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A Model Study of the Fructose Diphosphatase–Phosphofructokinase Substrate Cycle

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A fructose diphosphatase–phosphofructokinase substrate cycle has been reconstructed *in vitro* to provide a system that recycles fructose 6-phosphate and hydrolyses ATP to ADP and P_i. The concerted actions of glucose phosphate isomerase, phosphofructokinase, aldolase and triose phosphate isomerase catalysed the loss of ^3H from [5-^{3}H,U-^{14}C]glucose 6-phosphate. This was used as the basis of a method for the estimation of the fructose diphosphatase–phosphofructokinase substrate cycle. For the reconstructed cycle, the rate of decrease of the $^3H/^{14}C$ ratio in [5-^{3}H,U-^{14}C]hexose 6-phosphate was proportional to the rate of fructose 6-phosphate substrate cycling. A detailed theoretical treatment of this relationship is developed, which enables the rate of substrate cycling to be determined *in vivo*.

Metabolic pathways that can be either anabolic or catabolic have distinct enzymes to catalyse thermodynamically irreversible reactions. This is illustrated by the interconversion of fructose 6-phosphate and fructose 1,6-diphosphate where phosphofructokinase (ATP–D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) is required for glycolysis and fructose diphosphatase (fructose 1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) is required for gluconeogenesis. In a cell containing both of these enzymes, the simultaneous operation of phosphofructokinase (reaction 1) and fructose diphosphatase (reaction 2) constitutes an energetically wasteful substrate cycle (reaction 3).

Fructose 6-phosphate + $MgATP^{2-}$ → fructose 1,6-diphosphate + $MgADP^-$ (1)

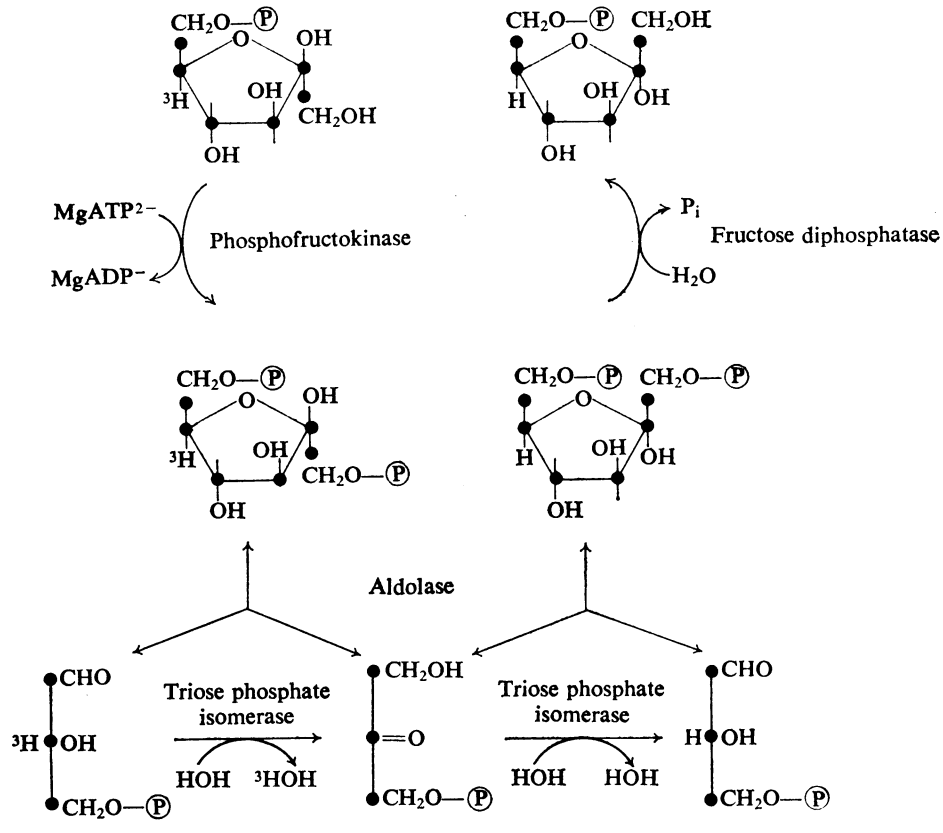
Fructose 6-phosphate + P_i ← fructose 1,6-diphosphate + H_2O (2)

