

STUDIES ON THE REGULATION OF  
TYROSINE AMINOTRANSFERASE IN RATS

ERNEST ALLAN LANE

THESIS

SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES IN  
PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF  
DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY  
UNIVERSITY OF OTTAWA

MAY 1972

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### ACKNOWLEDGEMENTS

To Dr. C. Mavrides, I would like to express my most sincere gratitude and appreciation for the direction given to this thesis.

I would also like to thank the Canadian Medical Research Council for financial support and the Government of Ontario for awarding me an Ontario Graduate Fellowship.

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LIST OF ABBREVIATIONSChemical compounds:

|            |                                   |
|------------|-----------------------------------|
| cyclic AMP | adenosine-3',5'-monophosphate     |
| dCMP       | deoxycytidine monophosphate       |
| DNA        | deoxyribonucleic acid             |
| DOPA       | L-dihydroxyphenylalanine          |
| GSH        | reduced glutathione               |
| IDP        | inosine-5'-diphosphate            |
| ITP        | inosine-5'-triphosphate           |
| NAD        | nicotinamide adenine dinucleotide |
| NADH       | reduced NAD                       |
| NADP       | NAD phosphate                     |
| PEP        | phosphoenolpyruvic acid           |
| pHPP       | p-hydroxyphenylpyruvic acid       |
| RNA        | ribonucleic acid                  |

Enzymes:

|       |                                   |
|-------|-----------------------------------|
| PEPCK | phosphoenolpyruvate carboxykinase |
| TAT   | tyrosine aminotransferase         |

Weights and measures:

|    |           |
|----|-----------|
| g  | gram      |
| kg | kilogram  |
| mg | milligram |
| nm | nanometer |
| μg | microgram |

Miscellaneous:

|        |                            |
|--------|----------------------------|
| c.p.m. | counts per minute          |
| d.p.m. | disintegrations per minute |
| HTC    | hepatoma tissue culture    |
| i.p.   | intraperitoneally          |
| s.e.m. | standard error of mean     |

### ABSTRACT

Three areas were investigated to determine factors responsible for the regulation of hepatic tyrosine aminotransferase. These were: 1) the effect of cycloheximide on the enzyme, 2) the diurnal rhythm of the enzyme and 3) the hormonal-dietary interactions and the possible role of gluconeogenesis in the regulation of this enzyme.

1. Cycloheximide was shown to prevent both the cortisol-mediated and the stress-mediated elevation of tyrosine aminotransferase. The administration of cycloheximide itself to adrenalectomized rats failed to stimulate the enzyme indicating that this antibiotic has no pseudohormonal action on tyrosine aminotransferase as had been proposed by others.
2. The diurnal rhythm of hepatic tyrosine aminotransferase was fully developed by the 21st day of age. The development of the rhythm could be prevented by constant light. Adrenalectomy at this time and return to a cyclic lighting schedule (lights off from 1800 and 0600 hours) prevented the normal appearance of the rhythm suggesting that the adrenal glands are important for its development. This was supported by experiments in which rats with an entrained rhythm were shifted to a constant light environment for 3 weeks to desynchronize the rhythm. Following adrenalectomy and 11 days on a cyclic lighting schedule the amplitude of the

enzyme rhythm was reduced by one-half compared to that of the sham-operated controls. These results indicated that the adrenal gland is important in the development and operation of the diurnal rhythm in rats and suggested that a relationship may exist between the diurnal rhythm of the enzyme and its ability to be stimulated by exogenously administered glucocorticoids. The study was extended to include other vertebrates to establish whether a similar relationship could be demonstrated. In both amphibia (tadpoles and frogs) and birds (pigeons) tyrosine aminotransferase displayed a diurnal rhythm although only in birds can the enzyme be stimulated by glucocorticoids. Corticosterone and cortisol were shown to have no effect on the enzyme of tadpoles and cortisol has been reported to have no effect in frogs. Thus although a relationship may exist for birds and rats this cannot be generalized for all species.

3. The infusion of microgram amounts of cortisol (35  $\mu$ g per 200 gm rat over a period of 2 hours) was found to be sufficient to stimulate hepatic tyrosine aminotransferase in adrenalectomized and hypophysectomized rats fed controlled protein diets. The percent stimulation increased as the protein content of the diet decreased. The stimulation was greater in hypophysectomized than adrenalectomized rats fed the same diet. A similar pattern emerged following glucagon infusion. Hepatic phosphoenolpyruvate carboxykinase, a key

gluconeogenic enzyme, was found to respond similarly following cortisol infusion, that is, with a greater stimulation in rats fed a protein-free diet than in those fed a high-protein diet. A diurnal rhythm was also demonstrated for this enzyme. The daily peak in phosphoenolpyruvate carboxykinase occurred at the same time or slightly before the daily peak in tyrosine aminotransferase. The drop in hepatic glycogen which coincided with the rise in phosphoenolpyruvate carboxykinase lends support to the suggestion that the rate of gluconeogenesis is increased during this time. As the protein content of the diet was increased, the rhythm of phosphoenolpyruvate carboxykinase grew more forceful indicating that the rats were relying more heavily upon gluconeogenesis for their needs for carbohydrate. These findings strongly suggest that a relationship exists between the rate of gluconeogenesis and the level of hepatic tyrosine aminotransferase.

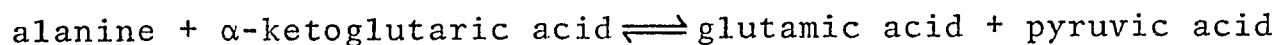
REVIEW OF LITERATURE

### A. Discovery of tyrosine aminotransferase.

Aminotransferases are enzymes which catalyze the reactions involving the transfer of an amino group from an  $\alpha$ -amino acid to an  $\alpha$ -keto acid, the overall process being termed transamination. The transamination reaction was first recognized by Needham (1930) who noticed that glutamic acid and aspartic acid disappeared when added to pigeon breast muscle, without a concomitant decrease in amino nitrogen.

Further evidence for the existence of the transamination reaction was provided by Szent-Gyorgyi (1936) who reported that the rate at which oxaloacetic acid disappeared was greatly increased when glutamic acid was added to pigeon breast muscle. He later confirmed the findings with an enzyme preparation from the same tissue (Szent-Gyorgyi, 1937).

The discovery, however, of the transamination reaction has been credited to Braunstein and Kritzmann (1937) who carried out the first detailed study of the reaction. They were able to isolate and chemically identify alanine and glutamic acid as the amino acid products in the reversible transamination reaction.



Braunstein and Kritzmann (1939) later termed the enzymes responsible for the transamination reaction "aminopherases", but this name was later changed to

"transaminases" by Cohen (1940) since this terminology was more consistent with enzyme nomenclature at that time. This term has since been changed to "aminotransferases" on the recommendations of the Commission on Enzymes of the International Union of Biochemistry (Florkin and Stotz, 1964).

All aminotransferase reactions require the cofactor pyridoxal phosphate. This was first shown by Schlenk and Snell (1945) who reported low aminotransferase activity in pyridoxine deficient rats. Similar findings were reported by Green et al. (1945), Lichstein et al. (1945), Schlenk and Fisher (1945) and Tryfiates (1971).

The first evidence for the existence of tyrosine aminotransferase (TAT) (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) was provided by Cammarate and Cohen (1950). While observing the action of tissue extracts on a number of amino acids either manometrically by measuring the CO<sub>2</sub> evolved or chemically by estimating the glutamic acid formed, they noted that tyrosine enhanced the formation of glutamic acid from  $\alpha$ -ketoglutaric acid. Feldman and Gunsalus (1950) reported a similar finding with a bacterial extract. Hird and Rowsell (1950) studied the reverse reaction using a rat homogenate and demonstrated the synthesis of tyrosine from glutamic acid and p-hydroxyphenylpyruvate (pHPP).

Further evidence was provided by La Du and Greenberg (1951) who showed that when tyrosine was oxidized to fumaric

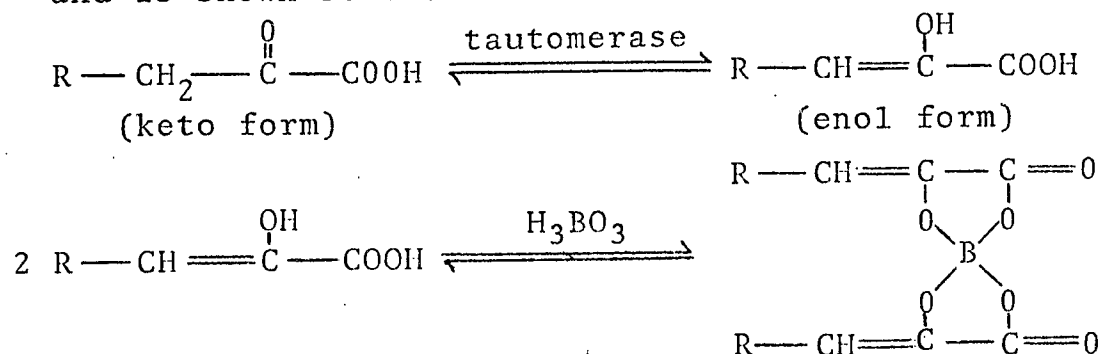
acid and acetoacetic acid (Fig. 1), no ammonia was produced as might have been expected were an amino acid oxidase present. In addition the number of moles of oxygen required to oxidize tyrosine to its two aforementioned products was approximately equal to what one might expect if an aminotransferase step was present.

Confirmation of the existence of the aminotransferase step was provided by Knox and Le May-Knox (1951) who followed the formation of pHPP spectrophotometrically at 320 nm under an atmosphere of nitrogen.

#### B. Assay of tyrosine aminotransferase.

Canellakis and Cohen (1956a) described an assay for TAT whereby the amount of pHPP formed was determined by a modified Briggs reaction (1922). Although the original assay was performed under nitrogen to prevent the disappearance of the product, Lin and Knox (1957) suggested that diethyl-dithiocarbamate be used instead since it inhibited the action of pHPP oxidase.

Another assay for TAT was reported by Lin and Knox (1957) in which the complex formed between the enol form of pHPP and boric acid was measured spectrophotometrically at 310 nm. This complex was described by Knox and Pitt (1957) and is shown below.



