)\}$. Now taking $X(t) = PY(t)$, where $Y(t)$ is a solution of 5.3.2, we get

$$X'' + \Lambda(t)D(t)X = 0. \tag{5.3.9}$$

Now using the conditions on $A(t)$ and observation (5.3.7) we conclude that equation (5.3.9) is oscillatory. Hence $Y(t)$ is an oscillatory solution of equation (5.3.2). □

In the case of a constant matrix A we have

COROLLARY 5.3.5. *Suppose equation (5.3.1) is oscillatory and assume that*

(i) Q *is functionally commutative;*

(ii) A *and* Q *commute;*

(iii) $A = A^* \geq I_n$;

then (5.3.2) is oscillatory.

5.4. Comparison results

In the following we no longer require functional commutativity for the coefficient matrix $Q(t)$. Using the Variational Technique, for vector systems, we have

THEOREM 5.4.1. *Suppose*

$$y'' + Q(t)y = 0 \tag{5.4.1}$$

is oscillatory. Then

$$y'' + AQ(t)Ay = 0 \tag{5.4.2}$$

is oscillatory for all constant matrices $A = A^* \geq I_n$.

PROOF: Since A is symmetric and $A \geq I$, there exists an $n \times n$ real orthogonal matrix P such that

$$P^{-1}(A - I)P = \Gamma \geq 0 \tag{5.4.3}$$

where Γ is a diagonal matrix. Hence we have

$$P^{-1}AP = I + \Gamma \tag{5.4.4}$$

which says that P also diagonalizes A. From the conditions on A it follows that the eigenvalues of A^2 are greater than or equal to 1, which in turn implies that the eigenvalues of A^{-2} are less than are equal to 1.

From Theorem [2.5.1], for some interval $[a, b]$ and for some u in $A_1(a, b)$ we have

$$J_Q(u) = \int_a^b \left[|u'|^2 - u^*Qu\right]dt < 0. \tag{5.4.5}$$

Taking $v = A^{-1}u$ we have, v is in the same space as u, and

$$\begin{aligned} |v'|^2 &= (v' \cdot v') \\ &= v'^* v' \\ &= u'^* A^{-2} u'. \end{aligned} \tag{5.4.6}$$

We also have

$$\begin{aligned} v^* AQAv &= u^* A^{-1} AQAA^{-1}u \\ &= u^* Qu. \end{aligned} \tag{5.4.7}$$

Using the above observations we get

$$\begin{aligned} J_{AQA}(v) &= \int_a^b \left[|v'|^2 - v^* AQAv\right]dt \\ &= \int_a^b \left[u'^* A^{-2} u' - u^* Qu\right]dt \\ &\leq \int_a^b \left[|u'|^2 - u^* Qu\right]dt < 0 \end{aligned} \tag{5.4.8}$$

and so by Theorem [2.5.1], the result follows.

A more general result is the following

THEOREM 5.4.2. *Let*

$$(P(t)y')' + Q(t)y = 0 \tag{5.4.9}$$

be oscillatory. Then

$$(P(t)y')' + AQ(t)Ay = 0 \tag{5.4.10}$$

is oscillatory for all constant matrices A for which

(i) $A = A^* \geq I_n$;

(ii) A *and* $P(t)$ *commute.*

□

For matrix systems we have the following result for nonconstant matrix multiplicative coefficients.

THEOREM 5.4.3. *Suppose*

$$(PY')' + QY = 0 \tag{5.4.11}$$

is oscillatory. Assume that B is symmetric and satisfies

(i) $BB' = B'B,\ B \geq I$;

(ii) $P^{\frac{1}{2}}B = BP^{\frac{1}{2}}$;

(iii) $PB' = B'P$;

(iv) $(PH')'H \geq 0$ *where* $H = B^{-1}$.

Then

$$(PZ')' + BQBZ = 0 \tag{5.4.12}$$

is also oscillatory.

PROOF: If equation (5.4.12) has a nontrivial prepared solution $Z = Z(t)$ with $\det Z(t) \neq 0$ for $t \geq t_0$ then taking $S(t) = -PZ'Z^{-1}$ we have

$$S'(t) = -(PZ')'Z^{-1} + PZ'Z^{-1}Z'Z^{-1}$$

$$= BQB + S(t)P^{-1}S(t).$$

Therefore $S(t)$ is a symmetric solution of the Riccati integral equation

$$S(t) = S(t_0) + \int_{t_0}^{t} BQBds + \int_{t_0}^{t} S(s)P^{-1}(s)S(s)ds. \tag{5.4.13}$$

By taking $R(t) = B^{-1}S(t)B^{-1}$ we note that

$$R'(t) = -B^{-1}B'B^{-1}S(t)B^{-1} + B^{-1}S'(t)B^{-1} - B^{-1}S(t)B^{-1}B'B^{-1}$$

$$= Q + B^{-1}S(t)P^{-1}S(t)B^{-1} - B^{-1}B'B^{-1}S(t)B^{-1} - B^{-1}S(t)B^{-1}B'B^{-1}$$

$$= Q + B^{-1}S(t)B^{-1}BP^{-1}BB^{-1}S(t)B^{-1} - B^{-1}B'R - RB'B^{-1}$$

$$= Q + RBP^{-1}BR - (RB'B^{-1} + B^{-1}B'R). \tag{5.4.14}$$

Integrating, we obtain

$$R(t) = R(c) + \int_{c}^{t} Q(s)ds + \int_{c}^{t} (RBP^{-1}BR - B^{-1}B'R - RB'B^{-1})ds$$

$$= R(c) + \int_{c}^{t} Q(s)ds + \int_{c}^{t} (RBP^{-1}BR - B^{-1}B'B^{-1}BR - RBB^{-1}B'B^{-1})ds.$$

That is,

$$R(t) = R(c) + \int_{c}^{t} Q(s)ds + \int_{c}^{t} (RBP^{-1}BR + (B^{-1})'BR + RB(B^{-1})')ds.$$

Now by taking $W(t) = R + PHH'$, where $H = B^{-1}$, we observe that

$$W(t)H^{-1}P^{-1}H^{-1}W(t) = (R + PHH')H^{-1}P^{-1}H^{-1}(R + PHH')$$

$$= RH^{-1}P^{-1}H^{-1}R + RH^{-1}P^{-1}H^{-1}PHH'$$

$$+ PHH'H^{-1}P^{-1}H^{-1}R + PHH'H^{-1}P^{-1}H^{-1}PHH'.$$

Using $PB = BP$ and $HH' = H'H$ we get

$$W(t)H^{-1}P^{-1}H^{-1}W(t) = RBP^{-1}BR + RH^{-1}H' + PH'P^{-1}H^{-1}R + P(H')^2.$$

Using $PB' = B'P$ we get

$$W(t)H^{-1}P^{-1}H^{-1}W(t) = RBP^{-1}BR + RH^{-1}H' + H'H^{-1}R + P(H')^2.$$

Using this in the above Riccati equation, we get

$$W(t) = PHH' - \int_c^t P(H')^2 + R(c) + \int_c^t Q(s)ds + \int_c^t W(s)H^{-1}P^{-1}H^{-1}W(s)ds$$

$$= \tilde{Q}(t) + R(c) + \int_c^t Q(s)ds + \int_c^t W(s)BP^{-1}BW(s)ds. \qquad (5.4.15)$$

We note that (5.4.15) is the Riccati equation corresponding to the matrix differential equation

$$(B^{-2}PZ')' + (\tilde{Q}' + Q)Z = 0. \qquad (5.4.16)$$

Since Riccati equation (5.4.15) has a solution we get that equation (5.4.16) is non oscillatory. Since P has symmetric square root which commutes with B and $B \geq I$ we get $B^{-2}P \leq P$. Since $(PH')'H \geq 0$, we get

$$(PHH')' \geq P(H')^2.$$

That is $\tilde{Q}' \geq 0$. Hence we get equation (5.4.11) is nonoscillatory. This contradiction implies that equation (5.4.12) is oscillatory.

5.5. Disconjugacy

Consider the second order vector differential equation

$$(P(t)y')' + Q(t)y = 0. \tag{5.5.1}$$

In addition to the usual assumptions on $P(t)$ and $Q(t)$ we assume that $P(t)$ is functionally commutative on the interval J. Then there exists a constant matrix T such that $TT^* = I$ and $P(t) = T^{-1}D(t)T$ where $D(t) = \text{diag}(d_1(t), d_2(t), \ldots, d_n(t))$, $d_i(t) > 0$. Taking $x(t) = Ty(t)$ we get that $x(t)$ satisfies the equation

$$(D(t)x')' + Q_1(t)x = 0 \tag{5.5.2}$$

where

$$Q_1(t) = TQ(t)T^{-1}. \tag{5.5.3}$$

Let $x(t) = col(x_1, x_2, \ldots, x_n)$ be a solution of (5.5.2). Taking $z(t) = col(\epsilon x, Dx')$ for some $\epsilon > 0$, we get

$$z' = \begin{bmatrix} \epsilon x' \\ -Q_1 x \end{bmatrix} = \begin{bmatrix} 0 & \epsilon D^{-1} \\ -\epsilon^{-1}Q_1 & 0 \end{bmatrix} \begin{bmatrix} \epsilon x \\ Dx' \end{bmatrix}.$$

That is

$$z' = C(t, \epsilon)z \tag{5.5.4}$$

where

$$C(t,\epsilon) = \begin{bmatrix} 0 & \epsilon D^{-1} \\ -\epsilon^{-1}Q_1 & 0 \end{bmatrix}. \tag{5.5.5}$$

Using a result of Nehari [47], we get that equation (5.5.4) is nonoscillatory on $[a,b]$ if

$$\int_a^b \|C\|dt < \frac{\pi}{2} \tag{5.5.6}$$

where $\|C\|$ denotes the matrix norm $\sup_{\|v\|=1} \|Bv\|$ and $\|v\|$ is the Euclidean norm of vector v.

Taking $u = col(z_1, z_2, \ldots, z_n)$ and $v = col(z_{n+1}, \ldots, z_{2n})$ we note that

$$\begin{aligned}
\|Cz\|^2 &= (Cz, Cz) \\
&= \left(\begin{bmatrix} \epsilon D^{-1}v \\ -\epsilon^{-1}Q_1 u \end{bmatrix}, \begin{bmatrix} \epsilon D^{-1}v \\ -\epsilon^{-1}Q_1 u \end{bmatrix} \right) \\
&= \epsilon^2 \|D^{-1}v\|^2 + \epsilon^{-2}\|Q_1 u\|^2 \\
&\leq \epsilon^2 \|D^{-1}\|^2 \|v\|^2 + \epsilon^{-2}\|Q_1\|^2 \|u\|^2 \\
&\leq \max\left(\epsilon^2\|D^{-1}\|^2,\ \epsilon^{-2}\|Q_1\|^2\right)\|z\|^2
\end{aligned}$$

hence

$$\|C\| \leq \max\left(\epsilon\|D^{-1}\|,\ \epsilon^{-1}\|Q_1\|\right). \tag{5.5.7}$$

If $x = (x_1, \ldots, x_n)^T$ is a solution of (5.5.2) then taking $w = \binom{x}{Dx'}$ we can reduce the second order equation (5.5.2) to the first-order system

$$w' + C_1 w = 0 \tag{5.5.8}$$

where

$$C_1 = \begin{bmatrix} 0 & -D^{-1} \\ -Q & 0 \end{bmatrix}.$$

If $x = (x_1, \ldots, x_n)$ is a nontrivial solution such that $x(a) = x(b) = 0$ then $x_i(a) = x_i(b) = 0$ for $i = 1, 2, \ldots, n$. By Rolle's theorem we get $y_i'(t_i) = 0$ for some t_i, $a < t_i < b$, $i = 1, \ldots, n$. This means that every component of x has a zero and hence equation (5.5.8) is oscillatory. Thus nonoscillation of (5.5.4) implies that equation (5.5.2) is right and left disfocal and hence

THEOREM 5.5.1. *If*

$$\int_a^b \max\left(\epsilon\|D^{-1}\|,\ \epsilon^{-1}\|Q_1\|\right)dt < \frac{\pi}{2}$$

for some $\epsilon > 0$, then equation (5.5.4) is nonoscillatory on the interval $I = [a, b]$.