Sum of reactions (1) and (2):

$$MgATP^{2-} + H_2O \rightarrow MgADP^- + P_i \quad (3)$$

Since these enzymes are subject to control by AMP [AMP activates phosphofructokinase (Passoneau & Lowry, 1962; Mansour, 1963; Underwood & Newsholme, 1965, 1967; Bloxham & Lardy, 1973) and inhibits fructose diphosphatase (Mendicino & Vasarhely, 1963; Taketa & Pogell, 1965; Underwood & Newsholme, 1965; Opie & Newsholme, 1967; Pontremoli & Horecker, 1971)], the possibility of cycling would appear to be minimized. However, recent evidence suggests that substrate cycling at the phosphofructokinase–fructose diphosphatase level may be important in a number of metabolic states, including the amplification of control signals (Newsholme & Gevers, 1967), the control of gluconeogenesis (Newsholme & Underwood, 1966; Williamson *et al.*, 1971) and the generation of heat in non-shivering thermogenesis (Newsholme & Crabtree, 1970; Newsholme *et al.*, 1972). This prompted us to develop a method that could be used to quantitate the phosphofructokinase–fructose diphosphatase substrate cycle. Measuring the hydrolysis of $MgATP^{2-}$ and the concomitant release of P_i is an obvious technique, but since $MgATP^{2-}$ and P_i are involved in numerous cellular reactions, this is a crude estimate at best.

A different approach makes use of the prediction that ^3H is lost to the medium when [5-^{3}H]fructose 1,6-diphosphate is a substrate for aldolase and triose phosphate isomerase because of the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (Rose, 1962). As shown in Scheme 1, substrate cycling of [5-^{3}H,U-^{14}C]fructose 6-phosphate should now generate species of fructose 6-phosphate with $^3H/^{14}C$ ratios lower than that of the initial substrate. Thus the decrease in $^3H/^{14}C$ ratio should represent a measure of the fructose diphosphatase–phosphofructokinase substrate cycle. In the present work it is established for a model system that the preceding approach can be used to evaluate substrate



Scheme 1. *Reactions of [5-^{3}H,U-^{14}C]fructose 6-phosphate in the fructose diphosphatase–phosphofructokinase substrate cycle*

The configuration designations of fructose 1,6-diphosphate and fructose 6-phosphate are in accord with the anomeric preference of fructose diphosphatase (Schray *et al.*, 1972) and of phosphofructokinase (Bloxham & Lardy, 1973).

cycling. The Appendix (Bloxham *et al.*, 1973) reports a detailed theoretical treatment of the relationship between substrate cycling and the decrease in the ^{3}H/^{14}C ratio in [5-^{3}H,U-^{14}C]glucose 6-phosphate, which enables the rate of substrate cycling to be determined *in vivo*. Application of this method to situations *in vivo* is discussed in the next paper (Clark *et al.*, 1973).

Experimental

Materials

Rabbit liver fructose diphosphatase (1.1 i.u./mg) was obtained from the Sigma Chemical Co., St. Louis, Mo., U.S.A. Phosphofructokinase (100 i.u./mg) was isolated from rabbit skeletal muscle (Ling *et al.*, 1965) and crystallized by the method of Parmeggiani *et al.* (1966). All other enzymes and substrates were obtained from the Boehringer Corp., New York, N.Y., U.S.A. The activities of individual enzymes were determined at saturating substrate concentrations.

Methods

Preparation of [5-^{3}H]glucose 6-phosphate. The reaction mixture contained 6.6mM-dihydroxyacetone phosphate, 200mCi of ^3H_2O, 4.8i.u. of triose phosphate isomerase, 15mM-$MgSO_4$ and 35mM-Tris–HCl, pH 7.4, in a total volume of 1.4ml at 25°C. After 20min, 9i.u. of aldolase and 5i.u. of fructose diphosphatase were added and dephosphorylation was allowed to proceed to completion. Excess of ^3H_2O was

removed by distillation under vacuum. The residue was resuspended in 20ml of 10mM-Tris–HCl, pH7.4, containing 10i.u. of glucose phosphate isomerase. After 10min, glucose 6-phosphate was isolated as described below.

Determination of metabolites. Glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate were determined as described in Bergmeyer (1963). P_i was determined by the reduction of phosphomolybdic acid with $FeSO_4$ (Taussky & Shorr, 1953).