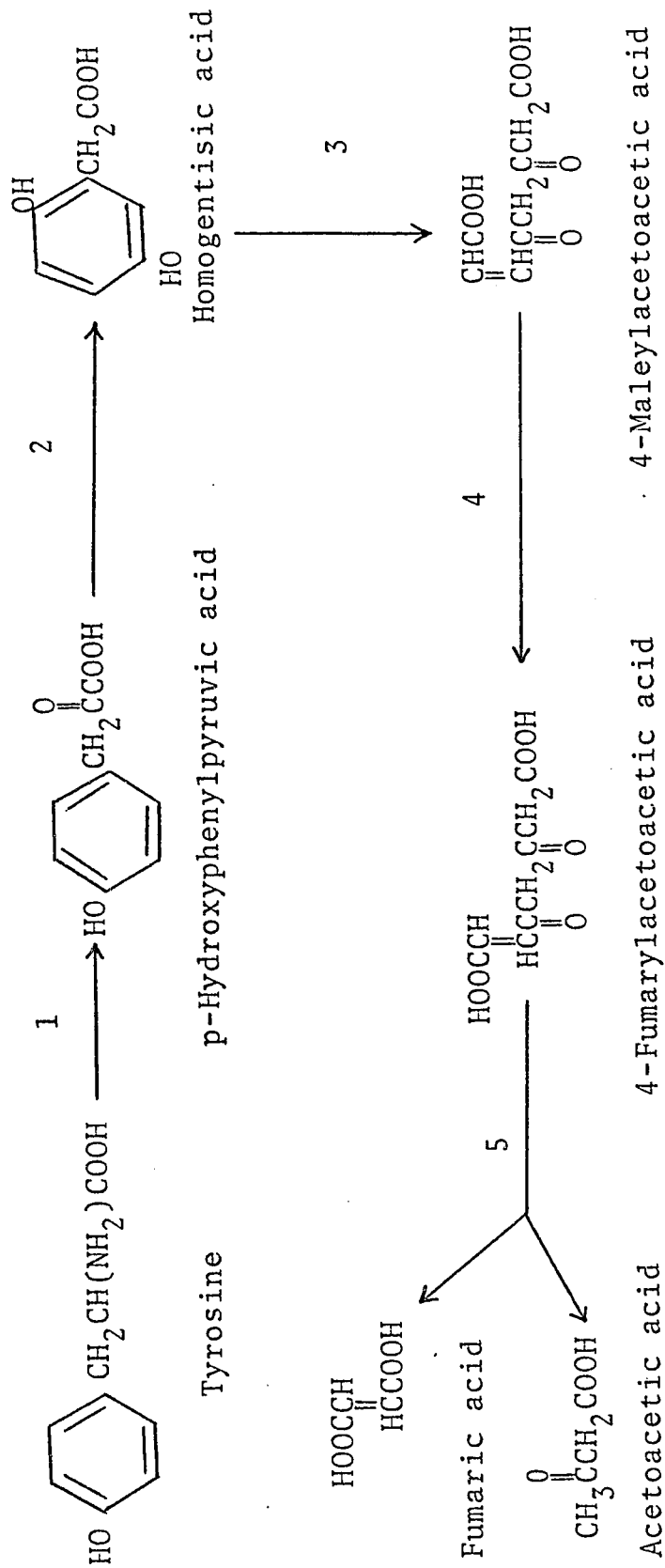


Fig. 1. Main metabolic pathway of tyrosine catabolism. Enzymes involved are: tyrosine aminotransferase (1), p-hydroxyphenylpyruvate hydroxylase (2), homogentisate oxygenase (3), maleylacetoacetate isomerase (4), fumarylacetoacetase (5).

The enzyme responsible for the rapid conversion of the keto form to the enol form is phenylpyruvate tautomerase (phenylpyruvate keto-enol-isomerase, EC 5.3.2.1) which has been purified free of any aminotransferase activity and thus can be added directly to the enzyme assay system (Knox and Pitt, 1957).

Diamondstone (1966) has described a relatively simple method for the measurement of TAT which is probably the most widely used today. It involves stopping the enzyme reaction with strong alkali which also converts pHPP to p-hydroxybenzaldehyde. The latter can be measured at 331 nm.

Several other methods have been described (Gabay and George, 1967; Weinstein et al., 1967; Litwack and Squires, 1968; Wurtman and Larin, 1968; Fellman et al., 1969; Schepartz, 1969) but these have never gained as wide acceptance as the previously mentioned methods.

#### C. Substrate specificity of tyrosine aminotransferase.

Canellakis and Cohen (1956b) partially purified the enzyme from dog liver and studied its substrate specificity. They reported that  $\alpha$ -ketoglutaric acid and L-tyrosine were by far the best substrates tested. No transamination occurred with D-tyrosine. Their studies revealed the importance of a phenolic hydroxyl, an  $\alpha$ -NH<sub>2</sub>, and an  $\alpha$ -COOH group in the substrate.

Jacoby and La Du (1964) reported a broader specificity for the enzyme purified from rat liver. Phenylalanine, tryptophan and several 3-substituted tyrosine derivatives were found to be substrates, to a limited extent, for this enzyme.

D. Product inhibition of tyrosine aminotransferase.

Canellakis and Cohen (1956b) reported that when pHPP was used as a substrate for the dog liver enzyme in the reverse reaction, the rate was not nearly as high as the rate of the forward reaction. Kenney (1959) confirmed these findings for the rat liver enzyme.

Rosenberg and Litwack (1970) later studied the effect of pHPP on the enzyme system and reported that this product was a linear noncompetitive inhibitor with respect to tyrosine or pyridoxal phosphate but was a linear competitive inhibitor with respect to  $\alpha$ -ketoglutaric acid. This was in contrast to an earlier paper by Civen et al. (1970) who reported no inhibition by pHPP. The inhibition of TAT by pHPP was also studied in the last stages of the present work and has been recently reported (Lane and Mavrides, 1971).

E. Effect of cortisol on tyrosine aminotransferase.

The induction\* of rat liver TAT by cortisol

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\* In this thesis the terms "induction" and "repression" will be used in the sense proposed by Hager and Kenney (1968) as the selective stimulation and inhibition, respectively, of the rate of enzyme synthesis.

(hydrocortisone;  $11\beta,17\alpha,21$ -trihydroxy-4-pregnene-3,20-dione) was first reported by Lin and Knox (1957). They showed that the enzyme activity began to rise two hours following the cortisol administration and reached a peak after 5 hours to a level at least 6 times greater than the initial activity. The enzyme activity returned to the normal level 12 hours after the hormone injection. The effect of the hormone was greater in adrenalectomized than in intact rats.

However the administration of tyrosine, the amino acid substrate of the enzyme, could only stimulate TAT in intact rats (Lin and Knox, 1957). Kenney and Flora (1961) studied the effect of administering tyrosine or other amino acids intraperitoneally to intact rats and reported that the relative efficiency of the various amino acids to induce TAT was roughly proportional to the extent to which they remained insoluble at the concentration injected. This was confirmed by the intraperitoneal administration of celite, an insoluble inorganic compound, which was as effective as tyrosine in stimulating TAT. Hence the injection of the insoluble material appears to be a stress to the rats and mediates the release of corticosterone from the adrenals (Geller et al., 1969). Stresses, such as immobilization (Hanninen and Hartiala, 1967), shaking in reciprocal shakers (Schapiro et al., 1966), laparotomy (Mavrides and Lane, 1967; Tsukada et al., 1968; Geller et al., 1969), or partial hepatectomy (Tsukada et al., 1968), have also been shown to stimulate the enzyme.

F. Cortisol stimulation of tyrosine aminotransferase occurs only in liver.

Lin and Knox (1958) demonstrated that the activity of TAT in rat liver greatly exceeded the activity found in either kidney or heart, both on the "per organ" and on the "per gram of tissue" basis. In addition, no appreciable amount of the enzyme was found in brain, intestine, lung, muscle or spleen. This agreed with what Canellakis and Cohen (1956a) had reported for TAT in the dog. In addition, Lin and Knox (1958) demonstrated that while the liver enzyme could be stimulated by cortisol, the kidney enzyme activity remained unchanged.

That the cortisol-stimulation of hepatic TAT is the result of a direct action of the hormone on the liver cells has been convincingly shown by several works. Goldstein et al. (1962) reported a substantial stimulation of TAT following cortisol perfusion of the isolated rat liver. This was confirmed by Barnabei and Sereni (1964). The enzyme has also been stimulated in isolated liver cells (Huang and Ebner, 1969) and in hepatoma tissue culture (HTC) cells (Thompson et al., 1966; Reel et al., 1970).

The possibility that the stimulation of TAT was due to the activation of pre-existing enzyme precursors was excluded by the work of Kenney (1962b). Using an immunochemical technique, he demonstrated that the increase in TAT following cortisol administration was matched by an equivalent increase

in the incorporation of  $^{14}\text{C}$ -amino acids into TAT protein. This increase was due solely to an increase in its rate of synthesis (Kenney, 1962c).

G. Permissive role of cortisol in the regulation of tyrosine aminotransferase.

The oral administration of an amino acid mixture to adrenalectomized rats has been shown to stimulate hepatic TAT (Korner and Labrie, 1967; Labrie and Korner, 1968a). If cortisol is administered at the same time as a casein hydrolysate mixture synergistic effects on the enzyme stimulation have been observed (Rosen and Milholland, 1968). It has also been shown that non-stimulating doses of cortisol (20  $\mu\text{g}$ ) further stimulated the elevation by casein hydrolysate in adrenalectomized, anesthetized rats (Mavrides and Lane, 1970). Glucocorticoids are known to increase plasma amino acid levels (Smith and Long, 1967) presumably by increasing the rate of protein catabolism in peripheral tissues (Long, Katzin and Fry, 1940; Beck and McGarry, 1962). These results suggest that perhaps one of the roles of cortisol is a permissive one associated with accelerating the rate of entry of amino acids into the liver (Noall et al., 1957). Schayer (1967) proposed that glucocorticoids may act by closing sphincters in the liver resulting in the liver cells being overnourished. Indeed circulatory alterations of the liver have been shown to increase RNA and protein

synthesis in the overperfused liver segment (Lieberman and Short, 1965). Thus cortisol may play a permissive role in the regulation of hepatic TAT.