□

THEOREM 5.5.2. *If for some $\epsilon > 0$,*

$$\int_a^b \max\left(\epsilon\|D^{-1}\|,\ \epsilon^{-1}\|Q_1\|\right)dt < \frac{\pi}{2}$$

then equation (5.5.1) is disconjugate and right - and left - disfocal on $[a, b]$.

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Mechanism of Bilirubin Toxicity in a Neural Cell Line.

BY

Yair Amit.

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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Mechanism of Bilirubin Toxicity in a Neural Cell Line.

submitted by - **Yair Amit**

in partial fulfillment of the requirements for the degree

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in - **Medical Sciences**

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Dedicated to my wife Ada

and my children Itai, Ido, and Yorai

ABSTRACT

The mechanism of bilirubin toxicity to the central nervous system has been the subject of numerous investigations over the past decade. The results of several clinical and experimental studies suggest that bilirubin is toxic to various cellular functions with reversibility of early stages of bilirubin encephalopathy. Yet, the major biochemical defect underlying bilirubin toxicity has not been completely elucidated. The difficulties in analyzing the results and the inability to point to a primary target of bilirubin toxicity stem from variations in experimental design, the use of different animal models and cell systems, and the use of unstable bilirubin mixtures. Spectrophotometric measurements demonstrated that bilirubin in tissue culture media, at concentrations of 35-125 μM and at bilirubin-to-albumin [B/A] molar ratios up to 3, is stable over a 24-hour period. The use of a neural cell line and the presence of appropriate albumin concentrations are advantageous. We have measured the interaction and toxic effects of bilirubin to N-115 cells, a murine neuroblastoma cell line. The results obtained point to a multistep interaction process between bilirubin and the plasma membrane. Bilirubin binding is dependent on bilirubin concentration, B/A molar ratios, temperature and pH conditions, and is partially reversible with the addition of albumin. Under appropriate B/A molar ratios, bilirubin was found to affect Na^+/K^+ ATPase activity, [^{3}H]-thymidine uptake, L-[^{35}S]-methionine incorporation into protein, and mitochondrial functions. The toxic effects seem

to be dependent again on B/A molar ratio, bilirubin concentration, and length of exposure. However, it is not possible to single out the primary target for bilirubin toxicity conclusively. In N-115 cells, once toxicity appeared, it was irreversible. Moreover, toxicity appeared long after removal of the bilirubin-containing media following a short-term exposure to bilirubin, during which toxicity was not manifest. We conclude that, under appropriate experimental conditions, the binding interaction between bilirubin and the cell plasma membrane is complex, and that bilirubin is toxic to several cellular functions in N-115 cells in a progressive and irreversible process.

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In 1984 I first came to Edmonton for a fellowship program in Neonatology. Shortly thereafter I became involved with Dr. Schiff's research on bilirubin. This thesis is the result of an intense laboratory work that would never have been completed without the help of many, to whom I wish to express my thanks and gratitude.

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B/A	-	Bilirubin to albumin molar ratio.
DMEM	-	Dulbecco's modified Eagle medium.
FCCP	-	Carbonyl cyanide -*p* - trifluoromethoxyphenylhydrazone.
HEPES	-	N-2-Hydroxyehtylpiperazine-N-2-ethanesulfonic acid.
HPLC	-	High performance liquid chromatography.
HSA	-	Human serum albumin.
MTT	-	3-(4,5-dimethylthiazol-y-yl)-2,5-diphenyl tetrazolium bromide.
PBS	-	Phosphate buffered saline.
PFM	-	Protein Free Medium.

CHAPTER 1

GENERAL INTRODUCTION

1. Introduction

Hyperbilirubinemia is a common occurrence in the newborn period. Bilirubin encephalopathy (kernicterus) is a major complication of the toxic effect of bilirubin on brain cells. Originally described in jaundiced newborns, it has been seen in recent years in premature infants suffering from a mild degree of hyperbilirubinemia.

The protection of the newborn's brain from bilirubin has been attributed to a number of factors, among them the capacity of albumin to bind bilirubin, the integrity of the blood brain barrier, and the integrity of the neural cells.

The mechanism of bilirubin encephalopathy has been extensively studied over the past decade. However, the primary target, the toxic manifestation and the nature of the interaction between bilirubin and neural cells remains unclear. Studies conducted on neural and on non-neural cells and tissues demonstrate that bilirubin may impair a large number of cellular functions. However, the use of bilirubin concentrations higher than those usually encountered in clinical situations, and the use of varying albumin concentrations may account for the multiplicity of effects and inconclusive results.

The use of bilirubin without the addition of albumin or at high bilirubin-to-albumin molar ratios causes rapid aggregation and precipitation, auto-oxidation, and decomposition of the pigment, as well as photoisomerization of the natural occurring

bilirubin IX-α isomer. Since bilirubin may be poisonous to cells, clearly it is important to establish appropriate experimental conditions under which bilirubin is maintained in solution throughout the time the cells are exposed to bilirubin.

The purpose of the work herein described was to establish the appropriate experimental conditions for studies related to bilirubin and its cellular interaction and to define the following:

1) The interaction between bilirubin and the neural cell.
2) The target and mechanism of bilirubin toxic effects.

In the following sections a number of subjects will be reviewed:

1) Neonatal jaundice.
2) Bilirubin metabolism, structure, and binding properties.
3) Bilirubin toxicity.
4) Kernicterus and bilirubin encephalopathy.
5) Studies on bilirubin toxic effects.

2. Neonatal Jaundice

Hyperbilirubinemia is a common occurrence during the neonatal period. Clinical hyperbilirubinemia is defined as a serum bilirubin concentration that exceeds 26 µmol/L, and is common to most newborn infants during their first week of life. In 10 to 15% of all normal-term babies, hyperbilirubinemia becomes sufficiently high to be visible as jaundice [1]. Although the majority of jaundiced full-term babies appear completely healthy, standard textbooks of newborn medicine mandate diagnostic investigation to rule out pathologic causes of jaundice in those infants whose serum bilirubin concentrations exceed a level of 170 to 220 µmol/L [2,3]. The incidence of serum bilirubin concentrations above 220 µmol/L ranges from 4.5% to 20% during the first week of life [4,5]. Although the presence of hyperbilirubinemia engenders some concern, 56% of infants whose serum bilirubin concentrations exceed the above levels show no cause for the jaundice [6].

There are many causes for neonatal hyperbilirubinemia unique to the fetus and the newborn. During the last stages of fetal life, removal of erythrocytes provides an increasing load of hemoglobin for catabolism. This results in an increase in bilirubin production [7]. The normal newborn produces more than double the bilirubin production of 3.6 mg/kg/day observed in the adult. Moreover, no rate-limiting step in hemoglobin catabolism and unconjugated bilirubin formation is recognized in the mammalian fetus [8,9,10,11].

The disposal mechanism for bilirubin in the fetus involves two pathways. The vast majority of unconjugated bilirubin is cleared via the placental circulation into the maternal circulation, where it is disposed of by the maternal liver [8,10]. The second pathway involves excretion by the fetal liver. This pathway is limited due to several factors. Foremost among these is a marked deficiency in hepatic uridine diphosphate glucuronyltransferase, noted in human as well as other mammalian fetuses [7]. As a result, the conjugating capacity of fetal liver is almost undetectable. Other factors associated with decreased hepatic clearance of bilirubin in the fetus are reduced hepatic blood flow and low levels of bilirubin binding proteins [9,11]. However, as a result of the different disposal processes, unconjugated hyperbilirubinemia is rarely evident at birth, even in severe cases of hemolytic anemia in the fetus.

The newborn infant, like the fetus, has several impairments in bilirubin metabolism and transport. These include increased bilirubin production [7], deficiency of hepatic bilirubin binding proteins and decreased glucuronyltransferase activity [12,13], as well as increased enterohepatic circulation of bilirubin [14]. Taken together, these factors usually result in the occurrence of increased concentrations of serum unconjugated bilirubin during the first days of life. Clinically, this is usually defined as "physiologic jaundice of the newborn" [15]. Yet, in certain groups of infants this phenomenon is exaggerated and the jaundice becomes pathological. A variety of conditions may result in unconjugated

hyperbilirubinemia : hemolytic disorders, polycythemia, increased extravasation of blood, increased enterohepatic circulation of bilirubin, defects in bilirubin metabolism, breast feeding, inherited metabolic disorders and prematurity [2,3].

There are two functionally distinct periods in physiologic jaundice of the newborn . The first is observed during the first 5 days of life and is characterized, in the full term infant, by a rapid rise in serum unconjugated bilirubin concentration to a peak of 100-120 μmol/L on the third day of life, and a rapid decline until the fifth day. In the premature infant, the peak value is higher and does not occur until the fifth to seventh day of life. The second period of physiologic jaundice is characterized by a relatively stable serum unconjugated bilirubin level of about 35 μmol/L that lasts until the end of the second week, in term infants, or for more than a month in preterm infants. After the second stage, serum unconjugated bilirubin concentrations decline to levels observed in normal adults [3,15,16,17,18,19].

Many studies of serum bilirubin concentrations in normal-term and in premature babies have provided guidelines for the diagnosis of "physiologic" and pathologic jaundice [4]. Pathologic jaundice is suspected whenever the following criteria are present:

1) Clinical jaundice in the first 24 hours of life.
2) Total serum bilirubin concentration increasing by more than 85 μmol/L per day.
3) Total serum bilirubin concentration exceeding 220 μmol/L in term infant and 255 μmol/L in prematures.

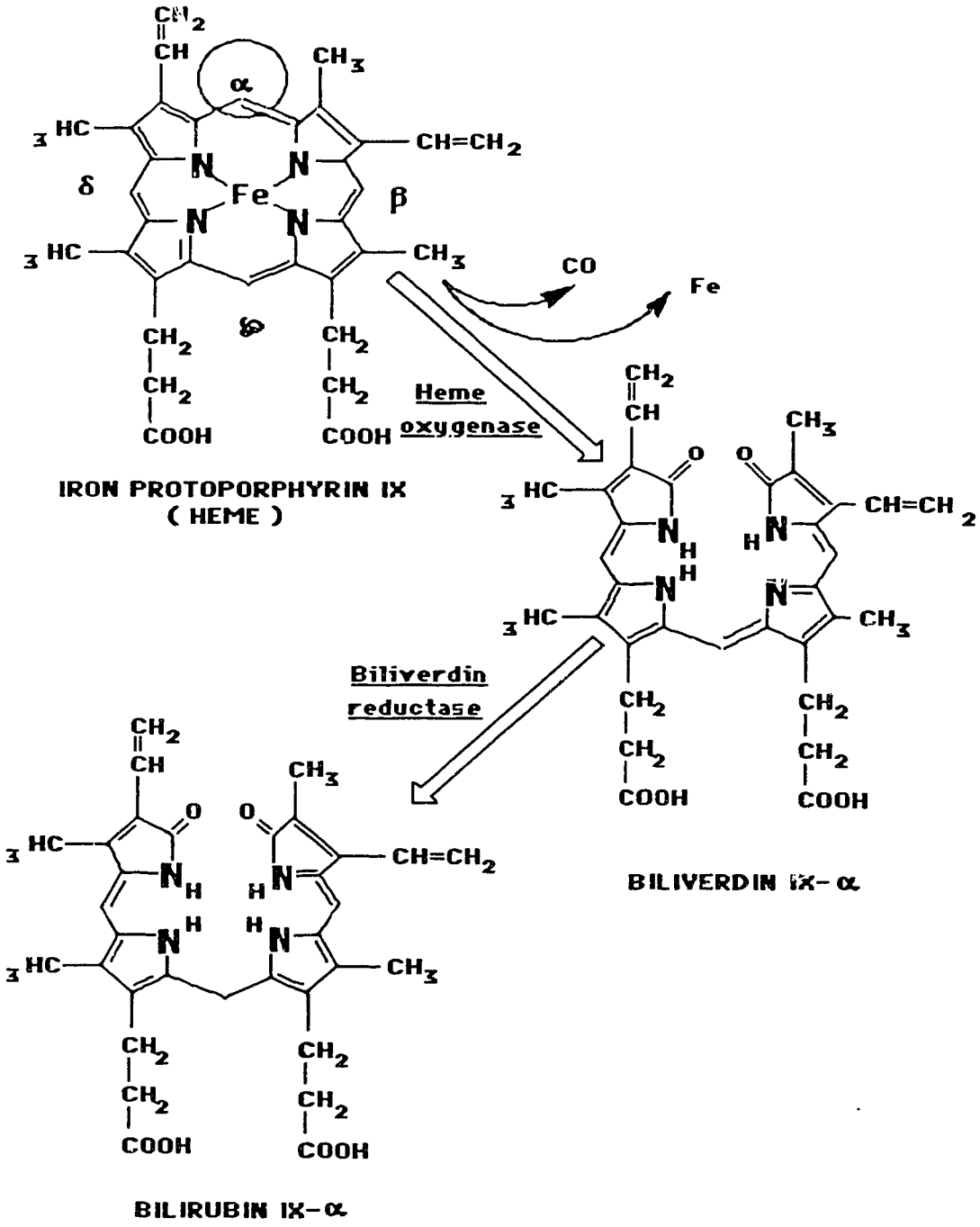
4) Direct serum bilirubin levels over 25-34 µmol/L.
5) Clinical jaundice lasting more than a week in a term baby, or two weeks in a premature infant [3].