Isolation of hexose phosphates. Aqueous samples containing hexose phosphates were applied to a column (0.5cm×6cm) of Dowex 50W AG resin (H^+ form; X8) and eluted with water (2×3ml). The combined eluates were concentrated and applied as a band to Whatman 3MM paper. The sugar phosphates were resolved by chromatography with the solvent system butan-1-ol–propan-1-ol–acetone–80% (w/v) formic acid–30% (w/v) trichloroacetic acid (8:4:5:5:3, by vol.). Authentic glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-diphosphate were used as markers. Each chromatogram was run twice in the same direction (ascending, 2×15h) and the sugar phosphates were stained with diphenylamine–aniline spray reagent (Smith, 1958). The band corresponding to each sugar phosphate was eluted with water. Traces of trichloroacetic acid in the eluate were removed by extraction with diethyl ether and the remaining aqueous fraction was evaporated to dryness.

Measurement of radioactivity. Samples were counted with a Packard scintillation spectrometer; ^3H was counted at 43.7% efficiency and ^{14}C at 52% efficiency. Ratios of $^3H/^{14}C$ were calculated from values corrected by using the method of internal standards.

Results and Discussion

Each cycle of fructose 6-phosphate through reactions (1) and (2) of the fructose diphosphatase–phosphofructokinase substrate cycle gives rise to the hydrolysis of 1 molecule of $MgATP^{2-}$ with the formation of 1 molecule each of $MgADP^-$ and P_i. The rate of substrate cycling established *in vitro* with rabbit liver fructose diphosphatase and rabbit muscle phosphofructokinase was determined by measuring the release of P_i caused by hydrolysis of $MgATP^{2-}$ in the presence of catalytic amounts of fructose 6-phosphate and fructose 1,6-diphosphate. Fig. 1 (curve A) shows that the rate of substrate cycling was proportional to the amount of fructose diphosphatase present until the ratio of fructose diphosphatase/phosphofructokinase reached a value of 2. In these experiments sufficient ATP was added to allow a maximum of 20 cycles of fructose 6-phosphate and substrate cycling was found to proceed until the ATP was completely utilized. P_i was not released when either phosphofructokinase or fructose diphosphatase was omitted.

The possible role of AMP in the control of substrate cycling was also evaluated. Fig. 1 (curve B) shows that the inclusion of AMP (50μM) and ADP (350μM) increased the ratio of fructose diphosphatase/phosphofructokinase required for maximal substrate cycling with only a slight decrease in the maximal rate. The further addition of myokinase to establish equilibrium between the adenine nucleotides caused a dramatic diminution in substrate cycling (Fig. 1, curve C). The demonstration of regulation of P_i release by AMP is in accord with the known regulatory action of AMP on fructose diphosphatase (Pontremoli & Horecker, 1971) and is a finding consistent with P_i release by substrate cycling.

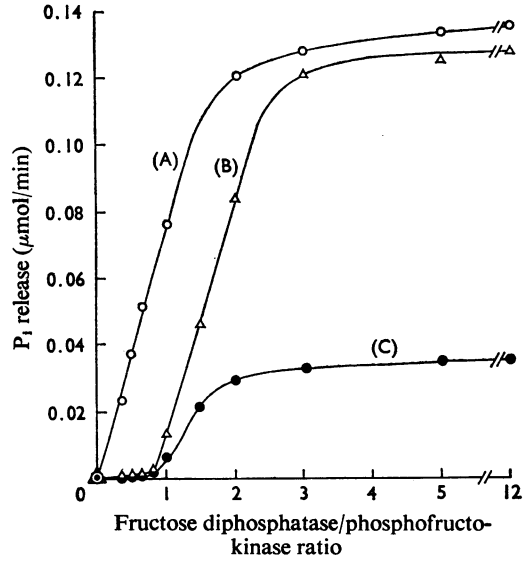


Fig. 1. *Effect of the fructose diphosphatase/phosphofructokinase ratio on the rate of ATP hydrolysis*

Reaction mixtures (volume 3ml) contained 100mM-Tris–HCl, pH7.4, 1mM-$MgSO_4$, 40mM-KCl, 1mM-dithiothreitol, 1mM-ATP, 50μM-fructose 6-phosphate, 50μM-fructose 1,6-diphosphate, 4.5i.u. of aldolase, 15i.u. of triose phosphate isomerase, 1.8i.u. of phosphofructokinase and fructose diphosphatase as indicated. Further additions were: none (○); 350μM-ADP and 50μM-AMP (△); 350μM-ADP, 50μM-AMP and 0.7i.u. of myokinase/ml (●). The reactions at 25°C were started by the addition of fructose diphosphatase. Samples (0.3ml) were removed at intervals of 1min and the reaction was terminated by heating at 100°C for 5min. The amount of ATP hydrolysed was determined by measurement of the P_i released.