#### H. Transcriptional control of tyrosine aminotransferase.

The fact that cortisol may have a direct gene action and enhance the synthesis of messenger RNA was investigated by several workers. Feigelson et al. (1962) were the first to report an increased incorporation of precursors in RNA following cortisol administration. This was supported by the work of Kenney and Kull (1963) and Kidson and Kirby (1964). RNA polymerase activity has also been shown to be increased at this time (Barnabei and Sereni, 1964; Lukacs and Sekeris, 1967; Schmid et al., 1967). Thus the induction of hepatic TAT might follow the general scheme of DNA  $\longrightarrow$  RNA  $\longrightarrow$  protein as proposed by Jacob and Monod (1961).

Yu and Feigelson (1969) reported that the first effect of cortisol was to stimulate the early synthesis in messenger RNA. Using  $^3\text{H}$ -uridine and  $^{14}\text{C}$ -guanine, they demonstrated that more than 59% of the  $^3\text{H}$ -uridine was incorporated into hepatic RNA within the first 30 minutes. The rate of incorporation of  $^{14}\text{C}$ -guanine into RNA was significantly inhibited during this period and only reached its peak incorporation between 3 to 7 hours after the start of the experiment. Darnell (1967) and Willems et al. (1968) had previously indicated that messenger RNA in higher

organisms was characterized by a high adenylic and uridylic acid content in contrast to ribosomal and transfer RNA which manifest a high guanylic and cytidylic acid content. The studies of Yu and Feigelson (1969) therefore were interpreted to suggest that during the first hour of hormone action, which preceeded the TAT induction, messenger RNA was selectively synthesized, followed later by the hormonal stimulation of ribosomal and transfer RNA synthesis.

Although an increase in the messenger RNA specific for TAT has never been demonstrated the conclusion that the hormonal enzyme induction might be preceeded by enhanced transcriptional activity is compatible with other studies in which the administration of actinomycin D prior to hormonal treatment prevented the stimulation of TAT (Greengard and Acs, 1962; Greengard et al., 1963; Barnabei and Sereni, 1964; Grossman and Mavrides, 1967; Hager and Kenney, 1968), whereas administration of this antibiotic one or two hours after the hormone did not prevent it (Garren et al., 1964b; Csányi et al., 1967; Grossman and Mavrides, 1967; Csányi and Greengard, 1968).

#### I. Translational control of tyrosine aminotransferase.

Garren et al. (1964b) postulated the existence of a cytoplasmic repressor capable of selectively inhibiting the enzyme synthesis at the translational level. They based their suggestion on the finding that inhibitors of RNA

synthesis, such as actinomycin D and 5-fluorouracil, could further stimulate the steroid-induced increase in the enzyme synthesis provided the inhibitors were injected at a carefully selected time after the induction was initiated by cortisol. The fact that this effect could only be observed with inhibitors of the transcriptional events of protein synthesis suggested that the repressor was probably another protein whose RNA template was about to be transcribed.

Tomkins et al. (1965) speculated from this that the messenger RNA for TAT was stable and the messenger RNA for the repressor as well as the repressor itself must turn over rapidly. They suggested that the stimulation of TAT synthesis by inhibitors of RNA synthesis resulted from inhibition of repressor messenger RNA formation. The translation of the stable TAT messenger would consequently be derepressed.

Kenney and Albritton (1965) reported data indicating that the decay of the enzyme activity after injection of tyrosine or celite to adrenalectomized rats followed first order kinetics with a half-life of approximately 2.5 hours, very near the estimated half-life of hepatic TAT (Lin and Knox, 1958; Kenney, 1962c; Berlin and Schimke, 1965; Grossman and Mavrides, 1967; Kenney, 1967; Boctor and Grossman, 1970; Levitan and Webb, 1970). This indicated that the synthesis of the enzyme was completely prevented by the repressor and was confirmed with immunochemical

studies which showed that practically no incorporation of  $^{14}\text{C}$ -leucine into TAT could be detected following the administration of a stressing agent, an effect which could be prevented with actinomycin D. Then the reaction to stress probably involved a transcriptional process leading to the formation of an active repressor.

Hypophysectomized rats, when stressed in the same manner as adrenalectomized rats, were able to maintain their normal hepatic levels of TAT and were thus seemingly incapable of forming the repressor (Kenney and Albritton, 1965). This was supported by the work of Kenney (1967a) who parabiotically joined an adrenalectomized hypophysectomized rat to an adrenalectomized rat. Only when tyrosine was administered (i.p.) to the rat with the intact pituitary could a repression in TAT activity be demonstrated in both rats. Growth hormone was shown to be the only pituitary hormone capable of causing a significant depression in this enzyme and this depression could be prevented with actinomycin D. Thus growth hormone must stimulate the synthesis of the repressor.

In support of this, Schapiro (1968) has shown that during times of stress the growth hormone content of the pituitary decreases. In addition, several workers (Grossman and Mavrides, 1967; Csányi and Greengard, 1968) have reported that the induction of hepatic TAT by cortisol is greater in hypophysectomized than in adrenalectomized rats. Others

have shown that pretreatment with growth hormone significantly reduces the cortisol stimulation in hypophysectomized and adrenalectomized rats (Holten and Kenney, 1967; Csányi and Greengard, 1968; Labrie and Korner, 1968b).

However, several workers have been unable to confirm the reported repression of the basal enzyme activity, either following growth hormone treatment of hypophysectomized rats (Labrie and Korner, 1968b; Schapiro, 1968) or following tyrosine administration to adrenalectomized rats (Grossman and Mavrides, 1967).

This discrepancy of results reported by the various workers suggests that perhaps the repression of hepatic TAT synthesis by growth hormone might be mediated by some unknown extra-hepatic factor. This would help explain the results of Hager and Kenney (1968) who have been unable to demonstrate a repression of TAT by growth hormone in the isolated perfused rat liver. Similarly, no effect of growth hormone on TAT could be observed in organ cultures of fetal liver (Wicks, 1968a).

J. Effect of turnover on the regulation of tyrosine aminotransferase.

Grossman and Mavrides (1967) reported that the inactivation phase in the induction cycle of TAT by cortisol might be due to the formation of an inactivator. The enzyme activity in incubated slices, biopsied from anesthetized

rats at various times during the cycle, was stable in those obtained during the early part of the cycle but disappeared rapidly in those obtained during the latter half of the cycle. They concluded that enzyme synthesis did not continue in the liver slices and that the decline in the enzyme activity in the slices taken during the latter half of the cycle might be explained by the presence of an inactivator formed approximately 4 hours after the administration of cortisol. This was further supported by Boctor and Grossman (1970) who showed that although the half-life of stimulated TAT is short (1.7 hours in their system), the half-life under basal conditions is longer (6.1 hours). Thus the turnover of the enzyme appears to be accelerated a few hours after hormonal stimulation and this increased turnover might be due to the formation of an inactivator. They suggested that the inactivator would not be formed as long as cortisol was present. This is supported by work in tissue culture where the enzyme activity remained high as long as the steroid was present (Kenney, 1965; Thompson et al., 1966).

K. Effect of pancreatic hormones on tyrosine aminotransferase.

Starvation has been reported to increase the enzyme activity in both intact (Rosen et al., 1963; Yuwiler et al., 1969) and adrenalectomized (Goswami and Chatagner, 1966) rats. However, Greengard et al. (1966) reported that the

activity was unchanged in the starved adrenalectomized rat although starvation did enhance the cortisol stimulation of the enzyme. Glucagon has been suggested to be responsible for this enhancement and indeed several workers (Greengard and Baker, 1966; Csányi et al., 1967) have demonstrated this. Glucagon alone has been shown to stimulate the activity of hepatic TAT (Greengard and Baker, 1966; Civen et al., 1967a; Csányi et al., 1967) but the rise and fall in the enzyme activity was much quicker than after treatment with cortisol.

Civen and co-workers (1967a) suggested that since the earliest detectable action of glucagon on the liver was a rapid increase in the level of cyclic AMP (Sutherland and Rall, 1960; Butcher et al., 1971) it was possible that cyclic AMP was actually the primary stimulator of TAT following glucagon administration. The report of Tryfiates and Litwack (1964) that TAT in the 15,000 x g supernatant could be activated about 59% by cyclic AMP in vitro supports this possibility.

The rapid decline in the enzyme activity following the glucagon stimulation might be the result of insulin secretion by the pancreas (Civen et al., 1967a). These workers showed a striking parallelism between the rise and fall of TAT and the blood glucose level. They concluded that since insulin reduced the level of cyclic AMP in vitro both in liver and in adipose tissue (Exton et al., 1966),

the secretion of insulin in response to the hyperglycemia may be responsible for the decline of TAT brought about by a decreasing level of cyclic AMP in the liver.

Civen et al. (1965) had earlier reported that large doses of glucose, administered in the diet or injected intravenously, repressed TAT 50 to 60% below control levels. Pitot and Peraino (1963) and Peraino and Pitot (1964) have extensively studied the carbohydrate repression of the rat liver amino acid-metabolizing enzymes, threonine dehydrase and ornithine aminotransferase, and found that in both cases oral glucose administration strongly inhibited the stimulation of these enzymes by casein hydrolysate. These effects of glucose could be due to a direct effect of glucose or its metabolites, or more indirectly brought about by insulin, the release of which would have been triggered by the large increase in circulating glucose. Thus physiologically elevated insulin levels could have been responsible for the lowering of TAT.

However, Holten and Kenney (1967) reported that insulin administration to adrenalectomized rats also stimulates TAT. The stimulation by either insulin or glucagon was found to be additive with the cortisol stimulation but not additive with each other suggesting that the protein hormones acted in a way different from cortisol. However, like cortisol, insulin and glucagon have been shown to act by increasing the synthesis of the enzyme (Holten and

Kenney, 1967). This was confirmed using the isolated perfused rat liver indicating that both hormones acted directly on the liver cell (Hager and Kenney, 1968).

Labrie and Korner (1969) confirmed the stimulation of TAT by insulin and glucagon in hypophysectomized rats but failed to find an additive effect with cortisol. This discrepancy might, however, be due either to the different hormonal status of the animals or to the fact that the animals were killed at a different time following the hormone injections.