3. Bilirubin Metabolism

Bilirubin is formed by the catabolism of different heme proteins including hemoglobin, myoglobin and heme-containing enzymes such as cytochromes, catalases and pyrolases [20]. Hemoglobin is the principal source of bile pigment in mammals, accounting for approximately 80% of the daily bilirubin production [21].

The metabolic pathway of heme catabolism has been clarified to a considerable extent [22]. Heme (Plate 1) is catabolized by a microsomal heme oxygenase localized primarily in the reticuloendothelial system [23], in tissue macrophages, and in the intestinal brush border membranes [24]. Inside the microsome, the porphyrin iron - located within the cyclic tetrapyrrole - is reduced, and an oxygen radicale is generated. Radicale attack and subsequent oxidation of the carbon atom at the α-methene carbon position, break the porphyrin ring. As a result, biliverdin IX-α is formed with loss of the iron atom and release of carbon monoxide. In mammals, biliverdin undergoes further reduction to bilirubin IX-α (Plate 2) [23]. The conversion is catalyzed by biliverdin reductase located in the cytosol [23].

Plate 1 Enzymatic oxidation of heme.



Bilirubin is a waste product and has no apparent function. Although the concentration of bilirubin in the serum is generally low, its concentration in the bile is significantly higher [20]. Five steps are involved in the transport of bilirubin from its sites of formation to the intestinal tract:

1) Transport in the plasma firmly bound to albumin [25].
2) Carrier-mediated transfer of bilirubin into the hepatocyte and binding to acceptor proteins located in the cytosol [26,27].
3) Hepatic conjugation that renders the pigment polar and water soluble [28].
4) Excretion of conjugated bilirubin into the bile [28]
5) Transport and elimination in the intestine [29,30].

Once inside the liver cell, bilirubin is transported to the smooth endoplasmic reticulum where the insoluble pigment is conjugated, thus converted into a water-soluble monoglucuronide pigment [29]. The final step in bilirubin metabolism, within the hepatocyte, is a second glucuronidation which takes place in the cytosol by a plasma membrane-bound enzyme [29]. Bilirubin mono- and diglucuronide are then excreted into the bile. When conjugated bilirubin reaches the sterile newborn intestine, the normal reduction of bilirubin to fecal stercobilinogen does not occur. Instead, a large proportion of the bilirubin is hydrolyzed by β-glucuronidase located in the brush border of the small intestine [29,30]. The resultant unconjugated bilirubin is reabsorbed in the gut and taken up by the portal system to start the disposal process

again [29,30], giving the so called enterohepatic circulation of bilirubin.

Disorders of bilirubin metabolism affect human beings from birth. The detrimental effects appear to arise chiefly from the virtual insolubility and instability of the pigment in aqueous solution at physiologic pH. Several bilirubin IX-α polar groups, namely, two carboxyl, two lactam, and two pyrrol groups render the substance soluble in water (Plate 2). The actual insolubility is explained by intra-molecular hydrogen bonding. In the hydrogen-bonded molecule (Plate 3), the hydrophilic polar COOH and NH groups are intimately associated and unavailable for interaction with polar groups in the environment. The insolubility of bilirubin-acid, with its two protonated carboxyl groups (Fig. 4), is considered the basis for its neurotoxicity. Understanding the conditions of bilirubin-acid formation is important for understanding the mechanism of its toxicity [31,32,33].

Bilirubin forms a saturated aqueous solution containing a very low concentration of the acid and a higher concentration of the dianion (Plate 4 & 5) [31,32]. Due to negative charges, the dianion is present in equilibrium with its dimer. The degree of dimerization is independent of pH, since hydrogen ions are not involved. However, with increasing hydrogen ion concentration some of the dimers and dianions take up protons from the medium, forming acid anions with fewer negative charges. The decrease in electric repulsion is followed by formation of large aggregates. During this aggregation the solution usually remains clear and

Plate 2: Bilirubin IX- α

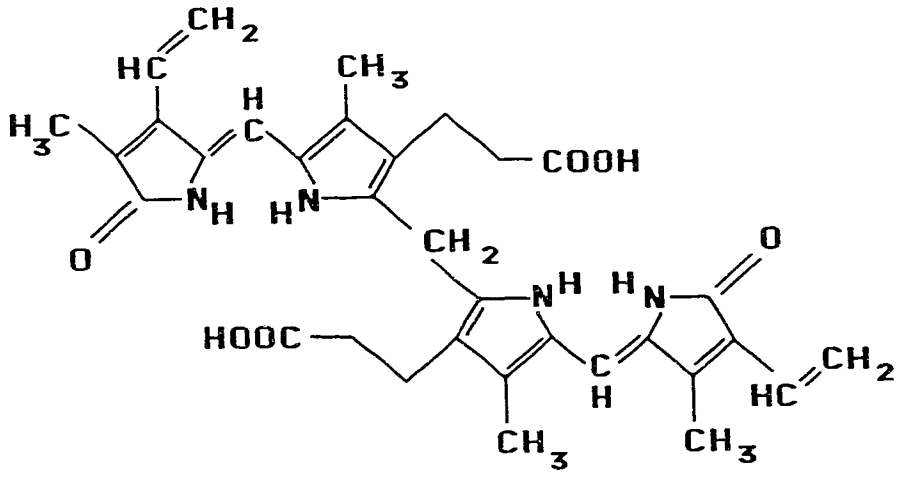


Plate 3: Bilirubin IX-α acid, intramolecularly hydrogen bonded.

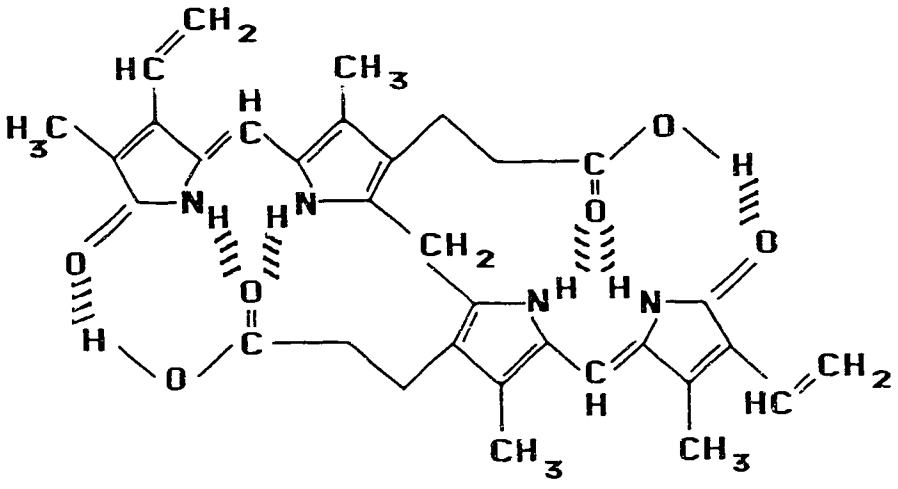


Plate 4: Bilirubin IX-α acid.

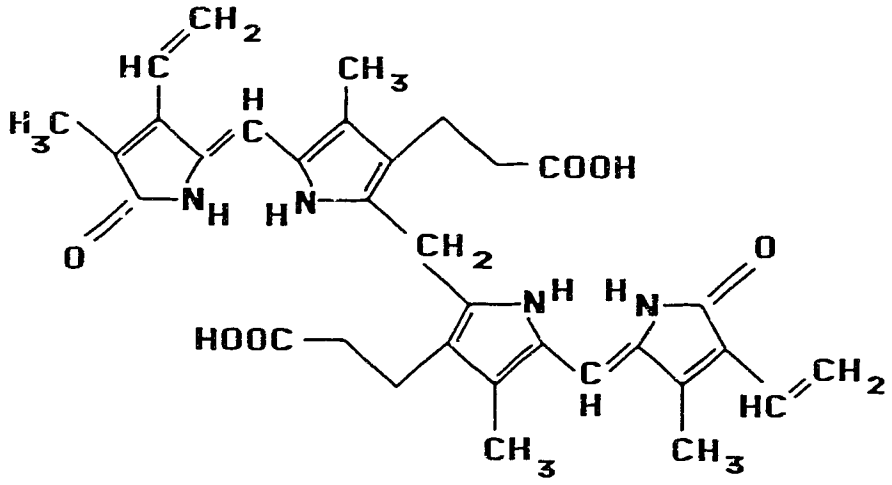
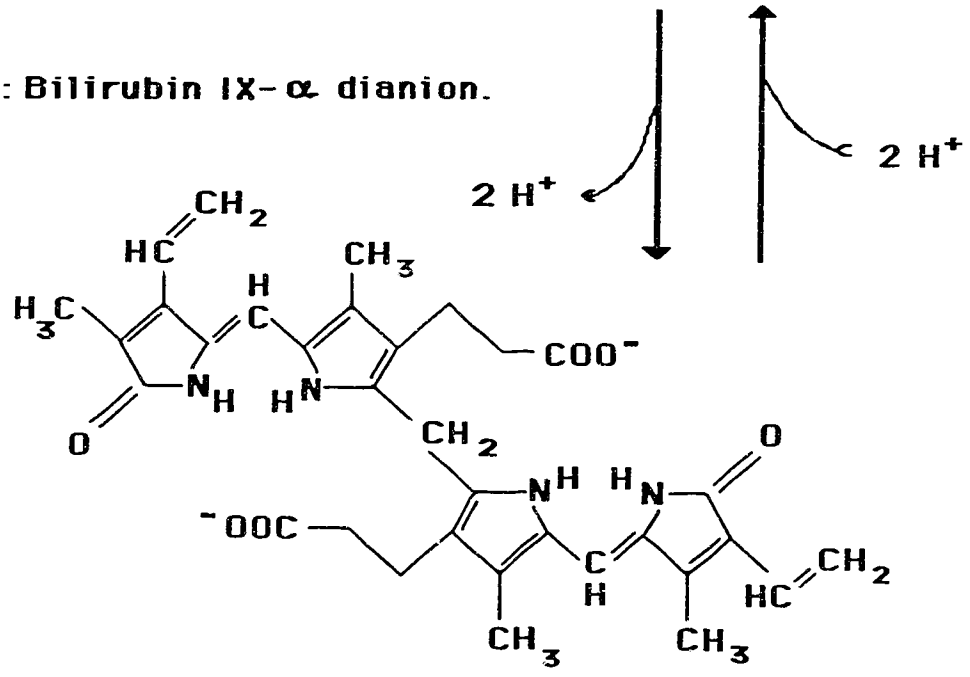


Plate 5: Bilirubin IX-α dianion.



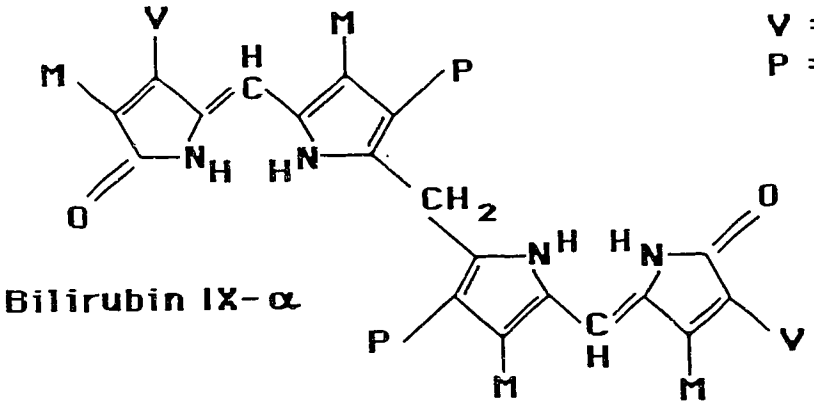
bright yellow when observed by the naked eye. The presence of strong light scattering indicates that a colloid suspension, and not a genuine bilirubin solution, is present [31,32,33,34].