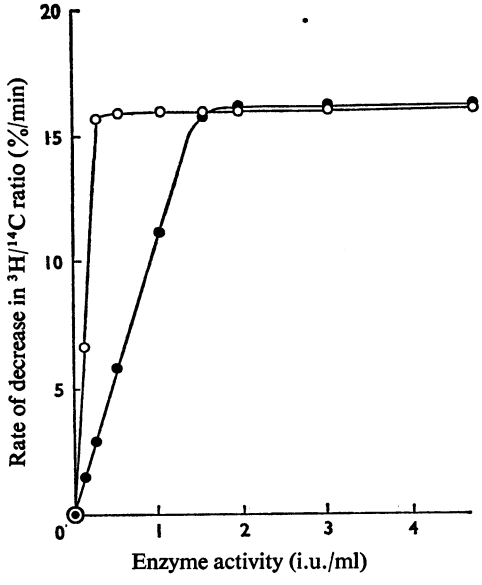


Fig. 2. *Enzyme-catalysed loss of ^3H from [5-^3H,U-^{14}C]glucose 6-phosphate*

Reaction mixtures (volume 0.5ml) contained 100mM-Tris–HCl, pH7.4, 1mM-$MgSO_4$, 40mM-KCl, 1mM-dithiothreitol, 1mM-ATP, 0.3mM-[5-^3H,U-^{14}C]glucose 6-phosphate (5.4×10^5c.p.m. of ^3H and 2.7×10^4c.p.m. of $^{14}C/\mu$mol), 7i.u. of glucose phosphate isomerase/ml, 2.2i.u. of phosphofructokinase/ml and the following concentrations of aldolase and triose phosphate isomerase: various amounts of triose phosphate isomerase and 1.5i.u. of aldolase/ml (○); various amounts of aldolase and 4.8i.u. of triose phosphate isomerase/ml (●). The rate of ^3H release was determined by removing samples (50μl) at various times and measuring the $^3H/^{14}C$ ratio of the combined hexose monophosphate and diphosphate pool.

It was now pertinent to demonstrate that ^3H was lost from the C-5 position of labelled glucose 6-phosphate by the concerted action of glucose phosphate isomerase, phosphofructokinase, aldolase and triose phosphate isomerase. This was investigated by incubating [5-^3H,U-^{14}C]glucose 6-phosphate in the presence of various concentrations of aldolase and triose phosphate isomerase at a fixed concentration of the other enzymes. Fig. 2 shows that in the absence of either aldolase or triose phosphate isomerase there was no significant release of ^3H. As the concentration of aldolase or triose phosphate isomerase was raised there was a progressive increase in the rate of ^3H release. Maximum rates were achieved at an aldolase concentration of 1.5i.u./ml

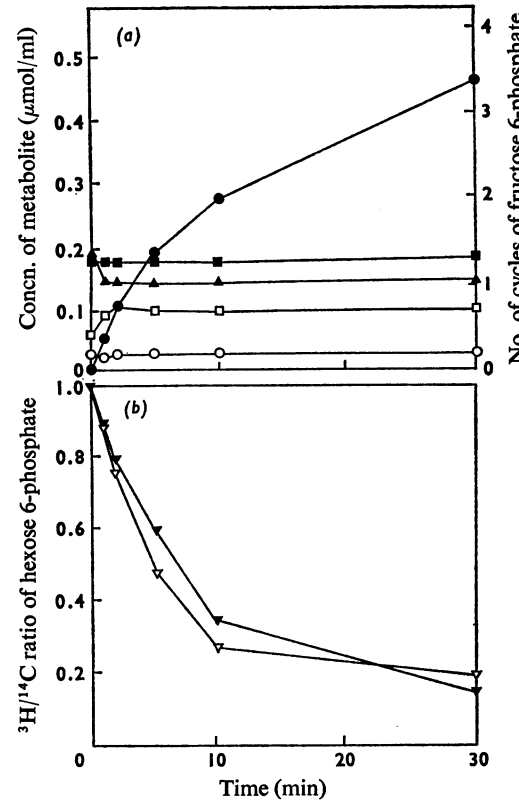


Fig. 3. *Relationship of substrate cycling of fructose 6-phosphate to the $^3H/^{14}C$ ratio of hexose 6-phosphates*