Brown and Civen (1969) suggested that perhaps the stimulatory effect of insulin on the enzyme reported by others might be due to the presence of a contaminant in the insulin preparations used. Indeed, Berson et al. (1965) have reported that crystalline bovine insulin actually shows several protein bands other than insulin after polyacrylamide disc-gel electrophoresis. Following the injection of an anti-insulin preparation to rats, Brown and Civen (1969) noted a rise in the enzyme and suggested that at physiological levels, glucagon appeared to be responsible for the elevation of TAT whereas insulin mediated its lowering. However, further work will have to be conducted to sort out this dilemma since insulin, and not glucagon, has been reported to induce TAT in HTC cells (Gelehrter and Tomkins, 1970; Lee et al., 1970; Reel et al., 1970).

L. Effect of cyclic AMP on tyrosine aminotransferase.

Both glucagon and catecholamines have been shown to increase adenyl cyclase activity (Makman and Sutherland, 1964; Exton and Park, 1966) resulting in increased cyclic AMP synthesis. Since both glucagon and catecholamines have also been shown to increase TAT synthesis (Wicks, 1968a), the question arose whether cyclic AMP might be an intracellular mediator of the action of these hormones on TAT synthesis.

Wicks (1968b; 1969) demonstrated that cyclic AMP could stimulate TAT in fetal rat liver maintained in organ culture. Similar findings were reported for the enzyme in adult rats (Wicks et al., 1969). Both actinomycin D and cycloheximide prevented the stimulation and Wicks (1969) has shown, using an immunochemical isotopic technique, that the effect of cyclic AMP on the enzyme was due to an increase in the synthesis of the enzyme. Theophylline was also an effective stimulator of the enzyme indicating that endogenous cyclic AMP was as effective as exogenous cyclic AMP. Theophylline has been shown to increase cyclic AMP in the liver cell by inhibiting phosphodiesterase, the enzyme responsible for its degradation (Sutherland et al., 1965).

Cyclic AMP had also been shown to increase PEPCK (GTP: oxaloacetate carboxy-lyase, EC 4.1.1.32) in the perfused rat liver (Exton and Park, 1966; Exton et al., 1966).

The effect of cyclic AMP, as well as of the adrenal and pancreatic hormones, on this enzyme were investigated in rat fetal liver explants in organ culture (Wicks, 1969) and in adult rat livers (Wicks et al., 1969). Although cyclic AMP and glucagon stimulated PEPCK, insulin and cortisol did not.

This contrasted with the results of other workers (Shrago et al., 1963; Foster et al., 1966; Shrago and Lardy, 1966; Ballard and Hanson, 1969; Usatenko, 1970) who have shown that cortisol does indeed stimulate PEPCK. However, the increase reported for PEPCK is, at best, 2 to 3 fold for normally fed rats and much less for fasted rats (Ray et al., 1964). Fasted intact or adrenalectomized rats have been reported to have an elevated level of PEPCK (Shrago et al., 1963; Young et al., 1964). The adrenalectomized rats used by Wicks et al. (1969) were fasted overnight which would result in the rats having a higher initial activity. Although they reported no elevation for PEPCK following cortisol treatment, their data indicates a slight elevation in the enzyme (151% of control) after cortisol administration, which may very well represent a significant increase. Indeed Barnett and Wicks (1971) have now reported that glucocorticoids stimulate PEPCK activity in Reuber H-35 cells. Thus most of the agents which stimulate TAT also stimulate PEPCK.

M. Stimulation of tyrosine aminotransferase by tryptophan.

Kenney and Flora (1961) reported that L-tryptophan increased hepatic TAT activity in intact rats, but not in adrenalectomized rats. In subsequent studies, Rosen and Milholland (1963) demonstrated that L-tryptophan, 5-hydroxytryptophan and serotonin could increase TAT activity in both intact and adrenalectomized rats. Similar findings were reported by Labrie and Korner (1968a) who demonstrated that the effect of tryptophan could be blocked with actinomycin D. Wurtman et al. (1968a) suggested that the diurnal rhythm in hepatic TAT might result from the cyclic changes in hepatic tryptophan content. Deguchi and Barchas (1971) reported that the indole amino acids must be decarboxylated to the corresponding amines to stimulate hepatic TAT. How the amines stimulate TAT is not known.

N. Possible mechanism for the regulation of tyrosine aminotransferase.

The stimulation of TAT by cortisol appears to be mechanistically quite different from that by glucagon or insulin as evidenced by the fact that the effect of the pancreatic hormones on TAT are additive with the cortisol effect. Kenney et al. (1968) suggested a possible mechanism for the regulation of TAT whereby cortisol would control the production of messenger RNA while insulin and glucagon

would control some post-transcriptional event (Fig. 2). Since cortisol administration increased the synthesis of RNA as well as TAT (Kenney and Kull, 1963), and the continued administration of the hormone was necessary to maintain the elevated enzyme level (Grossman and Mavrides, 1967), Kenney et al. (1968) proposed that the steroid stimulated the enzyme by promoting the selective transcription of the TAT messenger RNA and through mass action some of this messenger RNA passed into the cytoplasm where it became an active polysome for the translation of TAT. Kenney et al. (1968) proposed that the pancreatic hormones would stimulate either the formation of an active TAT messenger RNA from an inactive cytoplasmic messenger RNA or the transmission of the messenger RNA pool into the cytoplasm. The latter possibility was favored since much of the nuclear DNA-like RNA underwent turnover within the nucleus and never reached the cytoplasm (Georgiev, 1967). Thus these hormones would stimulate TAT by making available a component which was present in limited supply and which survived only a short while once it was activated. The fact that the stimulation of TAT by pancreatic hormones is additive with the cortisol stimulation supports this premise (Holten and Kenney, 1967). In addition insulin and glucagon elevate TAT for only 2 to 3 hours before the enzyme returns to the basal level regardless of whether the hormones are given as a single injection or repeatedly. However although

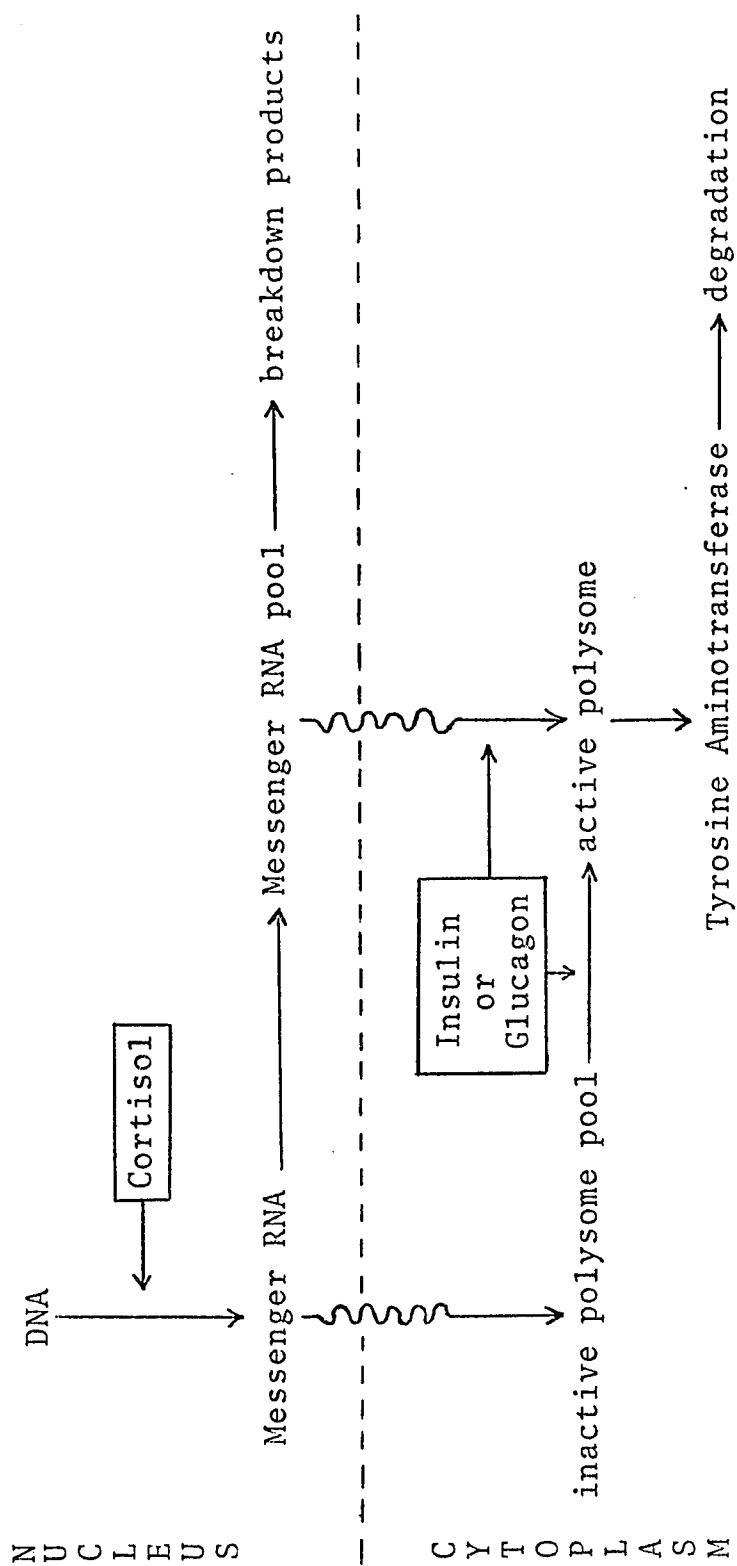


Fig. 2. Possible mechanism for the regulation of TAT in rat liver as adapted from Kenney et al. (1968).

insulin and glucagon appear to act by the same mechanism, some evidence has been reported to oppose this. The effect of glucagon has been proposed to be mediated by cyclic AMP (Wicks, 1968b; Wicks et al., 1969), whereas insulin has either no effect or possibly lowers the hepatic level of this compound (Exton and Park, 1967). In addition, only insulin has been shown to stimulate TAT in HTC cells (Gelehrter and Tomkins, 1970; Lee et al., 1970; Reel et al., 1970) casting some doubt as to whether glucagon is an "inducer".

O. Tyrosine aminotransferase studies in hepatoma tissue culture cells.

Several workers (Thompson et al., 1966; Reel and Kenney, 1968; Mendelson et al., 1969; Boctor and Grossman, 1970) have now extended their investigations on the regulation of TAT to cells derived from a multicellular differentiated organism. Since it was virtually impossible to propagate normal liver cells in culture, Pitot et al. (1964) adapted a malignant tumor of rat liver parenchymal cells to grow in cell culture. These originated from a minimal deviation tumor arising from aminoacetylfluorence treatment in studies by Reuber (1961). Several cell lines exist, some of which are HTC, Reuber H-35 and Novikoff cell lines.