When colloid formation is expected but fails to take place, the bilirubin solution is said to be in a supersaturated state. Under acidic pH conditions, supersaturation with extensive aggregation and precipitation of the insoluble protonated acid will occur [33]. Phospholipids accelerate the aggregation of bilirubin at acidic pH conditions as well as co-precipitation of bilirubin and phospholipids [34]. When a neutralized supersaturated solution of bilirubin is mixed with a suspension of erythrocyte membranes or mitochondria, and with liposomes or phospholipids ***in vitro*** , a process of binding and aggregation of bilirubin starts immediately and proceeds rapidly [33]. The end result is similar to colloid aggregates with the aggregates remaining attached to the membranes [34]. Since the same cellular structures are present in intact cells, it is reasonable to assume that the same process will occur ***in vivo.***

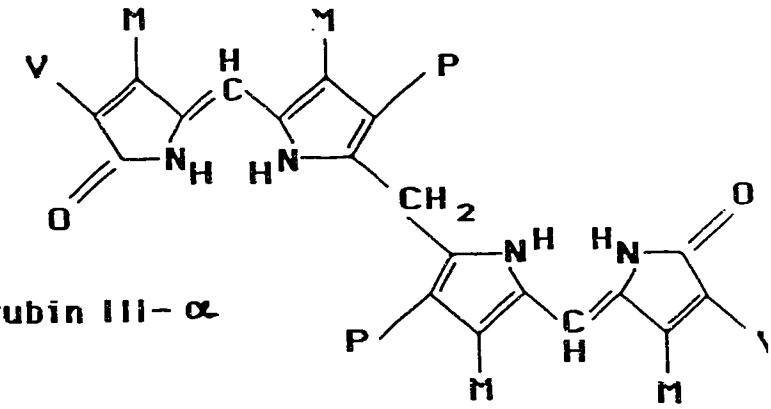
Besides its insolubility and tendency to aggregate and form colloids, bilirubin is unstable in solution and tends to auto-oxidize and decompose. Hydrogen-bonded bilirubin, dissolved in oxygenated alkaline aqueous solution, is unstable and may undergo rearrangement and auto-oxidation [35]. Furthermore, over a pH range from 7.4 to 12 [36] or in the presence of acid [37], bilirubin IX-α is cleaved at the central methylene bridge with subsequent rearrangement of the separate units to give a mixture of bilirubin III-α, and bilirubin XIII-α in addition to the natural IX-α isomer (Plate 6) [36,37].

Plate 6: Bilirubin isomers.

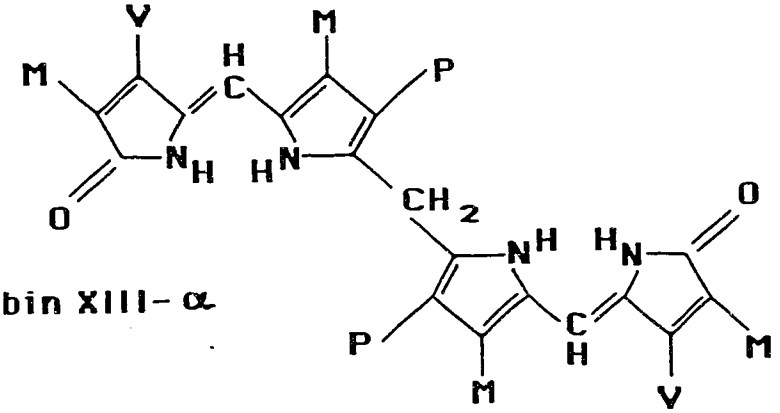
M = CH_3
V = CH=CH
P = CH_2CH_2COOH



Bilirubin IX-α



Bilirubin III-α



Bilirubin XIII-α

4. Bilirubin Binding to Albumin

In order to prevent bilirubin precipitation and toxicity, it is necessary to bind the pigment to a carrier. Serum albumin serves as a universal carrier, reversibly binding a large number of substances including bilirubin [32,38,39]. The importance of the interaction between bilirubin and albumin was demonstrated by several investigators. Bowen et al, demonstrated the protective role of albumin against unconjugated bilirubin injected into puppies [40]. Mustafa et al, found that one mole of albumin binds one mole of bilirubin and detoxifies it [41]. Odell [42] and Silverman et al [43] described increased bilirubin toxicity as a result of a dissociation of the pigment from its albumin binding site, caused by the use of different drugs.

In the blood, unconjugated bilirubin dianion is bound to a high affinity binding site on albumin with smaller amounts located at one or two lower affinity sites [44]. The binding process is fast, occurring in a matter of milliseconds [45], and is pH-independent within a pH range between 7 and 9.

Nevertheless, the distribution of bilirubin, ***in vivo***, between serum albumin and tissues is highly sensitive to pH changes, where acidosis favors a shift of the pigment from albumin to fat [46]. The shift is readily explained by a change in the solubility of the unbound pigment, formation of aggregates with lipoid membranes and lipids, and a shift in the bilirubin-albumin binding equilibrium [46]. Formation of the bilirubin-albumin complex is

reversible and is associated with protection of the pigment from degradation by various processes - e.g. photochemical degradation, isomerization, auto-oxidation and enzymatic reduction [47,48,49].

The same type of binding takes place ***in vitro*** , when a solution of albumin, at a slightly alkaline pH condition, is mixed with a solution of bilirubin dissolved in sodium hydroxide. Another type of binding occurs if a solution of albumin, ***in vitro*** , is mixed with a molar excess of bilirubin at pH 7.4 or below. Under such conditions a slow process of association takes place whereby large aggregates, consisting of large numbers of albumin and bilirubin acid molecules, are formed. This process results in co-crystallization of albumin and bilirubin with little bilirubin left in the solution [50]. Binding of bilirubin acid to albumin is pH-dependent since a high number of hydrogen ions are involved. Increasing the pH conditions of the solution towards an alkaline pH disintegrates most of the aggregates, and an equilibrium of binding of the anion is re-established [32,50].

5. Bilirubin Binding to Other Proteins

Although the only plasma protein with strong affinity for bilirubin is albumin, bilirubin can also bind to other blood components [51]. Binding to non-albumin proteins in the serum is important only when bilirubin concentrations exceed those of albumin and when the available primary binding sites on albumin are saturated. Bilirubin can bind to proteins such as serum β-lipoproteins and α-globulin, but when bilirubin is present in

the serum, the pigment distribution is always in favor of albumin [51,52,53,54].

Of physiological importance is bilirubin binding to proteins located in the cytosol, especially in hepatocytes. These proteins probably function as carriers for bilirubin within the cell and facilitate the uptake of the pigment [13]. Whether the binding is necessary for protection of the cell content against the pigment is unknown .

Other cellular and tissue components such as erythrocyte membrane [55,56,57], pulmonary hyaline membrane [58,59], mitochondria from heart and brain cells [60,61], glycolipids [62] , lipids and phospholipids [61,63,64] have been shown to bind bilirubin. Binding of the pigment to non-albumin proteins and other cellular components is of lesser importance and negligible when albumin exceeds bilirubin molar concentrations.

6. Bilirubin Interaction With Lipids .

The interaction of bilirubin with lipids has been studied by several investigators. Mustafa and King [61] suggested that bilirubin, in supersaturated solutions, is capable of binding to a variety of native membrane lipids as inferred by spectral changes. The changes observed were rapidly reversed by washing the liposomes with albumin, suggesting a loose binding of bilirubin to lipids. Weil and Menkes [62] have demonstrated that bilirubin interacts with gangliosides ***in vitro*** . In another study [63] bilirubin, at physiologic pH conditions, quenched a fluorescent

probe located within the lipid bilayer of membranes. Talafant [64] has found different binding qualities between the pigment and different phospholipids.

Of major importance is the knowledge of the interaction between bilirubin and the lipid bilayer. Eriksen et al [34] and Cestaro et al [65] demonstrated that bilirubin may be incorporated within the hydrophobic hydrocarbon domains of the bilayers, but migrate to the surface as equilibrium is achieved. On the other hand, Tipping et al [66] and Hayward et al [67], in a more recent study, were able to demonstrate that bilirubin is capable of passive diffusion across the lipid bilayer into liposomes. However, since no complete extraction of bilirubin from the liposomes was demonstrated, an interaction between the lipid bilayer and the pigment cannot be excluded.

The properties of bilirubin with regard to its effect on monolayers were demonstrated in two studies . In 1939 Stenhagen and Rideal [68] explored the interaction between bilirubin and various lipids and proteins. The results obtained suggest an interaction of the carboxyl groups of the pigment with the primary amide groups of lipids and proteins. Another series of monolayer experiments was carried out by Notter et al [69], exploring the effect of bilirubin on dynamic surface tension forces. Under acidic pH conditions, bilirubin-acid intercalates with the phospholipid acyl-fatty acid chains. At higher pH values, the more soluble bilirubin interacts with water away from the hydrophobic core of the lipid bilayer. Overall, it was shown that under acidic pH

conditions bilirubin is a highly surface-active material at the interface, and is capable of influencing the spreading behavior of membrane lipids [69].

7. Bilirubin Interaction With Membranes

The interaction of bilirubin with the central nervous system should consist of three steps: a) the entry of bilirubin into the brain from blood, b) the binding of bilirubin to the neural cell surface with or without subsequent internalization, and c) the interaction of bilirubin with plasma membrane, leading to alteration of membrane properties, or with intracellular targets.

Studying the interaction of bilirubin with synaptosomal plasma membrane, Vazquez et al [70] proposed a three step model for the interaction : 1) a rapid initial complex formation between anionic forms of bilirubin and the polar lipid head groups on the membrane surface, 2) a slow inclusion of bilirubin into the hydrophobic core of the lipid bilayers, and 3) the formation of bilirubin acid aggregates, by the remaining bilirubin molecules, on the surface of the plasma membrane [70]. While Vazquez demonstrated a multi-step interaction between bilirubin and the synaptosomal membrane, Leonard et al [71] suggested a different model for interaction. According to their results the interaction of bilirubin with model or biological membranes depends on the sizes of the free volumes, located within the membrane. These pools of free volumes varied according to the lipid composition and the presence or absence of proteins in the membranes. Bilirubin

appears to interact with neither the polar nor the apolar regions of the membrane but to partition with the free spaces in the apolar region of the lipid bilayer [71].

8. Bilirubin Toxicity

Despite the extensive knowledge of the chemical and biochemical properties of bilirubin, the question whether bilirubin is poisonous or only potentially toxic to the living organism has not been completely elucidated. While adults produce up to 250 mg of bilirubin daily without any harm and large doses have been injected intravenously into adults [72] and newborn babies [73] with no apparent ill effects, hyperbilirubinemia in newborn infants [44] and newborn rats [74] may cause bilirubin encephalopathy. Furthermore, studies in experimental animals indicate that unconjugated hyperbilirubinemia impairs liver mitochondrial function [41]. In contrast, no toxic hepatic effects have been seen in humans or Gunn rats suffering from prolonged unconjugated hyperbilirubinemia due to hepatic glucuronyltransferase deficiency [75,76].

A variety of pathologic conditions may result in severe or prolonged jaundice characterized by increased serum concentration of unconjugated bilirubin [77]. In several studies, bilirubin has been shown to be poisonous to neural and non-neural cells and tissues both ***in vitro*** and ***in vivo*** [78,79,80]. Bilirubin toxicity usually manifests as central nervous system damage which occurs almost exclusively during the early neonatal period [77]. Passage of

unconjugated bilirubin from the intravascular space - across the blood brain barrier - into the brain is thought to be the cause of kernicterus and bilirubin encephalopathy (see below).