The reaction mixture (volume 3ml) contained 100mM-Tris–HCl, pH7.4, 1mM-$MgSO_4$, 40mM-KCl, 1mM-dithiothreitol, 1mM-ATP, 0.1mM-[5-^3H,U-^{14}C]glucose 6-phosphate (8.1×10^5c.p.m. of ^3H and 3.3×10^4c.p.m. of $^{14}C/\mu$mol), 0.1mM-fructose 6-phosphate, 0.2mM-fructose 1,6-diphosphate, 3.5i.u. of glucose phosphate isomerase/ml, 1.5i.u. of aldolase/ml and 4.8i.u. of triose phosphate isomerase/ml. The reaction was started by the addition of 1.5i.u. of fructose diphosphatase/ml and 0.45i.u. of phosphofructokinase/ml. Samples (0.4ml) were removed before the addition of the fructose diphosphatase–phosphofructokinase mixture and, at the times indicated, for (*a*), the analysis of glucose 6-phosphate and fructose 6-phosphate (■), dihydroxyacetone phosphate (▲), glyceraldehyde 3-phosphate (○), fructose 1,6-diphosphate (□) and P_i (●), and for (*b*), the determination of the $^3H/^{14}C$ ratio of glucose 6-phosphate (▼) and fructose 6-phosphate (▽).

and a triose phosphate isomerase concentration of 0.1i.u./ml at a phosphofructokinase concentration of 2.2i.u./ml. The ability of cells to catalyse the rapid

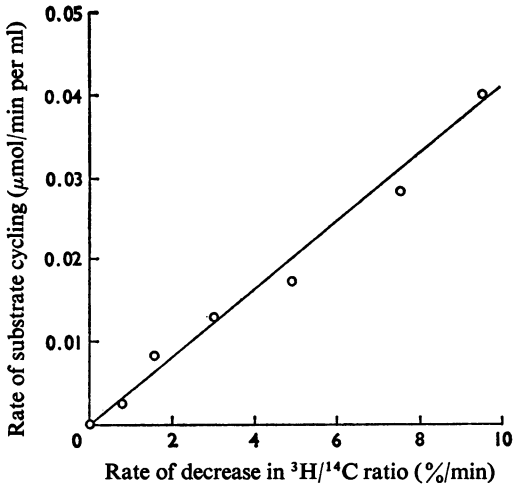


Fig. 4. *Proportionality of the rate of substrate cycling and the rate of decrease of $^3H/^{14}C$ ratio of [5-^{3}H,U-^{14}C]glucose 6-phosphate*

The values for this plot were obtained by differentiating the rate curves for orthophosphate release and the decrease in the $^3H/^{14}C$ ratio of glucose 6-phosphate shown in Fig. 3 (*a* and *b*).

loss of ^3H from [5-^{3}H,U-^{14}C]glucose 6-phosphate will depend on both the absolute amounts of aldolase and triose phosphate isomerase and their relative activities compared with that of phosphofructokinase. A general consideration of the concentration of these enzymes (e.g., Shonk & Boxer, 1964) shows that in many tissues the activities of aldolase and triose phosphate isomerase are in excess of the minimum required activity shown in Fig. 2 and that the ratios of aldolase/phosphofructokinase and triose phosphate isomerase/phosphofructokinase exceed those of Fig. 2.

Having established the relation between enzyme concentration and the rate of loss of ^3H, the relationship between the rate of substrate cycling and the rate of change of the $^3H/^{14}C$ ratio of glucose 6-phosphate was investigated. For these studies it was essential that no net carbon flux occurred. The final choice of experimental conditions that fulfilled this requirement was decided after several reconstructed systems were tested. Fig. 3(*a*) shows that after 2min a constant concentration of each of the metabolites in the desired proportion was achieved by a fructose diphosphatase/phosphofructokinase ratio of 3.33. For the observed concentration of fructose 6-phosphate (0.426μmol/3ml), the release of 1.404μmol of P_i (at 30min) corresponded to 3.3 complete cycles of fructose 6-phosphate. Concomitant with the measured increase of P_i there was a marked decrease in the $^3H/^{14}C$ ratios of glucose 6-phosphate and fructose 6-phosphate (Fig. 3*b*). The rate of decrease of the $^3H/^{14}C$ ratio for each substrate was similar and closely resembled the rate of release of P_i. A plot of the first differential of the P_i release versus the rate of change of the $^3H/^{14}C$ ratio in glucose 6-phosphate was linear (Fig. 4).