Although several disadvantages exist when using these malignant cells for study, such as the increase in chromosome

number and the malignancy itself, the advantages of working with such a system are obvious. The generation time of about 24 hours is also quite convenient.

1) Stimulation of tyrosine aminotransferase.

Thompson et al (1966) reported that following the addition of dexamethasone ( $9\alpha$ -fluoro- $11\beta$ , $17\alpha$ , $21$ -trihydroxy- $16\alpha$ -methyl- $1,4$ -pregnadiene- $3,20$ -dione) to the hepatoma cells in tissue culture, there was a 2 hour lag, just as observed in rat liver. The activity of the enzyme then rose for the next 5 to 8 hours to a plateau level which was about 10 times the baseline level. They noted that this level could be maintained as long as the steroid was present in the medium. If, however, the maximally stimulated cells were gently washed with the same medium but without the steroid, and allowed to incubate further, the enzyme activity returned in a few hours to its baseline level. This has also been observed by Kenney (1965) in Reuber H-35 cells.

Granner et al. (1968b) reported that the increased TAT activity was due to an increased rate of synthesis and not a decreased rate of degradation. They also reported that the elevation could not be due to an activation of precursor TAT since their immunological studies revealed that the stimulated and basal enzymes had the same ratio of catalytic to antigenic activity. This was later confirmed by Granner et al. (1970).

2) Characteristics of tyrosine aminotransferase from tissue culture cells.

Kenney (1962a) had reported that  $\alpha$ -ketoglutarate protected TAT from brief exposures of 55 to 60°C. Thompson et al. (1966) reported similar findings with TAT from HTC cells. They also reported strong similarities between the basal and stimulated enzymes from the cultured cells. Both enzyme preparations have identical temperature stability curves, both in the absence and in the presence of pyridoxal phosphate and  $\alpha$ -ketoglutarate. The kinetics of the heat denaturation at 76°C were also identical. Finally when the stimulated and basal enzymes were purified by heating at 60°C for one hour in the presence of pyridoxal phosphate and  $\alpha$ -ketoglutarate and separated by electrophoresis they were found to have identical migration rates.

Perhaps the strongest evidence that the enzymes were the same, whether from rat liver or hepatoma culture cells, stimulated or basal, was reported by Granner et al. (1968b) who, using rabbit anti-transaminase serum, demonstrated the immunological identity of the enzymes.

3) Synchronized hepatoma tissue culture cells.

Martin et al. (1969b) succeeded in synchronizing HTC cells with colcimid, a drug which blocked the mitotic division of metaphase. They then studied the effects of dexamethasone addition during the generation cycle following

the removal of colcimid. The cycle included a mitotic, G1, S and G2 phase with the entire cycle lasting approximately 24 hours. They reported that TAT could be only induced during the latter 2/3 of the G1 phase and the S phase, and concluded that the transcription of the entire genome was probably repressed during the G2, mitotic and early G1 phase.

#### 4) Inhibition of tyrosine aminotransferase induction.

Similar to what had been reported by Greengard et al. (1963) for TAT in rat liver, the induction in HTC cells was inhibited by actinomycin D or mitomycin C (Thompson et al., 1966). Peterkofsky and Tomkins (1967) reported that this inhibition was directly related to the inhibition of RNA synthesis as measured by <sup>3</sup>H-uridine incorporation. In addition, the removal of actinomycin D from the cells by the addition of DNA to cell cultures reversed the inhibition of both RNA synthesis and the enzyme stimulation to a similar extent. They also reported that actinomycin D derivatives, such as antinocin, which do not inhibit RNA synthesis, failed to prevent the stimulation. They concluded on the basis of these inhibitor studies that the synthesis and accumulation of enzyme-specific messenger RNA was required for the stimulation of TAT, although Gelehrter and Tomkins (1967) had earlier reported that no gross changes in the RNA synthesis occurred after the addition of dexamethasone to the HTC cells.

Peterkofsky and Tomkins (1968) later reported that the stimulation of TAT occurred in the absence of dexamethasone when the cells were preincubated for 1.5 hours with dexamethasone and cycloheximide and then washed free of these agents. When actinomycin D was included in the preincubation mixture with dexamethasone and cycloheximide, the TAT elevation after the wash was prevented. However if the cells were first preincubated with dexamethasone and cycloheximide, washed, and actinomycin D then added to the culture medium, the synthesis of the enzyme was not inhibited indicating that further transcription was not required to increase TAT. They concluded that one of the actions of the steroid was the stimulation of messenger RNA accumulation.

In addition, Tomkins and collaborators (1966) had earlier reported that the addition of actinomycin D to pre-stimulated HTC cells, which had been washed free of the dexamethasone, resulted in a more rapid synthesis of the enzyme, than in control cells without actinomycin D, as measured by  $^{14}\text{C}$ -amino acid incorporation studies. They proposed that the rate of translation of the TAT messenger was probably limited by a labile substance similar to what they had previously referred to as a "cytoplasmic repressor" (Garren et al., 1964b; Tomkins et al., 1965).

##### 5) Model for the regulation of tyrosine aminotransferase.

To explain these and other findings which will be discussed later, Tomkins (1968) proposed a model which was

later revised (Tomkins et al., 1969) in the light of more recent data. The scheme (Fig. 3) included both a structural and a regulatory gene which were responsible for the control of the synthesis of TAT. The structural gene was the template for the TAT specific messenger RNA while the regulatory gene was the template for the repressor. These authors assumed that the repressor was a protein and that both the repressor and its messenger RNA were very labile relative to TAT and its messenger. The function of the repressor is to reversibly inhibit the translation of the messenger into TAT. The messenger can only be degraded when its translation is inhibited by the repressor. Thus the repressor both inhibits the translation of the messenger and promotes its degradation. The role of the steroid was to prevent the action of the repressor.

#### 6) Application of the model.

The stimulation of TAT in HTC cells following the addition of dexamethasone (Tomkins et al., 1966; Granner et al., 1968b) could be explained on the basis that the steroid prevented the action of the repressor resulting in the accumulation of TAT messenger RNA and increased synthesis of TAT. The model would also explain why the constant presence of the steroid was necessary to maintain the enzyme at the elevated level. The removal of the steroid would allow the repressor to facilitate the degradation of the messenger

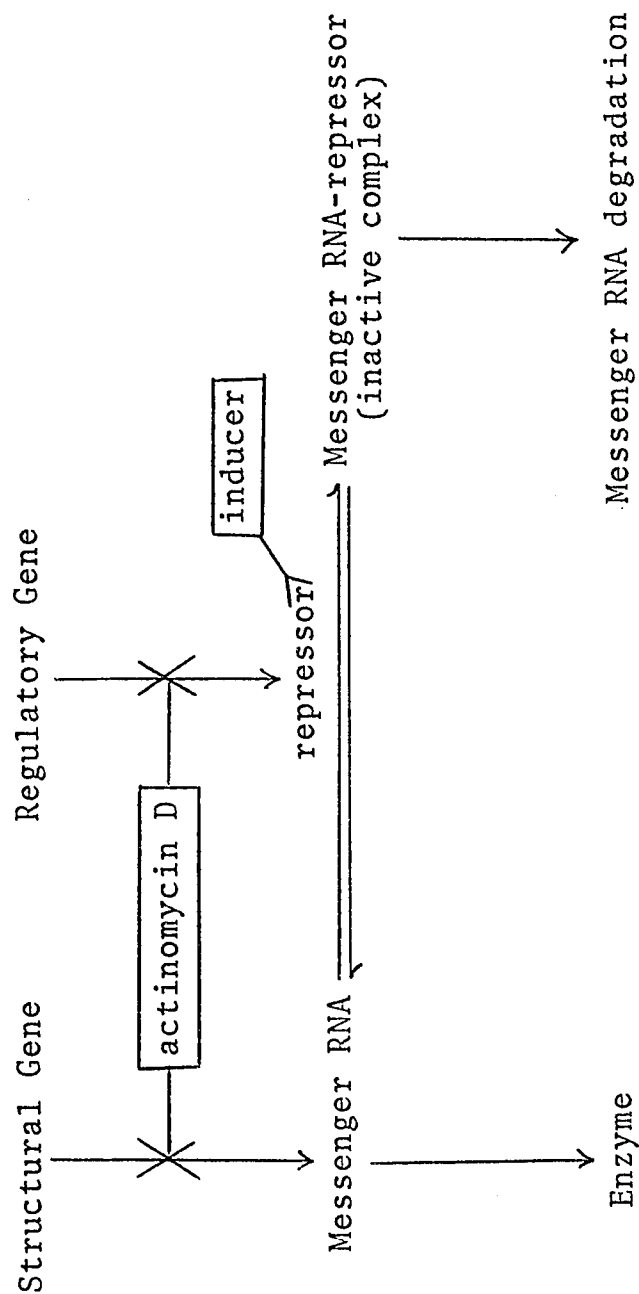


Fig. 3. Model scheme for the regulation of enzymes in mammalian cells as adapted from Tomkins et al. (1969). Symbols:  $\overline{\longrightarrow}$  refers to repressor inactivation,  $\overline{\longleftarrow}$  refers to pathway inhibition.

resulting in a decrease in the translation and an ultimate drop in the enzyme.

The experiment of Tomkins and co-workers (1966) described earlier in which actinomycin D added to cells preincubated for 2.5 hours with dexamethasone resulted in a more rapid synthesis of TAT can be explained by the scheme. The antibiotic prevented the formation of the repressor as well as the TAT messenger RNA allowing any messenger already formed to be translated at its maximum rate. Without the antibiotic the repressor would have inactivated the messenger and facilitated its degradation.

In another experiment described earlier, Tomkins et al. (1966) found that the addition of actinomycin D to stimulated cells, washed free of the steroid, prevented the fall in the enzyme. In fact the enzyme rose above the previously stimulated level. They referred to this effect by actinomycin D as "superinduction". They reasoned that actinomycin D prevented the transcription of both the structural gene and the regulatory gene thus inhibiting the formation of the labile repressor. Messenger RNA which had been inactivated by previously formed repressor, but not yet degraded, was reactivated as the repressor broke down. The net effect was a greater amount of enzyme being synthesized (Tomkins et al., 1969).