9. Kernicterus and Bilirubin Encephalopathy

In 1903 Schmorl [81] coined the term kernicterus to describe the characteristic yellow staining of subcortical nuclei of the brain, that was commonly observed in jaundiced infants who died from severe erythroblastosis fetalis. The term was selected specifically to differentiate it from a more diffuse yellow staining of periventricular tissues and hemisphere surfaces, a condition considered secondary to passive diffusion of bilirubin following tissue necrosis [83]. Kernicterus, originally used as a pathologic term, is now associated with a particular clinical picture which varies from subtle neurologic changes such as high tone deafness to more extreme forms of severe choreoathetosis, mental retardation and, in some cases, to immediate death of the infant [82,83,84,85,86]. Moreover, in infants who survive the acute stages of hyperbilirubinemia but subsequently die, the staining may no longer be present, yet the basal ganglia display microscopic evidence of cell injury, neuronal loss and glial replacement [87,88,89]. Bilirubin encephalopathy is a more appropriate term to describe the clinical picture associated with the diffuse staining of the brain, the neuronal damage and the neurological picture associated with hyperbilirubinemia.

It is generally accepted that unconjugated bilirubin deposited in the brain is responsible for the yellow staining and the neurologic dysfunction characterizing bilirubin encephalopathy. To be toxic to the nervous system, unconjugated bilirubin has to cross the blood-brain barrier and specifically interact with vulnerable neural cells. The blood-brain barrier is a complex structure consisting of tight junctions cementing brain capillary endothelial cells plus adjoining foot processes of astroglial cells. Soon after contact with the astrocytes, continuous tight junctions seal the endothelial cells together and polar molecules no longer readily enter the brain by simple diffusion. Essential molecules such as glucose, organic acids and amino acids, therefore, require specific transporters to mediate their passage into the brain. Functionally, the blood-brain barrier comprises a series of carriers and transport mechanisms for various substances [90]. Permeation of the blood-brain barrier may result from changes in the anatomy and/or the function of its constituents.

The blood-brain barrier of the neonate is immature and thus may be more permeable [91]. Whether immaturity and increased permeability are responsible for the passage of free bilirubin into the neonatal brain is not clear. Many different factors, besides immaturity of the blood-brain barrier, account for the development of kernicterus. Among them are relative hypoalbuminemia, hypoxia, acidosis, hyperosmolarity, hypothermia, sepsis and drugs competing for bilirubin binding sites on albumin [92,93,94]. Endothelial cells of brain capillaries, as other cells, are

susceptible to injury by toxins and other abnormal metabolic conditions. Most evidence supports a passage of free bilirubin across the blood-brain barrier but a transfer of the albumin-bilirubin complex has not been excluded [95]. In normal infants, the restrictive nature of the blood-brain barrier is very well preserved despite immaturity of the endothelial cells composing the barrier. In infants with an intact blood-brain barrier, bilirubin will leave the blood to enter the brain only when the pigment is uncoupled from albumin and other plasma proteins. However, if brain endothelial cells are damaged, the altered barrier will then permit bilirubin, uncoupled from or complexed with albumin, to enter and damage the brain cells [95].

Another factor, the selective affinity of bilirubin for specific brain sites, complicates the picture of bilirubin encephalopathy. The vulnerability of specific brain areas to bilirubin toxic effects may be patterned by the blood flow to the brain [96,97,98] or affected by the different bilirubin binding affinities to various brain phospholipids [62,63,71].

Brodersen has suggested the possible existence of a bilirubin oxidase enzyme within the neural cells, which might play a role in protecting the cells by oxidizing the unbound bilirubin [99]. The presence of such an enzyme remains speculative.

Thus, protection of the newborn's brain from bilirubin may be attributed to a number of factors :

1) The interaction of bilirubin with albumin and /or different phospholipids [37,38,62,63,100].
2) The integrity of the blood brain barrier and of the brain cell membrane [95,98,101,102,103,].
3) The possible presence of a bilirubin oxidase enzyme [99].

The classical form of bilirubin encephalopathy , which was generally observed in term infants with hemolytic diseases, is virtually unknown today. This is a result of an improved and aggressive therapy directed at controlling hyperbilirubinemia with phototherapy, exchange transfusion, and prenatal management of the mother and fetus [104,105].

Unfortunately, kernicterus is still being observed at autopsies [105]. Small premature babies are the population at greatest risk for the development of bilirubin encephalopathy. In these infants, kernicterus has been found at bilirubin levels that are considered to be within the normal and "safe" range for the mature newborn [106,107,108]. Several potentiating factors that affect albumin binding of bilirubin or enhance tissue uptake of bilirubin have been suggested. Among these are low birth weight, hypothermia, asphyxia, acidosis, hypoalbuminemia, sepsis, meningitis and the use of drugs that displace bilirubin from its albumin binding sites [109,110]. To date, there is no proof for a direct relationship between the potentiating factors and the presence or absence of kernicterus and bilirubin encephalopathy [110,111,112]. The question as to what is affecting the newborn infant, still remains open. Is hyperbilirubinemia <u>per se</u> toxic, or is hyperbilirubinemia

an associated factor with the compounding effect of the other risk factors [113] ? Despite the uncertainty, measures have been taken to reduce the risk of bilirubin encephalopathy by adjusting the critical bilirubin concentrations to birth weight, gestational age and clinical situations at which medical intervention is indicated [114].

10. Studies on Bilirubin Toxicity

That bilirubin might be toxic to neural cells stems from the clinical association between the neurological picture and hyperbilirubinemia. However, despite a fairly detailed understanding of the chemistry and biochemistry of bilirubin there have been very few studies designed to define the interaction between bilirubin and the central nervous system. The mechanism by which bilirubin enters the cell has been studied in many non-neural cells and subcellular fractions [78]. Specific kinetic studies carried out in hepatocytes [115,116,117,118,119,120] and human erythrocytes [121], have suggested the existence of saturable bilirubin binding sites. In other studies, the effects of pH and albumin on bilirubin binding to endothelial cells [122], fibroblasts [48,123], and isolated mitochondria [124] have been demonstrated. Our understanding of the interaction between bilirubin and neural cells is based on studies in which either the brain was exposed to bilirubin through opening of the blood brain barrier [95,98], or brain slices were exposed directly to bilirubin [125]. Both approaches present a relatively crude assessment of this interaction. To have a clear understanding of the mechanism of

bilirubin toxicity to the neural cell, knowledge of the interaction between bilirubin and the cell is critical.

Results of several studies indicate that bilirubin interferes with various cell functions [78,79,80]. Bilirubin toxicity to non-neural cells has been investigated extensively over the past years in fibroblasts [126,127,128,129], hepatocytes [130,131], erythrocytes [132,133,134,135], leukocytes [136,137], platelets [138] and Ehrlich ascites cells [139,140]. Toxic manifestations of bilirubin were demonstrated by non specific effects on cell viability and growth [126,127,128,129], cell morphology [135], and cell behavior [137,138]. More specific effects were observed when ATP synthesis [127] and membrane enzymes [133,134,139,140] were investigated.

Studies conducted on neural tissue demonstrated that bilirubin may impair a large number of cell functions such as changes in energy metabolism [41,141,142], alteration in the physical structure and function of cell membranes [61,62,63,64,65], changes in key intracellular enzymes [143,144,145,146,147], inhibition of both DNA [148,149] and protein synthesis [150,151,152,153], changes in carbohydrate metabolism [154,155] and modulation of neurotransmitter synthesis [156] and release [157]. Most of the work done on bilirubin toxicity in neural tissues can be divided into two major groups. In one group, Gunn rats which suffer from hereditary unconjugated hyperbilirubinemia, served as a model [145,148, 150,151,152,153,155]. In the other, brain cells from normally developed animals were used

[144,146,147,154,155,157]. There is a major difference between the two. The use of the Gunn rat as a model for bilirubin encephalopathy is based on the assumption that the damage seen is primarily due to bilirubin. Although extensive damage to the nervous system in the Gunn rat can be attributed to bilirubin, a genetically determined bilirubin-independent abnormality in these animals cannot be excluded [158,159].

Bilirubin toxicity of the central nervous system is thought to occur in two stages : 1) an early reversible stage, sometimes referred to as subclinical and transient bilirubin-induced neurotoxicity, and 2) a later stage initiated when the sequelae become irreversible [80,160,]. Clinical studies in hyperbilirubinemic neonates have shown reversibility of the acute toxic bilirubin-induced changes in auditory nerve and brainstem responses [161,162,163]. Cowger demonstrated that bilirubin toxicity in an L-929 cell line was reversible with the addition of albumin [127]. Recently, Hansen et al demonstrated a similar phenomenon in hippocampal slices [157], and Wennberg provided evidence for the reversibility of bilirubin toxicity and mitochondrial uptake of bilirubin in erythrocytes [164]. On the other hand, working in a cell free system, Sano et al demonstrated that bilirubin inhibition of protein kinase C activity is irreversible [147].

A major concern when experimenting with a bilirubin-to-albumin molar ratio that exceeds one, is the instability of bilirubin

leading to the formation of bilirubin aggregates and co-aggregates of bilirubin and albumin [33,37,38,165]. Once aggregates are formed, changes in free bilirubin concentration occur, giving rise to experimental variability. This problem has not been fully addressed in experiments dealing with bilirubin toxicity ***in vitro***. The frequent use of non-physiological bilirubin concentrations in ***in vitro*** studies, the addition of varying albumin concentrations with alteration of bilirubin-to-albumin molar ratios, and variations in the cells investigated, are among the major reasons for inconclusive results.

To date, few studies have been carried out in cultured neural cells. The question as to whether bilirubin is indeed toxic to the brain cell or whether the yellow staining of the brain is a coincidental finding has been raised. Schiff et al [149], reported recently that bilirubin toxicity in N-115, a murine neuroblastoma cell line, was dependent on bilirubin concentration, bilirubin to albumin molar ratio and time of exposure to bilirubin.

The present work will define the specific ***in vitro*** conditions under which bilirubin, when added to cells in media, is stable and remains so during the entire experiment. Working under these conditions and using N-115, a murine neuroblastoma cell line in culture, the present studies will attempt to characterize the following:

1) The interaction between bilirubin and the cell.
2) The target and the mechanism of bilirubin toxicity at the cellular level.

3) The possible reversibility of the toxic effects.

4) The delayed bilirubin effects after short-term bilirubin exposure during which no evidence of toxicity is manifested.

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CHAPTER 2

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Bilirubin - Neural Cell Interaction: Characterization of Initial Cell Surface Binding Leading to Toxicity in the Neuroblastoma Cell Line N-115.

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Introduction

Hyperbilirubinemia and bilirubin encephalopathy are well known occurrences in the newborn period [1,2]. It has been suggested that the protection of the newborn's brain to bilirubin toxicity may be due to a number of different factors. These include: a) the interaction of bilirubin with albumin and/or different phospholipids [3-9] and b) the integrity of the blood-brain-barrier and the brain cell membrane [10-14]. The fact that bilirubin might be toxic to neural cells stems from the clinical association of the neurologic picture that has emerged and the associated hyperbilirubinemia [15].

In spite of a fairly detailed understanding of the chemistry and biochemistry of bilirubin, there have been very few studies designed to define the interaction of bilirubin with the nervous system. The mechanism by which bilirubin enters the cell has been studied in many non-neural cells and subcellular fractions. Specific binding and kinetic studies carried out on hepatocytes [16-21] and human erythrocytes [22] have suggested the existence of saturable bilirubin binding sites. Other studies have demonstrated the effect of pH and albumin on the binding of bilirubin to L-929 cells [4], endothelial cells [23], fibroblasts [24] and isolated mitochondria [25].

The interaction of bilirubin with the central nervous system should consist of three steps, (i) the entry of bilirubin into the brain from the blood, (ii) the binding of bilirubin to neural cell surface with or without a subsequent internalization, and (iii) the interaction of bilirubin with intracellular targets (in the case of

internalization) or the alteration of plasma membrane properties leading to the toxic effect. There are two different views as to the mechanism of bilirubin entry into the brain. Some studies suggest that though bilirubin exists as a complex with albumin in the blood, only free bilirubin crosses the blood-brain-barrier (free bilirubin hypothesis) whereas other studies suggest that under certain conditions such as hyperosmolality, the blood-brain-barrier will be opened and bilirubin enters the brain as a bilirubin-albumin complex [10,11]. Once bilirubin enters the brain, the toxic effects will be determined by the interaction of bilirubin with the individual neurons.