When phosphofructokinase was omitted from the reaction mixture there was no decrease in the $^3H/^{14}C$ ratio of glucose 6-phosphate. Measurement of the $^3H/^{14}C$ ratios of hexose 6-phosphates was impractical for the system lacking fructose diphosphatase since the substrate, [5-^{3}H,U-^{14}C]glucose 6-phosphate, was rapidly converted into fructose 1,6-diphosphate and measurements of the $^3H/^{14}C$ ratio in glucose 6-phosphate or fructose 6-phosphate could not be obtained after 2min.

Conclusion

It is demonstrated that for the reconstructed fructose diphosphatase–phosphofructokinase substrate cycle the rate of decrease of the $^3H/^{14}C$ ratio in [5-^{3}H,U-^{14}C]hexose 6-phosphate is proportional to the rate of substrate cycling. Provided that aldolase and triose phosphate isomerase catalyse the rapid loss of ^3H from C-5 of fructose 1,6-diphosphate *in vivo*, this technique can be used to estimate substrate cycling *in vivo*.

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APPENDIX

The Theoretical Estimation of Substrate Cycling *in vivo*

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In the main paper (Bloxham *et al.*, 1973), it was established that the decrease in the $^3H/^{14}C$ ratio of [5-^{3}H,U-^{14}C]glucose 6-phosphate is a measure of substrate cycling of fructose 6-phosphate *in vitro*. In this Appendix a mathematical expression for this relationship is derived. For systems *in vivo*, determination of the rate of substrate cycling by measurement of the $^3H/^{14}C$ ratio in glucose 6-phosphate requires that radioactive isotope is applied as [5-^{3}H,U-^{14}C]glucose and that glucose 6-phosphate is labelled by reactions of glycolysis.

Scheme 1 represents a possible model for substrate cycling *in vivo* and includes a list of the notations used in this work. Under steady-state conditions:

$$k_1 a = k_2 b - k_3 c = k_4 c = \text{rate of glycolysis} \quad (1)$$

By definition, a, b, and c are constant.

The following assumptions were made: (i) a^κ and a^τ are constant for the duration of the experiment; (ii) glucose phosphate isomerase maintains radioisotopic equilibrium between glucose 6-phosphate and fructose 6-phosphate; (iii) the ^{3}H from [5-^{3}H,U-^{14}C]fructose 1,6-diphosphate is replaced by unlabelled H immediately; (iv) other reactions leading to the loss of ^{3}H from [5-^{3}H,U-^{14}C]glucose 6-phosphate are neglected (see Clark *et al.*, 1973). The rate of labelling of b and c with ^{3}H or ^{14}C is given by:

$$\frac{dbb^\tau}{dt} = k_1 aa^\tau - k_2 bb^\tau \quad (2)$$

$$\frac{dbb^\kappa}{dt} = k_1 aa^\kappa - k_2 bb^\kappa + k_3 cc^\kappa \quad (3)$$

$$\frac{dcc^\kappa}{dt} = k_2 bb^\kappa - (k_3 + k_4) cc^\kappa \quad (4)$$

Combining equations (2), (3) and (4) with (1) gives:

$$\frac{db^\tau}{dt} = -k_2 b^\tau + \frac{k_2 a^\tau}{(1 + k_3/k_4)} \quad (5)$$

$$\frac{db^\kappa}{dt} = -k_2 b^\kappa + \frac{k_2 c^\kappa}{(1 + k_4/k_3)} + \frac{k_2 a^\kappa}{(1 + k_3/k_4)} \quad (6a)$$

$$\frac{dc^\kappa}{dt} = (k_3 + k_4) b^\kappa - (k_3 + k_4) c^\kappa \quad (6b)$$

The method of deriving the solution of eqn. (5) and the simultaneous solutions of eqns. (6a) and (6b) can be found in standard texts on differential equations (e.g. Brauer & Nohel, 1967). Eqns. (7), (8a) and (8b) can be checked by substitution into (5), (6a) and (6b):

$$b^\tau(t) = \frac{a^\tau}{(1 + k_3/k_4)} - \frac{a^\tau e^{-k_2 t}}{(1 + k_3/k_4)} \quad (7)$$

$$b^\kappa(t) = a^\kappa - \frac{\delta(\beta - \alpha) e^{-(\gamma - \beta)t/2}}{\gamma - \beta} - \frac{\delta(\alpha + \beta) e^{-(\beta + \gamma)t/2}}{\beta + \gamma} \quad (8a)$$