Similar paradoxical phenomena with actinomycin D have been reported in the literature and may be explained by

Tomkins' scheme if this is taken as a general model for regulation. Such effects have been reported for interferon synthesis in rabbit kidney cell cultures (Vilcěk et al., 1969), alkaline phosphatase in the intestinal mucosa (Moog, 1964), dCMP aminohydrolase in sea urchin eggs (Scarano et al., 1964), thymidine kinase of Hela cells (McAuslan, 1963), aryl hydroxylase of hamster fetus cells (Nebert and Gelboin, 1968), hepatic gulonate NADP oxidoreductase (Stubbs and Haufreer, 1968) and the total clearing-factor lipase activity of rat adipose tissue (Wing and Robinson, 1968).

Reel and Kenney (1968) however claimed that the real reason for the apparent "superinduction" after actinomycin D administration was an almost complete shutdown of the enzyme degradation while some TAT messenger was still being translated. However Tomkins' group (Auricchio et al., 1969; Martin et al., 1969a; Peterkofsky and Tomkins, 1967) claimed that the rate of enzyme degradation was not affected by the actinomycin D treatment. Similarly, Feldman and Yagil (1969) reported that cycloheximide did not inhibit the rate of degradation of general cell protein in cultured cells. These results indicated that neither actinomycin D nor cycloheximide inhibited the degradation of TAT. This was supported by the work of Jervell and Segler (1969) who showed that in the isolated perfused rat liver cycloheximide did not block the degradation of the stimulated enzyme,

and by Rosen and Milholland (1968) who reported that cycloheximide did not prevent the degradation of casein hydrolysate-stimulated TAT. In addition, Grossman and Mavrides (1967) reported that puromycin had no significant effect on the inactivation phase of the cortisol-stimulated TAT in adrenalectomized rats.

Auricchio et al. (1969) conducted nutritional studies with HTC cells in an effort to resolve this apparent discrepancy. They found that if induced cells were transferred to a medium containing no nutrient, such as buffered saline, then the enzyme was degraded faster than if they were transferred to the medium minus only the steroid. In addition, both actinomycin D and cycloheximide were effective in slowing this enhanced degradation of the enzyme. They proposed that there are two modes of TAT degradation, a slower, "normal", process not requiring concomitant RNA or protein synthesis and an "enhanced" degradation, evoked by step-down, which requires concurrent macromolecular synthesis.

Auricchio and collaborators (1969) concluded that since the animals in the experiments of Kenney (1967b) and Levitan and Webb (1969a,b) were fasted for considerable periods of time before being killed, a nutritional step-down was an intrinsic part of their experimental design. The same applies to the tissue culture study of Reel and Kenney (1968). Thus perhaps the inhibitor-sensitive TAT degradation observed by these workers might in fact have

been "enhanced" degradation evoked by a step-down or its equivalent.

Support for the existence of a gene responsible for the regulation of TAT has been reported by Schneider and Weiss (1971). Hybrid cells (between rat hepatoma cells and mouse fibroblasts) have low activity and can not be stimulated by steroids. Heat inactivation curves have demonstrated that the hybrid cells contain both parental forms of the enzyme, and therefore contain the parental structural genes responsible for transcription of the enzyme. The presence of the hybrids of detectable amounts of rat TAT and the absence of inducibility suggest that a second gene is involved in the regulation of TAT, and that this gene is not expressed in the hybrids.

#### 7) Effect of pancreatic hormones on tyrosine aminotransferase.

Gelehrter and Tomkins (1970) and Reel et al. (1970) have recently shown that the addition of small amounts of insulin to tissue culture cells resulted in the stimulation of TAT. Gelehrter and Tomkins (1970) found that in dexamethasone-stimulated HTC cells a 2- to 3-fold increase in the enzyme occurred 2 hours after insulin addition and then the enzyme fell back toward the stimulated plateau level. They also reported that insulin stimulated an almost immediate increase in the enzyme in basal cells of about the same magnitude and duration as in stimulated cells. Reel et al.

(1970) reported, on the other hand, that in basal Reuber H-35 hepatoma cells, a 5-fold increase in the enzyme activity occurred within 7 hours after addition of insulin. The activity of TAT then gradually declined. Both groups found that the peak level could be maintained by continually resupplementing the medium with insulin. The addition of the isolated A and B chains of insulin had no effect on the enzyme activity (Reel et al., 1970).

Gelehrter and Tomkins (1970) reported that although cycloheximide prevented the insulin stimulation, actinomycin D did not, suggesting that insulin acted by enhancing the translation of pre-existing TAT messenger RNA. They also reported that addition of cycloheximide alone or insulin and cycloheximide resulted in identical rates of enzyme degradation. This indicated that insulin does not stimulate TAT by decreasing its rate of degradation but rather by increasing its rate of synthesis. This was supported by the findings of Reel et al. (1970) and Lee et al. (1970).

Thus, contrary to what has been reported for the insulin stimulation in the perfused liver (Hager and Kenney, 1968) or in the intact liver in vivo (Holten and Kenney, 1967), the stimulation in tissue culture cells is not inhibited by actinomycin D. The insulin stimulation did not appear to be secondary to an uptake of glucose or amino acids since Gelehrter and Tomkins (1970) found that the stimulation

was not proportional to the concentration of either glucose or amino acids in the medium. In addition, Dickson and Potter (1968) reported that insulin did not affect the amino acid uptake by either Novikoff or Reuber hepatoma cells in tissue culture.

Kenney et al. (1969) reported that glucagon also stimulated TAT but when insulin-free glucagon was used no effect on the enzyme activity was observed (Reel et al., 1970). The failure of glucagon to induce TAT in cells in tissue culture agreed well with the findings of Granner et al. (1968a) who reported that neither adenyl cyclase nor cyclic AMP were present in HTC cells. However, this also meant that the insulin effect in the tissue culture cells did not involve the adenyl cyclase-cyclic AMP system. These workers (Gelehrter and Tomkins, 1970; Lee et al., 1970; Reel et al., 1970) concluded that insulin acted at some post-transcriptional or translational step in the synthesis of TAT and Gelehrter and Tomkins (1970) went on to suggest that insulin might in some way overcome the action of the proposed repressor.

P. Development of tyrosine aminotransferase in the neonatal rat.

Before the existence of the diurnal rhythm for hepatic TAT became known, Sereni and collaborators (1959) studied the development of the liver enzyme from fetal to adult rat. They reported that the enzyme in the fetal rat

liver was only 10% to 20% of that found in the adult liver. The enzyme rose sharply two hours after birth and reached a peak after 12 hours at a level double the adult level. The enzyme then fell over the next 36 hours to adult levels where it remained. Similar findings were reported by Auerbach and Waisman (1959) and confirmed by Franz and Knox (1967) and Msuya and Schepartz (1969). Kretchmer et al. (1956) had earlier reported an almost complete absence of the tyrosine oxidizing system in the livers of premature infants while the activity was present in the livers of the newborn infants and notably greater in the livers of adults.

Although Sereni et al. (1959) reported that there was no appreciable difference between newborn animals fasted for 12 hours and fed rats, Reynolds and Potter (1971) claimed that the observed rise in newborn rats was actually artifactual in nature, presumably resulting from stresses associated with lack of food when the newborn rats were taken from their mother. They reported that if newborn rats were returned to a mother, not necessarily their own, within 2 hours after birth, no rise occurred in their enzyme. They concluded that the rise in the enzyme was the result of a release of glucagon brought about by hypoglycemia (Unger et al., 1962), as glucagon has been observed to stimulate TAT in both fetal and neonatal rats (Greengard and Dewey, 1967). In the case of newborn rats returned to a nursing mother

shortly after birth, the milk consumed may have provided a supply of carbohydrate sufficient to counteract the hypoglycemia. Indeed Greengard and Dewey (1967) and Holt and Oliver (1968) have shown that following the injection of glucose to neonatal rats, the postpartum rise in TAT was prevented.

Holt and Oliver (1968) also reported that galactose, fructose and mannose were equally as effective in preventing the rise in TAT although these sugars had previously been shown not to prevent the drop in blood glucose which occurred in newborn rats 2 hours after birth (Yeung and Oliver, 1968). Although these findings seemed to contradict the idea that the rise in TAT was due to hypoglycemia, Yeung and Oliver (1968) also reported similar findings for PEPCK which, like TAT, was practically absent in fetal liver and developed rapidly immediately after birth (Ballard and Hanson, 1967; Yeung et al., 1967). This was confirmed by Philippides and Ballard (1969) who demonstrated that following the injection of substrate amounts of lactate into newborn rats, an increase in the concentration of PEP occurred in their livers. In addition a large increase in TAT (Holt and Oliver, 1968) and PEPCK (Yeung and Oliver, 1967) occurs in fetuses delivered prematurely by uterine section.

The role of the adrenal gland in the development of TAT was investigated by Sereni et al. (1959) who first administered amphenone intraperitoneally and observed a

decrease in the enzyme below controls during the first 4 hours after birth. However, by the sixth hour the enzyme had caught up with their controls. Amphenone is known to cause adrenal insufficiency for only a limited amount of time (Tullner et al., 1956).

Sereni and co-workers (1959) then studied the role of the adrenal gland more closely by adrenalectomizing newborn rats immediately after birth. Eight and twelve hours after the operation (i.e., after birth) the enzyme remained depressed to about 25% of that found in the control and sham-operated litter mates. In addition the administration of cortisol to the adrenalectomized newborn rats was quite effective in preventing the decrease in the enzyme resulting from the removal of the adrenal glands. These observations indicated that the release of the adrenal steroid after birth was responsible for the sharp increase in TAT which occurred at this time.

However, Sereni et al. (1959) were unable to stimulate fetal TAT by parenteral administration of either cortisol, adrenocorticotrophic hormone or tyrosine. This was confirmed by Greengard and Dewey (1967) although Yeung et al. (1967) reported a stimulation in fetal TAT following the administration of triamcinolone ( $9\alpha$ -fluoro- $11\beta$ , $16\alpha$ , $17\alpha$ , $21$ -tetrahydroxy- $1,4$ -pregnandiene- $3,20$ -dione  $16\alpha$ , $21$ -diacetate). Glucagon is also effective in stimulating the enzyme in fetal liver

(Greengard and Dewey, 1967; Holt and Oliver, 1968; Wicks, 1968a).