Different approaches have been made to study the interaction of bilirubin with neural cells. There are studies that exposed either the whole brain [10,11] or brain slices to bilirubin [26]. These studies give a relatively crude assessment of the interaction because the exposure as well as the washing after the exposure will not be complete in a tissue and data are expressed in terms of total bilirubin uptake per gram of brain tissue. Another approach has been to characterize the interaction using membrane fractions and lipids of nervous tissue including components like sphingomyelin and gangliosides [9,27,28]. But these systems are far removed from the actual physiological situation with respect to the target as well as the form of bilirubin solution used. These studies employ supersaturated solutions of bilirubin, whereas in plasma, bilirubin is believed to be present predominantly as a complex with albumin. A better approach to the problem is to use a neural cell line under

the normal conditions of tissue culture in the presence of albumin as a model system. Such studies are almost lacking in the literature.

The present study examines the nature of the interaction of bilirubin with the murine neuroblastoma cell line N-115. The cells were exposed to bilirubin at different concentrations and different bilirubin to albumin molar ratios (B/A). The cellular uptake of bilirubin was characterized in terms of the kinetics, apparent equilibration (limiting values) and the effect of pH and temperature on the equlibration. The results indicate that the "free" form of bilirubin is the reactive species, and it interacts with the plasma membrane through a multistep binding process.

Materials and Methods

Materials. All reagents were of analytical grade and were purchased from Sigma Chemical Co. (USA). Bilirubin purity was verified by high performance liquid chromatography (HPLC), as indicated below, and was found to contain 92% IX-α isomer, 4.8% XIII-α isomer, and 2.8% III-α isomer. No other bile pigments were detected. Since all measurements of bilirubin extracted from cells were performed on HPLC, no further purification was carried out. [^{3}H]-bilirubin was prepared by ***in vivo*** labelling in rats using δ-amino [3,5(N)-^{3}H] levulinic acid (New England Nuclear) as the precursor [8]. [^{3}H]-bilirubin was purified from the bile as described by McDonagh [3], and was found to contain more than 98% bilirubin IX-α by HPLC (absorption at 454 nm), with specific activity of 1710 CPM/nmole bilirubin.

HSA (fraction V, Essentially Fatty Acid Free) obtained from Sigma Chemical Co. (St. Louis, MO), Dulbecco's Modified Eagle Medium (DMEM) and phosphate buffered saline (PBS) and fetal calf serum (FCS) were obtained from GIBCO (Canada). Solvents used were of HPLC grade (JT Baker Chemical Co.).

Bilirubin treatment of cells. The murine neuroblastoma cell line N-115 was seeded at a density of 3 x 10^6 cells/plate in 10 cm culture dishes (Falcon) and grown in standard DMEM plus 10% FCS, pH 7.4 at 37°C in a 5% CO_2 humidified atmosphere for 10-12 hours. The media was then removed, the cells washed twice with sterile PBS, and reincubated in 10 mL of protein-free media [29] containing human serum albumin plus 25 mM N-2-hydroxy-ethylpiperazine-N-2 ethanesulfonic acid (HEPES) to maintain a pH of 7.4 for another 12 hours, before the experiments with bilirubin were started. The albumin concentration was varied in different experiments to meet the required final B/A ratios. Three or four culture dishes were used in each experimental condition. These dishes were seeded with cells, as above, with bilirubin being added to two or three of them. The remaining dish contained experimental media plus bilirubin, but no cells - a measure of non-specific binding of bilirubin to the plate.

A stock solution of bilirubin was made by dissolving 2 mg bilirubin in [illegible] mL of N_2-purged 0.1 N NaOH. Bilirubin was added to the culture [illegible] to achieve the appropriate experimental conditions, followed immediately by the addition of an amount of 0.1 N HCl equivalent to the amount of NaOH added to restore the pH of the

culture media to 7.4. Under the experimental conditions, bilirubin-albumin mixtures were found to be stable when measured spectrophotometrically for a minimum of three hours and is reported elsewhere [30].

All procedures involving addition, incubation and extraction of bilirubin were carried out in a dimly lit room to avoid bilirubin photodegradation.

Measurement of bilirubin uptake by cells. At the end of the incubation period, the media was removed and saved for pH measurement. The cells were washed four times with ice cold PBS and then dislodged from the plate with a rubber policeman in 1.5 mL PBS and transferred into an Eppendorf Test tube. The cell suspension was then vortexed and 0.1 mL aliquots were taken for DNA analysis [31] and cell viability as measured by the nigrosin exclusion technique [32]. The remainder was spun down in a microfuge (Eppendorf) for 5 minutes and the supernatant removed. Bilirubin was extracted from the pellet by adding 0.9 mL of methanol:chloroform (1:2, v/v) followed by sonication for 10 minutes, and centrifugation for 10 minutes in an Eppendorf microfuge. The supernatant was dried under N_2 and kept at -20°C until HPLC analysis was performed [33].

Bilirubin extracts from the cells were analyzed by reverse-phase HPLC (Beckman Altex Ultrasphere IP, 5 µm, C-18, 25 x 0.46 cm column with Beckman Altex IP precolumn 4.5 x 0.46 cm) using 0.1 M di-n-octylamine acetate in methanol, pH 7.7, as eluant with a flow

rate of 1.0 mL/min - and the detector set at 454 nm [33]. Quantitation of peak areas was performed on a Gilson Data Master reporting integrator, using preweighed bilirubin (Sigma, Lot No. 25F-0584) as external standard.

The experiments with [^{3}H] bilirubin were also performed as described above except that instead of extracting cell-bound bilirubin with organic solvents and analysis by HPLC, the cells were suspended in 0.1 mL of 0.2 N NaOH and neutralized with 0.1 mL of 0.2 N HCl. The radioactivity was measured by liquid scintillation counting after adding 15 mL of aqueous counting scintillant (Amersham).

Results

In this study, uptake is defined as the total amount of bilirubin associated with the cells including both surface bound and internalized bilirubin. The results are the mean of the net uptake (total minus non-specific) of the two or three experimental dishes. The non-specific uptake was always less than 1.7% of the total uptake. If not mentioned otherwise, the bilirubin concentration refers to the total (input) concentration. The term "free bilirubin" is used to denote the bilirubin remaining after saturating the high affinity primary binding sites of albumin and as such include both "free bilirubin" in solution and the bilirubin loosely bound to albumin. The bilirubin-albumin solution were found to be stable for the time periods used in this study [31] and the isomeric composition of the bilirubin extracted from the cells was found to

be the same as the input bilirubin within error limits (1-2% increase in photoisomers).

Fig. 1 gives the time course of bilirubin uptake by N-115 cells when the cells are incubated with 100 μM bilirubin at different B/A ratios. The bilirubin uptake at a B/A ratio of 3 in 10 min is 80 pmole/μg DNA and plateaus at 125 pmole/μg DNA in 40 min. , whereas at a B/A ratio of 0.8 the rate is much slower and levels achieved are much less, < 5 pmole/μg DNA in 90 minutes. Thus, there is a sharp increase in the initial rate as well as the extent of uptake with increasing B/A ratio even though the input (total) concentration of bilirubin is held constant. The results support the idea that the "free" rather than the albumin-bound form of bilirubin is responsible for toxicity. Since the stoichiometry of albumin-bilirubin is 1:1 the concentration of "free bilirubin" will increase drastically as the B/A ratio increases from 0.8 to 3.0.

The effect of varying the bilirubin concentration on the initial rate of uptake of bilirubin by the neuroblastoma cell is given in Fig. 2. At a B/A ratio of 3, increasing the bilirubin concentration from 12.5 μM to 100 μM shows no evidence of saturation. A similar result was obtained at a B/A ratio of 1.5 with concentrations ranging up to 250 μM bilirubin. The apparent absence of saturation kinetics in either case likely rules out the possibility of carrier-mediated transport across the plasma membrane implicated in the uptake of bilirubin by hepatocytes [17-22]. The concentration of "free bilirubin" can also be varied by varying the B/A ratio at a constant total bilirubin concentration. The initial uptakes under

these conditions are plotted in two different forms in Fig. 3. As expected, the initial rate decreases rapidly with increasing albumin concentration almost linearly (probably) up to B/A = 2 and then very slowly (Fig. 3A). The same data plotted as a function of "free bilirubin" concentration calculated from the bilirubin-albumin stability constant of $3.2 \times 10^7 M^{-1}$ [4] is given in Fig. 3B, and shows no saturation up to 80 μM of "free bilirubin". (The curve suggests the possibility of saturation at higher bilirubin concentrations and a possible explanation for this is that at high B/A ratios the free bilirubin concentration is so high that it might form small aggregates, the reactivity of which might be less than that of the monomeric form. The results in Figs. 2 and 3 along with the known binding of bilirubin to lipids such as sphingomyelin and gangliosides with the affinity in the range of 10^5 - 10^6 M^{-1} [9,27,28] argue against the notion of a bilirubin carrier in N-115 cells.

The apparent equilibrium uptake (limiting values in Fig. 1) as a function of bilirubin concentration at B/A ratios of 1.5 and 3 are shown in Fig. 4. The curves are neither linear, expected for passive diffusion, nor hyperbolic, expected for a normal receptor-ligand system. The curves are parabolic (or rather part of a sigmoidal curve) suggestive of cooperative binding of bilirubin to the cells (At B/A = 1.5, a reasonable linear fitting can be done as shown by the solid line. However, there is considerable deviation from a linear extrapolation of early points as shown by the dotted lines). The uptake of [^{3}H] bilirubin by N-115 cells given in Table 1, also agree

with the non linear behavior seen in Fig. 4 effectively rulling out a diffusion mechanism .

To further characterize the binding we tested reversibility of binding by trying to extract cell-bound bilirubin with fresh albumin. Extraction was performed after incubating the cells with bilirubin for different time intervals and the results are given in Table 2. The uptake is partially reversible and the fraction reversible (extracted) decreases with an increasing period of incubation of cells with bilirubin. This indicates that the binding cannot be described by a simple receptor ligand system. The effect of temperature on bilirubin binding is given in Table 3. The temperature insensitivity of bilirubin uptake at B/A ratio of 1.5 suggests a specific binding to the cell because non-specific binding is expected to increase with increasing temperature due to increased concentration of "free bilirubin" in equilibrium with albumin at higher temperatures [3]. The difference in behavior at B/A ratios 1.5 and 3 could be a reflection of a complex binding process.

The effect of pH on bilirubin uptake by N-115 cells at a B/A ratio of 1.5 is given in Fig. 5. The uptake increases rapidly with decreasing pH - almost a 10 fold increase in uptake as the pH of the medium is lowered by 1 unit from pH 8.0 to pH 7.0. Changes in pH are reported to affect bilirubin deposition in the brain, erythrocytes and mitochondria [13,14,16,26,34]. One of the factors likely to contribute to this pH effect is the increased concentration of "free bilirubin" resulting from the decreased affinity of bilirubin for albumin with decreasing pH. Decreasing the pH from 7.4 to 7 leads to

a 4 fold increase in bilirubin uptake by N-115 cells whereas the expected change in "free bilirubin" concentration is negligible (16.729 μM at pH 7.4 and 16.738 μM at pH 7 calculated from binding constants of 3.2 x 10^7 M^{-1} at pH 7.4 and 2.8 x 10^7 M^{-1} at pH 7 [4]) suggesting that factors other than "free bilirubin" concentration may be responsible.

Discussion

The mechanism of bilirubin toxicity to the nervous system has been the subject of numerous investigations over the last few decades, yet the area is dominated by speculation rather than concrete ideas. This is mainly due to the peculiar properties of the bilirubin molecule. The molecule is neither hydrophilic nor hydrophobic, as indicated by its very poor solubility in aqueous media at neutral pH and poor to moderate solubility in organic solvents [6]. This has given rise to considerable limitation in experimentation as well as the interpretation of experimental data. It has also led to the use of a variety of model systems consisting of bilirubin solutions of varying kinds from supersaturated solutions at alkaline pH to bilirubin-albumin mixtures of different ratios and a range of targets from pure lipids and proteins to the whole brain. Though these studies have provided valuable information on different aspects of bilirubin action, a complete picture is still lacking. An important piece of information missing is the nature of bilirubin interaction with the plasma membrane. Studies with purified proteins and subcellular fractions have shown that bilirubin at micromolar concentrations can affect the activity of many

enzymes of cytosolic, mitochondrial and microsomal origin [2]. The relevance of these findings in relation to bilirubin toxicity ***in vivo*** requires an understanding of whether bilirubin can cross the plasma membrane and if so, what intracellular concentrations can be achieved under clinically relevant conditions. An integrated approach consisting of the quantification of bilirubin uptake and the measurement of consequent changes in some biochemical parameters of toxicity in the same system is desirable. Using a neural cell line we have shown recently that bilirubin affects mitochondrial function, protein synthesis and DNA synthesis in intact N-115 cells and the toxicity is determined by the concentration of bilirubin, B/A ratio and the period of exposure [35,36]. The complementary studies on the cellular uptake of bilirubin are presented here. In the clinical situation it is assumed that a B/A of less than one is safe, as the majority of bilirubin is bound to the primary "tight" binding site of the albumin molecule. In order to assess bilirubin interaction with the cell, we have used a B/A greater than 1 which would make available free bilirubin and/or loosely bound bilirubin [36].