These findings suggest that shortly after birth when the plasma corticosterone concentration rises, the elevation of TAT may be due to the synergistic action of the steroid hormone and glucagon (Holt and Oliver, 1968).

Q. Diurnal rhythm of tyrosine aminotransferase.

1) Development of the enzyme rhythm.

Honova et al. (1968) reported that a recognizable periodicity in TAT, with a maximum at 1000 hours and a minimum at 2200 hours (the only 2 points measured), occurred within 48 hours after birth. They also presented evidence that sometime between the 2nd and 21st day after birth the cycle reversed itself, i.e., the peak now occurred at 2200 hours. They concluded that this may simply be a consequence of the change from the diffuse feeding patterns of the newborn (Gustaffson, 1948) to the more regular one of the adult (Seigel and Stackey, 1947; Zigmond et al., 1969).

2) Factors responsible for the generation of the rhythm.

A diurnal rhythm is a recurring phenomenon which takes place at roughly 24-hour intervals (Harker, 1958). Such a phenomenon has been shown to exist for TAT in rat liver. Potter et al. (1966) were the first to demonstrate the existence of the diurnal rhythm for this enzyme. Since

the rat is a nocturnally active animal (Richter, 1965), Potter et al. (1966) provided their rats with food only during the dark hours (0600 to 1800 hours). The protein content of the diet ranged from 0 to 90% for several groups of rats. The enzyme peaked at 2400 hours in all animals and the amplitude of the rhythm varied directly with the protein content in the diet. They concluded that TAT might be repressed at low levels of protein by the glucose content in the diet. This effect was similar to what has been reported by Pitot and Peraino (1963) and Peraino and Pitot (1964) with threonine dehydrase and ornithine aminotransferase. The existence of the rhythm for TAT was firmly established by Wurtman and Axelrod (1967), Civen et al. (1967b) and Shambaugh et al. (1967).

A rhythm for plasma corticosterone had been shown to exist in rats (Guillemin et al., 1959b; McCarthy et al., 1960; Civen et al., 1967b; Wurtman and Axelrod, 1967) with a peak occurring between 1600 hours and 1800 hours, approximately 4 hours before the peak in TAT. In addition corticosterone, as well as other glucocorticoids, have been shown to be excellent inducers of TAT (Lin and Knox, 1958). For this reason several workers proceeded to investigate whether the adrenal gland was essential for the generation of the rhythm. The reports of these workers were conflicting, Civen et al. (1967b), Wurtman and Axelrod (1967) and Black and Axelrod (1968b) claiming that adrenalectomy or

hypophysectomy had no effect on the enzyme rhythm whereas the results of Shambaugh et al. (1967) indicated that adrenalectomy or hypophysectomy significantly decreased the amplitude of the rhythm.

Several explanations have been posed to explain the continuation of the enzyme rhythm following adrenalectomy. Black and Axelrod (1968a) examined the possibility that neural mechanisms might be responsible for the generation of the enzyme rhythm. They reported that the depletion of norepinephrine stores by reserpine, a depletor of biogenic amines (Glowinski and Axelrod, 1966), caused a 5-fold rise in TAT. They also demonstrated a similar elevation in the enzyme following the administration of  $\alpha$ -methyl-p-tyrosine, an inhibitor of tyrosine hydroxylase.  $\alpha$ -Methyl-p-tyrosine also reduced the brain norepinephrine content while the administration of DOPA, the precursor of norepinephrine, partly restored its concentration following the depletion. DOPA administration also returned the enzyme to its basal level. Thus low brain norepinephrine levels seem to correlate with high TAT activities.

Axelrod and Black (1968) later reported that the normal diurnal rhythm in TAT could be abolished by the administration of  $\beta$ -phenylisopropylhydrazine, a monoamine oxidase inhibitor, which also resulted in the doubling in the brain norepinephrine content. In addition the administration of DOPA resulted in a 15% increase in the

norepinephrine content of the brain as well as a decrease in the amplitude of the TAT diurnal rhythm.

Thus diurnal changes in brain stem norepinephrine concentrations (Walker, 1967) were thought to be involved in the diurnal rhythm of TAT. Norepinephrine may alter TAT by direct interaction with the enzyme systems or may operate indirectly through a number of mediators. Epinephrine has been shown to inhibit TAT in vitro (Canellakis and Cohen, 1956b) and recently Black and Axelrod (1969) have demonstrated a similar effect with norepinephrine and related catecholamines. The enzyme inhibition correlates well with the ability of norepinephrine to form a complex with pyridoxal phosphate and suggests that TAT and norepinephrine may compete for the cofactor.

Wurtman et al. (1968b) disagreed with the notion that oscillating levels of brain norepinephrine controlled the enzyme rhythm. They reported that following the administration of reserpine twice daily for 3 days prior to killing the rats, the normal diurnal rhythm of the enzyme was not changed, even though the brain norepinephrine concentration was reduced to 25% its normal level. In addition, Scheving et al. (1968) were unable to demonstrate a diurnal rhythm in brain norepinephrine.

Another possible reason for the presistence of the diurnal rhythm after adrenalectomy might be the cyclic feeding habits of the rats. Zigmond et al. (1969) have

presented evidence that the rats start to eat toward the end of the light period and suggested that this indicated that the ingestion of food preceeded the daily rise in TAT. As the rate of food ingestion increases, the liver becomes perfused with relatively large amounts of amino acids through the portal circulation, which in turn cause the activity of TAT to rise. Tryptophan has been suggested to be of special significance in producing this effect (Labrie and Korner, 1968a; Wurtman et al., 1968a). Cohn et al. (1970) later reported that when rats were fed at hourly intervals instead of ad libitum, the rhythm was significantly reduced in the intact rat and completely abolished in the adrenalectomized rat. Wurtman et al. (1968a) concluded that, since no diurnal rhythm could be demonstrated in intact rats fed a protein-free diet, access to dietary protein was an absolute requirement for the production of the TAT rhythm.

Fuller and Snoody (1968) have reported that when rats were allowed food only during the early light hours (lights on from 0700 to 1700 hours, food allowed from 0800 to 1200 hours) the daily rhythm shifted, resulting in the peak in enzyme activity occurring at 1200 hours, four hours after the rats were allowed food. Although this would seem to indicate that the ingestion of food was responsible for the rise in TAT, a more careful examination of their

data revealed that the rise in enzyme activity preceeded the time when food was allowed.

A similar finding was reported by Black and Axelrod (1968b) who demonstrated that although adrenalectomized rats had been fasted for 10 hours, the peak in the enzyme activity still occurred, and with almost as great an amplitude as found in fed adrenalectomized rats. They concluded that the afternoon increase in food consumption appeared to be unrelated to the evening peak in TAT.

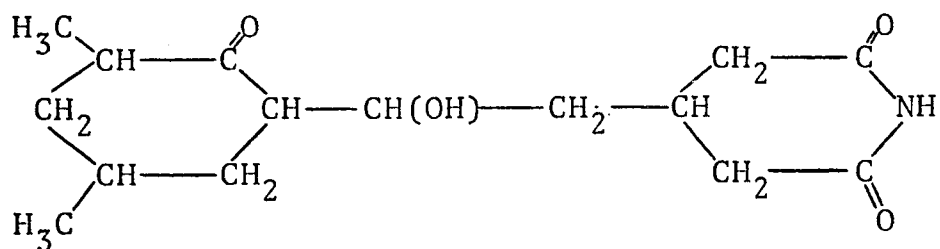
As will be shown later in this thesis the magnitude of the TAT rhythm was found to be significantly reduced upon adrenalectomy in properly controlled experiments.

PART I

THE EFFECT OF CYCLOHEXIMIDE ON TYROSINE AMINOTRANSFERASE

## INTRODUCTION

Cycloheximide (Actidione;  $\beta$ -[2(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-glutarimide), an antibiotic first isolated in crystalline form from culture filtrates of Streptomyces griseus by Leach et al. (1947), has been shown to be an inhibitor of protein synthesis in vivo



Structure of Cycloheximide

(Siegel and Sisler, 1964; Trakatellis et al., 1965; Morris, 1966) and in vitro (Ennis and Lubin, 1964; Wettstein et al., 1964; Stanners, 1966). Although the actual site of inhibition is not definitely known, evidence suggests that cycloheximide might be inhibiting the translocation reaction (McKeehan and Hardesty, 1969).

Since the stimulation of enzymes such as tryptophan pyrrolase (Fiala and Fiala, 1966) and  $\beta$ -galactosidase (Craeser, 1955) had been shown to be inhibited by cycloheximide, the report by Fiala and Fiala (1966) that cycloheximide "induced" TAT 300% in 4 hours was interesting. These workers reported that the effect could be observed

in both intact and adrenalectomized rats and the increase could be blocked in adrenalectomized rats by puromycin, another inhibitor of protein synthesis. Moreover, their data also indicated that the antibiotic enhanced the stimulation of TAT by cortisol.

Repression of TAT synthesis at the translational level by a repressor with a very rapid turnover had been proposed by Garren et al. (1964b) and Kenney and Albritton (1965) as a possible control mechanism for the regulation of this enzyme. If this model was correct, the paradoxical stimulation of TAT by cycloheximide might be explained in terms of a differential inhibition of repressor formation versus enzyme synthesis, favoring the latter. The net effect would be an early transient increase in the enzyme such as reported by Fiala and Fiala (1966). The following experiments were designed to confirm the work of Fiala and Fiala (1966).

### MATERIALS AND METHODS

#### 1. Chemical sources.

L-Tyrosine,  $\alpha$ -ketoglutaric acid, pyridoxal phosphate, Fraction V Bovine Serum Albumin and cycloheximide (Actidione) were obtained from Nutritional Biochemicals Corporation (Cleveland, Ohio). Sodium diethyldithiocarbamate was obtained from Eastman Kodak Chemicals (Rochester, New York). Buffer salts were obtained from Fisher Scientific Company

(St. Louis, Missouri). Sodium pentobarbital in a sterile aqueous solution containing 20% propylene glycol and 10% ethanol (Nembutal) was obtained from Abbott Laboratories Limited (Montreal, Quebec). Cortisol, as the sodium succinate ester (Solu-Cortef), was a gift from Dr. E.L. Masson, The Upjohn Company of Canada (Don Mills, Ontario). Tautomerase was obtained from Sigma Chemical Company (St. Louis, Missouri).