The results in Figs. 1-4 clearly indicate that the uptake of bilirubin by N-115 cells increases with increasing period of exposure, B/A ratio and bilirubin concentration at a given B/A ratio consistent with our earlier results on the measurements of toxicity parameters under the same experimental conditions [35,36]. While this suggests that bilirubin enters the cell, the data presented here are not consistent with a simple transport mechanism. The data can be explained in terms of a multistep binding with the plasma

membrane similar to that proposed for the interaction of bilirubin with rat brain synaptosomal plasma membrane vesicles [28]. According to this model the interaction occurs in three steps: (i) bilirubin binding to the polar head group region of the membrane, (ii) insertion of the surface-bound bilirubin into the hydrophobic core of the membrane, and (iii) membrane induced aggregation of bound bilirubin on the surface of the membrane.

The unusual rate curve for bilirubin uptake at B/A = 1.5 (Fig. 1) could be a reflection of the multistep binding process. The effect is seen at bilirubin concentrations of 50 and 100 μM. Similar rate curves have been reported for the interaction of bilirubin with synaptosomal plasma membrane vesicles and liposomes made of lipids and proteins extracted from these vesicles [28]. The very low concentration of free bilirubin at B/A = 0.8 and a much faster uptake due to a high concentration of "free bilirubin" at B/A = 3 might explain the apparent normal behavior under these conditions. A multistep binding mechanism is also supported by the concentration-dependence of limiting uptake given in Fig. 4 and Table 1. The parabolic or probably sigmoidal curve is indicative of a cooperative process reflecting the aggregation of bilirubin on the membrane at high concentrations. The partial reversibility of bilirubin uptake, as assessed by the extraction with albumin (Table 2) also favors a multistep mechanisms. The bilirubin displaced from N-115 cells by albumin mainly represents the bilirubin bound to the cell surface (polar head groups), the initial step, because the fraction reversed decreases with increasing period of exposure. The

remaining non-extractable portion need not be irreversible in the thermodynamic sense because the dissociation of bilirubin aggregates and the desorption of bilirubin from the hydrophobic core of the membrane could be very slow processes as in the case of some lipids. The half-life for the desorption of membrane components such as phospholipids and glycolipids is in the order of days [37,38]. The difference in the effects of temperature on uptake at B/A ratios of 1.5 and 3 (Table 3) could be a further reflection of a multistep mechanism. At 50 μM bilirubin and B/A = 1.5, the "free bilirubin" concentration will be low so that the cell-bilirubin interaction is likely to be dominated by the initial step(s) whereas at 100 μM bilirubin and B/A = 3 the aggregation step is likely to be dominant. The step(s) following the initial binding is entropy driven as suggested by the increase in uptake with increasing temperature at B/A = 3. The most probable explanation for this is the penetration of bilirubin into the hydrophobic interior of the bilayer causing a disordering of acyl chains (increasing the fluidity). A recent study has suggested that the entropy gain may be due to the partitioning of bilirubin into free spaces in the bilayer [39]. The increased uptake with decreasing pH (Fig. 5) is also suggestive of hydrophobic interaction. As the pH is decreased the concentration of bilirubin monoanion will increase at the expense of bilirubin dianion and because of the reduced charge on the monoanionic form, penetration into the hydrophobic interior of the membrane will be favoured. A multistep binding mechanism including an aggregation of bilirubin on the surface has been suggested earlier for the interaction of

bilirubin with lipid vesicles and erythrocyte ghosts [22,27,28 40,41].

It is difficult to conclude from the present data on the question whether bilirubin crosses the plasma membrane and reaches intracellular targets. Some of the possibilities to be considered follow. Bilirubin may be confined to the plasma membrane and elicit the intracellular response by membrane-mediated transduction of information. Another possibility is that a fraction of the (plasma) membrane-bound bilirubin is transported into the cytosol by partitioning into a cytosolic carrier molecule. The ability of albumin to extract partially the cell-bound bilirubin (Table 2) and our earlier finding that bilirubin trapped in lipid vesicles can be extracted with albumin [42] support the idea. However, the presence of such carrier molecules for bilirubin has not yet been demonstrated in the nervous system though proteins such as Z-protein, glutathione-S-transferase and ligandin have been implicated to have such a role in the liver [20,43]. Finally, the possibility that bilirubin in the plasma membrane reaches intracellular membranes through membrane recycling or aqueous diffusion of the monomer as proposed for phospholipids and cholesterol [37,44] should also be considered. Experiments including subcellular fractionation of bilirubin-treated cells are in progress to obtain further insight into the mechanism of bilirubin transport across the plasma membrane.

Table 2-1. [^{3}H] bilirubin uptake by N-115 cells at 37°C. Cells were incubated with the indicated concentrations of bilirubin containing a constant amount of [^{3}H] bilirubin (28,000 CPM/dish) for the indicated periods and the cell-bound radioactivity was measured. The values are given as Mean ± S.E. of three dishes of cells.

B/A	Conc. of Bilirubin (μM)	Period of Incubation (min.)	[^{3}H]bilirubin Uptake (CPM/μg DNA)
1.5	5	5	7.86 ± 0.59
1.5	150	5	12.32 ± 1.26
1.5	5	60	12.78 ± 0.28
1.5	150	60	49.66 ± 1.12
3.0	5	60	19.46 ± 2.91
3.0	50	60	58.61 ± 1.80
3.0	75	60	68.58 ± 1.62
3.0	100	60	67.84 ± 2.03

Table 2-2. Reversibility of bilirubin uptake by N-115 cells at 37°C. For each case, 6 dishes of cells were treated with 100 μM bilirubin (B/A=3) at 37°C for the indicated period. Three dishes were subjected to uptake measurements by HPLC as usual (Control). To the remaining three dishes after a bilirubin washout, 33 μM of HSA was added and incubated at 37°C for 30 min. and then bilirubin remaining bound to the cell was measured as usual and this represented the uptake after extraction with albumin (Residual). The difference between control and residual uptakes gives the bilirubin extracted with albumin which represents the readily reversible portion of uptake. All uptake values are Mean ± S.E. from three dishes.

Period of Bilirubin Treatment	Bilirubin Uptake (pmole/μg DNA)		Bilirubin Extracted with Albumin	
(min.)	Control	Residual	pmole/μg DNA	% Control
3	26.6 ± 1.0	11.3 ± 1.6	15.5	57.8
10	80.5 ± 3.1	48.0 ± 1.8	32.5	40.4
20	72.5 ± 5.2	43.3 ± 4.7	29.2	40.3
40	124.6 ± 5.9	104.6 ± 3.4	20.0	16.1
60	132.8 ± 8.7	93.0 ± 4.6	39.8	30.0

Table 2-3. Effect of temperature on bilirubin uptake by N-115 cells. The cells were maintained at the indicated temperature for 2 hours and pH was maintained at 7.4 by adding appropriate amounts of 40 mM HEPES to the media. Bilirubin (100 μM at a B/A=3 and 50 μM at a B/A=1.5) was then added to the cells and incubated at the respective temperature for an additional 60 min. (B/A= 3) and 90 min. (B/A=1.5). Cell-bound bilirubin was extracted and measured by HPLC. The values at B/A= 3 are Mean ± S.E from three dishes while the values at B/A=1.5 are means from two dishes. The values in parenthesis give the pH of the medium at the end of incubation with bilirubin.

Temperature °C	Bilirubin Uptake (pmole / μg DNA) B/A = 1.5	B/A = 3
4	15.4 (7.68)	30.7 ± 1.8 (7.84)
15	9.2 (7.70)	52.1 ± 10.4 (7.74)
25	15.9 (7.75)	59.6 ± 4.4 (7.98)
37	14.4 (7.70)	94.0 ± 10.7 (7.88)

Figure 2-1. Time course for the uptake of bilirubin by N-115 cells at 37 C. Cells were incubated with bilirubin for different time intervals at 37 C and the cell-bound bilirubin was extracted and measured by HPLC. Each point represents the Mean ± S.E. from three dishes of cells. 100 μM bilirubin at B/A=0.8 (x), B/A=1.5 (o), and B/A=3 ( ), 50 μM bilirubin at B/A=1.5 (Δ). The curves for B/A=0.8 & 3 are drawn as rectangular hyperbolas, whereas the curves for B/A=1.5 are drawn as smoothed interpolations because the fitting to rectangular hyperbola results in a straight line and the deviations are considerable.

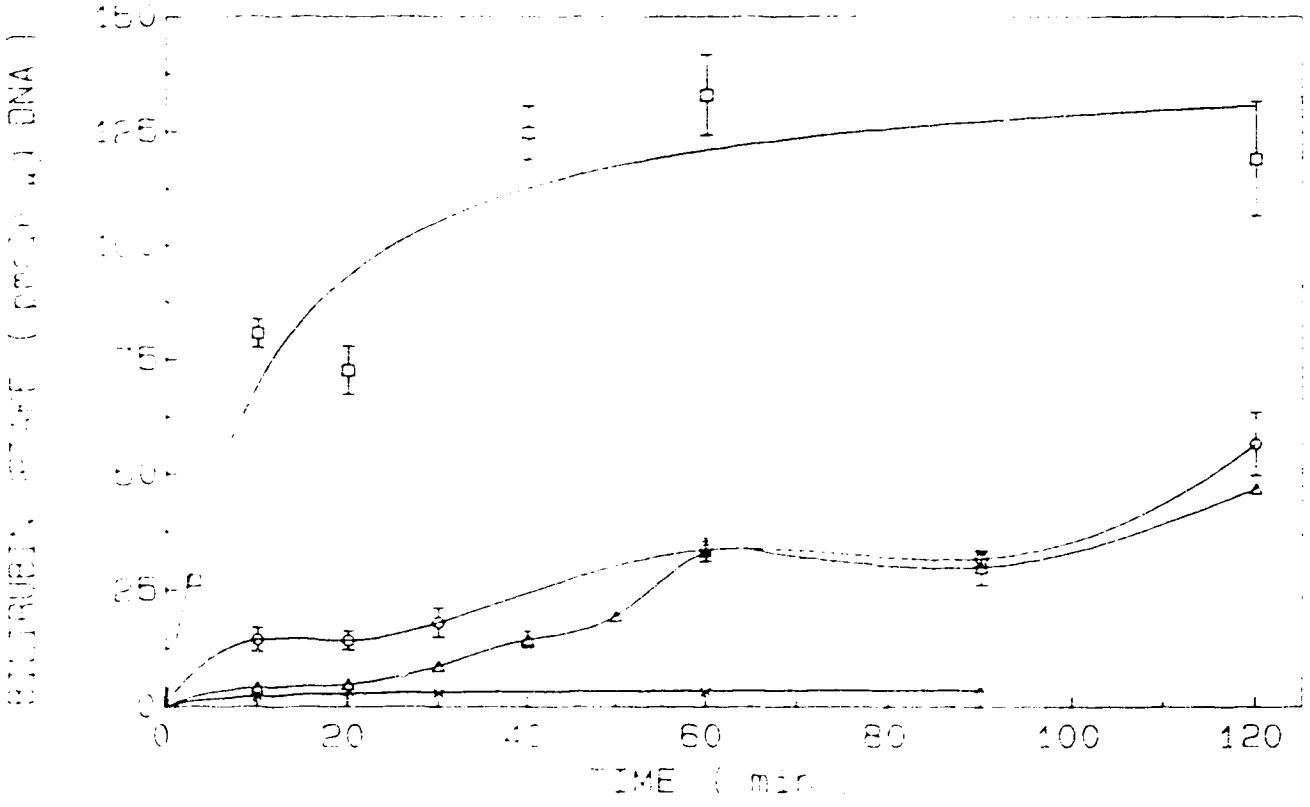


Figure 2-2. Initial rate of uptake of bilirubin by N-115 cells as a function of bilirubin concentration at constant B/A ratio. Cells were incubated with indicated concentrations of bilirubin for 10 min. at 37°C and the cell-bound bilirubin was extracted and measured by HPLC. Each point represents the Mean ± S.E. from three dishes of cells for B/A=3 (Δ), and the mean of duplicates (which differ by <18%) for B/A=1.5 (□).

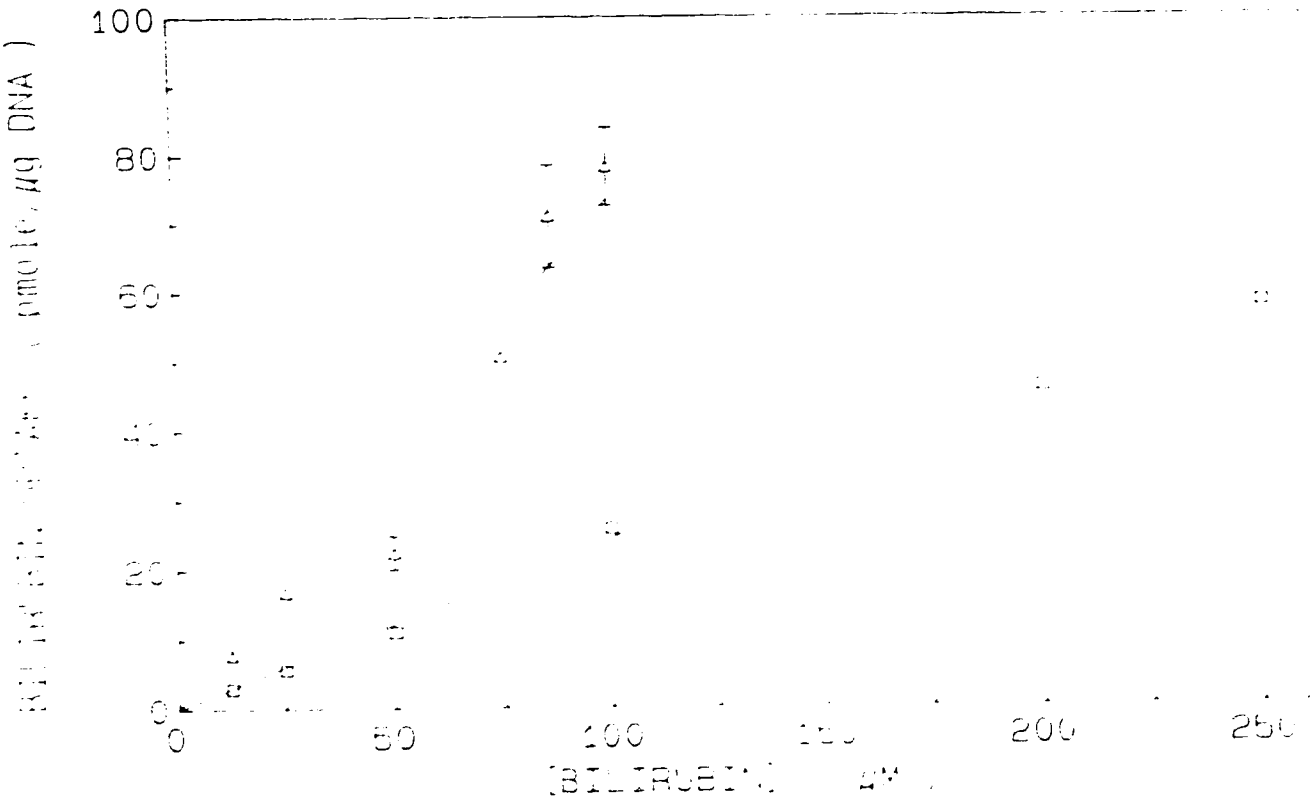


Figure 2-3. Initial rate of uptake of bilirubin by N-115 cells as a function of B/A ratio at a constant concentration of bilirubin. Cells were incubated with 100 μM bilirubin (and varying albumin concentration) for 10 min. at 37°C and the cell-bound bilirubin was extracted and measured by HPLC. Cellular uptake of bilirubin is plotted as a function of albumin concentration (A) and as a function of free bilirubin concentration (B). Concentration of free bilirubin was calculated assuming a bilirubin-albumin binding constant of 3.2 x 10^7 M^{-1} [4]. Each point represents the Mean ± S.E. from three dishes of cells.

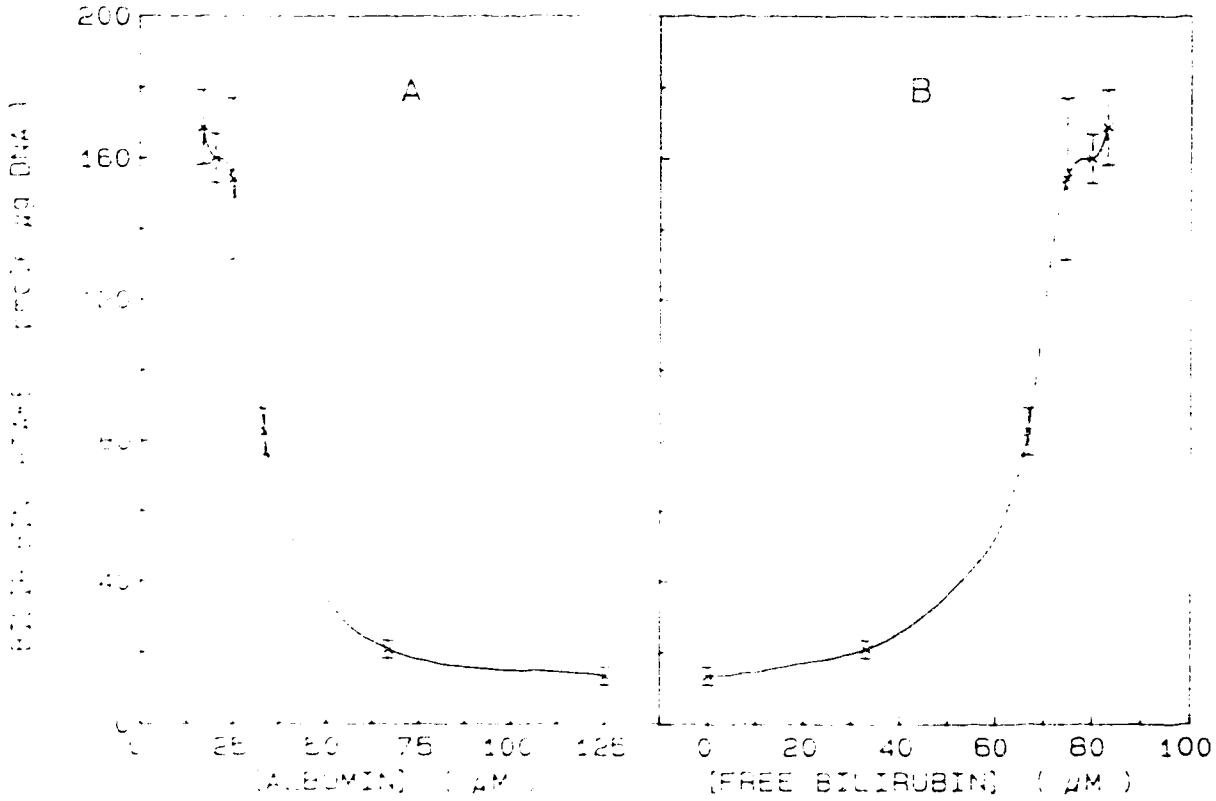
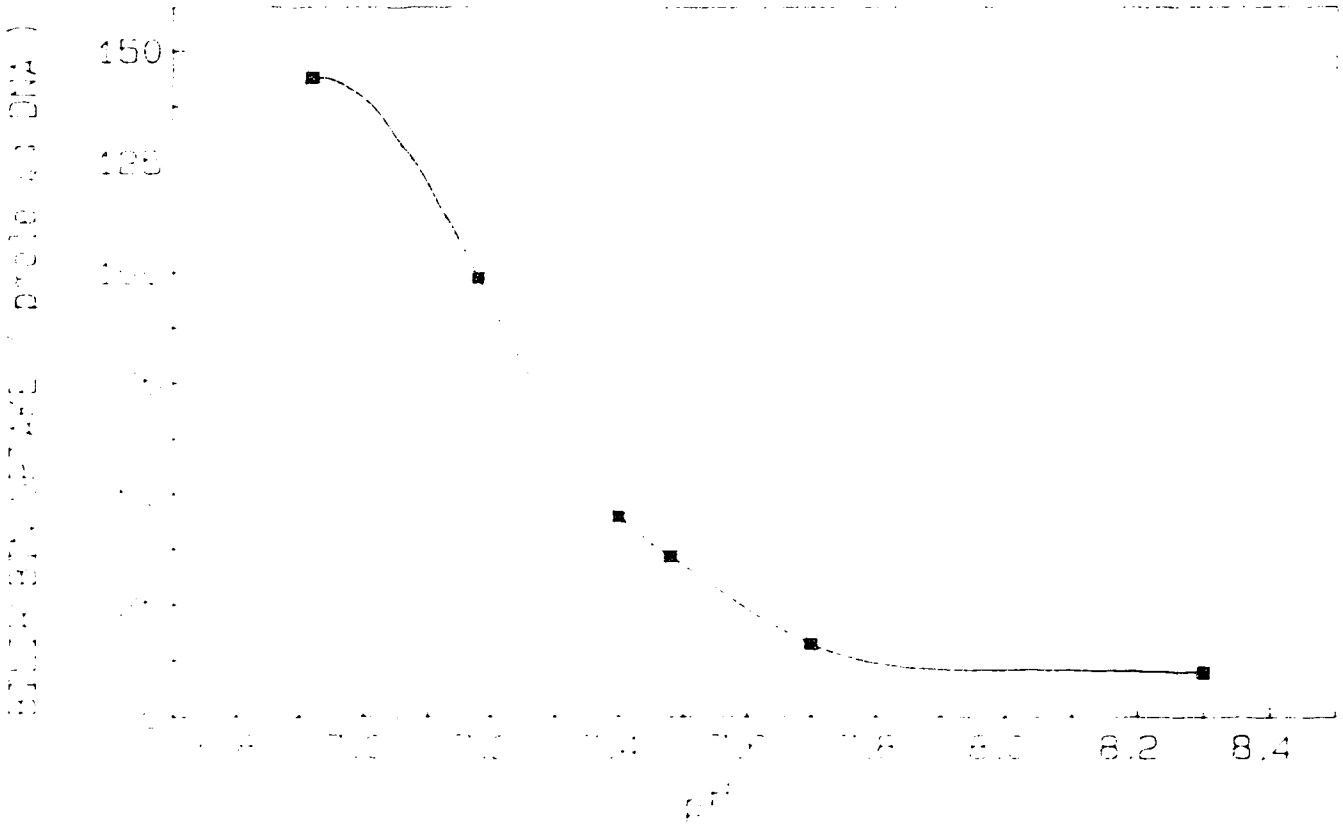


Figure 2-4. Limiting (apparent equilibrium) uptake of bilirubin by N-115 cells as a function of bilirubin concentration. Cells were incubated with indicated concentrations of bilirubin for 2 hours at 37°C and the cell-bound bilirubin was extracted and measured by HPLC. Each point represents the Mean ± S.E. from three dishes of cells for B/A=1.5 (x) and the mean of duplicates (which differ by < 14%) for B/A=3 (□). The dotted lines are linear extrapolations from points with bilirubin concentrations < 50 μM.

Figure 2-5. Effect of pH on bilirubin uptake by N-115 cells. Cells were grown as usual, the media was aseptically removed, 100-200 µL of sterile 1N HCl or 1N NaOH was added to achieve the desired pH and the media was gently poured back into the culture dish. Cells were incubated for 1 hour at 37°C and then 50 µM bilirubin at B/A=1.5 was added. After an additional 90 min. of incubation, cell-bound bilirubin was extracted and measured by HPLC. Each point represents the mean of duplicates (which differ by < 13%).



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CHAPTER 3

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Bilirubin Toxicity in a Neuroblastoma Cell Line N-115: I. Effects on Na^+ K^+ ATPase, [^{3}H]-Thymidine Uptake, L-[^{35}S]-Methionine Incorporation, and Mitochondrial Function.

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