## 2. Animals.

Male, intact or bilaterally adrenalectomized rats of the Sprague-Dawley strain, weighing between 165 and 220 grams, were supplied by the Canadian Breeding Laboratories (St. Constant, Quebec). Adrenalectomized rats were received 2 to 3 days after the operation and were allowed 0.9% saline as drinking water. All animals were maintained at a controlled temperature ( $23 \pm 1^{\circ}\text{C}$ ) and were allowed food and water ad libitum. All experiments were started at approximately 0900 hours and were terminated either in the afternoon or evening, depending upon the duration of the experiment.

## 3. Treatment of animals.

a) In experiments in which the rats were used without prior anesthesia and surgery they were killed by decapitation, the livers were quickly removed, placed in a beaker and chilled in ice.

b) Sodium pentobarbital was used to anesthetize the rats. The dose ranged from 10 mg per kg body weight (for hypophysectomized rats, i.p.) to 30 mg per kg body weight (for intact rats, i.p.). The rats were usually fully anesthetized within 5 minutes and were then tied on their backs on specially constructed boards. The biopsy technique employed was that described by Grossman and Mavrides (1967). A one-inch midline incision was made into the upper abdominal wall and small liver samples (approximately 50 mg) were taken at various times. Relatively little bleeding occurred at the cut liver surfaces and precautions against hemorrhage were not necessary. The control value was obtained from the first biopsy sample, taken just before administration of the substance under study.

#### 4. Homogenization of the tissue samples.

Each liver was blotted and approximately 50 mg tissue were weighed on a torsion balance. All subsequent procedures were carried out at 0° to 3°C. The weighed portion of the liver was placed in a hand-operated Ten Broeck homogenizer kept in an ice bucket and containing 1 ml of 0.1M sodium phosphate buffer, pH 7.5. Homogenization was achieved with 10 strokes of the plunger. The homogenate was then transferred to a chilled heavy-walled centrifuge tube and centrifuged at 27,000 x g for 15 minutes in a

Servall RC-2 refrigerated centrifuge maintained at  $0^{\circ}$  to  $3^{\circ}\text{C}$ . The resulting supernatant fluid was kept on ice until the enzyme activity and protein content could be analyzed, usually within 2 hours. As a general rule all livers were weighed and homogenized within 15 minutes after excision.

#### 5. Assay of tyrosine aminotransferase.

The spectrophotometric method of Lin and Knox (1957) was used for the measurement of TAT. The principle of the method is described in the Review of the Literature, section B. The formation of the enol-form of pHPP was assured by the inclusion of excess tautomerase in the assay system.

The substrate L-tyrosine was suspended in the borate-phosphate buffer while  $\alpha$ -ketoglutaric acid, sodium diethyldithiocarbamate and pyridoxal phosphate were dissolved in the sodium phosphate buffer. The  $\alpha$ -ketoglutaric acid was neutralized with 5N sodium hydroxide before adjusting to the final volume.

The assay mixture contained the following in  $\mu\text{moles}$ , unless otherwise specified; borate phosphate buffer, pH 7.8, 250; tyrosine, 8.3;  $\alpha$ -ketoglutaric acid, 80; sodium diethyldithiocarbamate, 7.5; pyridoxal phosphate, 0.12; and the enzyme extract. The total volume was 3.0 ml and was contained in a quartz cuvette (10 mm light path). A thermostatically controlled ( $25^{\circ}\text{C}$ ) Unicam SP 800 recording

spectrophotometer complete with a SP 825 programmer for four simultaneous analyses was used for the measurement of the increase in the absorbance at 310 nm. The sample mixture was measured against a blank in which tyrosine was omitted.

## 6. Calculations.

The concentration of pHPP formed during one minute in the linear part of the assay was calculated from the following equation:

$$c = \frac{d}{\epsilon}$$

where c is the concentration of pHPP in moles per liter, d is the difference in absorbance units per minute for a cuvette with a one-cm light path and  $\epsilon$  is the molar extinction coefficient for pHPP at 310 nm which has been reported to be  $9.85 \text{ cm}^{-1}\text{M}^{-1}$  (Lin et al., 1958). The concentration is multiplied by  $3 \times 10^3$  to correct for the 3 ml assay volume and to express the results in  $\mu\text{moles}$ . Thus the equation used is actually:

$$\frac{d \times 3 \times 10^3}{9.85} = \mu\text{moles/minute or units}$$

## 7. Protein analysis.

The method of Lowry et al. (1951) was used for the determination of protein in the samples. Fraction V Bovine Serum Albumin was used as a standard for protein.

## 8. Statistical analysis.

The standard deviations of the groups of results were calculated using the following formula:

$$S = \frac{\Sigma x^2 - (\Sigma x)^2/n}{n - 1}$$

where S is the standard deviation, x is the enzyme specific activity and n is the number of results in the group.

The s.e.m. of the groups of results was calculated using the following formula:

$$\text{s.e.m.} = \frac{S}{\sqrt{N}}$$

The experimental results were analyzed for statistical significance using the Student t-test. The formula is shown below:

$$t = \frac{x_1 - x_2}{\sqrt{\frac{(n_1 - 1)S_1^2 + (n_2 - 1)S_2^2}{n_1 + n_2 - 2}}} \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$$

where t is the value for the comparison of differences,  $x_1$ ,  $S_1$ , and  $n_1$  are the mean, standard deviation and number of results respectively in the 1st group and  $x_2$ ,  $S_2$ , and  $n_2$  are the mean, standard deviation and number of results respectively in the 2nd group.

Results were expressed as percent of the control (zero time) value.

## RESULTS

### 1. Effect of cycloheximide on hepatic TAT in adrenalectomized rats.

The response of TAT to cycloheximide is shown in Table I. Each experiment represents one group of animals as shipped by the supplier. The group was divided into saline controls and cycloheximide-treated animals which were killed simultaneously. The rats of the cycloheximide-treated group received 1 mg cycloheximide per kg body weight (i.p.) at the start of the experiment. Since cycloheximide had been dissolved in saline to a concentration of 2 mg per 5 ml, the volume received by each rat was 2.5 ml per kg body weight (i.p.). The saline controls received (i.p.) the same volume of saline. All animals were killed 4 hours after administration of the antibiotic. Only in experiment III was a significant elevation in the enzyme observed in the cycloheximide-treated rats. It must be noted that approximately 50% of the animals treated with cycloheximide died 2 to 3 hours after treatment.

### 2. Effect of cycloheximide on hepatic TAT in anesthetized adrenalectomized rats.

Cycloheximide (1 mg per kg body weight, i.p.) had no stimulatory effect on the enzyme over a 6-hour period (Table II). None of the cycloheximide-treated animals survived beyond this time. The enzyme activity in the livers of the

Table I

The effect of cycloheximide on hepatic TAT  
in adrenalectomized rats.

| EXPERIMENT<br>NUMBER | TAT activity*      |                             | SIGNIFICANCE<br>BETWEEN<br>GROUPS |
|----------------------|--------------------|-----------------------------|-----------------------------------|
|                      | SALINE<br>CONTROL  | CYCLOHEXIMIDE-<br>TREATED** |                                   |
| I                    | 4.54 ± 1.80 (4)*** | 3.76 ± 0.52 (6)             | P < 0.70                          |
| II                   | 2.87 ± 0.23 (4)    | 3.41 ± 0.35 (3)             | P < 0.25                          |
| III                  | 3.06 ± 0.46 (4)    | 6.64 ± 1.03 (6)             | P < 0.05                          |
| IV                   | 5.16 ± 2.62 (3)    | 4.41 ± 0.24 (4)             | P < 0.80                          |

\* : units/mg protein x 10<sup>3</sup> ± s.e.m.

\*\* : Rats killed 4 hours after administration of 1 mg  
cycloheximide/kg body weight.

\*\*\*: Numbers in parentheses indicate the number of rats.

Table II  
Effect of cycloheximide (1 mg/kg body weight, i.p.) on hepatic TAT  
in anesthetized adrenalectomized rats.

| TIME<br>(hours) | SALINE CONTROLS      |                  |                                   | CYCLOHEXIMIDE-TREATED |                  |                                   |
|-----------------|----------------------|------------------|-----------------------------------|-----------------------|------------------|-----------------------------------|
|                 | number<br>of<br>rats | TAT<br>activity* | percent of<br>initial<br>activity | number<br>of<br>rats  | TAT<br>activity* | percent of<br>initial<br>activity |
| 0               | 8                    | 3.59 ± 0.57      | 100                               | 7                     | 2.59 ± 0.33      | 100                               |
| 2               | 8                    | 2.19 ± 0.40      | 62 ± 7                            | 7                     | 2.13 ± 0.24      | 83 ± 4                            |
| 4               | 8                    | 3.12 ± 0.40      | 93 ± 10                           | 7                     | 2.14 ± 0.26      | 87 ± 9                            |
| 6               | 8                    | 2.75 ± 0.18      | 89 ± 14                           | 3                     | 1.77 ± 0.09      | 76 ± 21                           |
| 8               | 4                    | 4.21 ± 0.87      | 105 ± 25                          |                       |                  |                                   |

\* : units/mg protein x 10<sup>3</sup> ± s.e.m.

saline controls closely followed the enzyme activity in the cycloheximide-treated rats.

3. Effect of cycloheximide on the cortisol induction of hepatic TAT in anesthetized adrenalectomized rats.

Cortisol (30 mg per kg body weight, i.p.) alone resulted in a 24-fold increase in the enzyme activity which peaked between 6 to 8 hours after administration of the hormone (Fig. 4). Cycloheximide (1 mg per kg body weight, i.p.) delayed the cortisol-stimulated increase in the enzyme allowing only a 6-fold elevation which peaked between 10 to 12 hours after the start of the experiment.

4. Effect of cycloheximide on hepatic TAT in anesthetized intact rats.

Although the saline control group received only 2.5 ml saline per kg body weight, the enzyme increased for at least 10 hours at which time it was nearly 8 times its initial level (Fig. 5). This rise could be prevented with cycloheximide (1 mg per kg body weight, i.p.).

5. Effect of cycloheximide on the cortisol stimulation of hepatic TAT in anesthetized intact rats.

The administration of cortisol (30 mg per kg body weight, i.p.) resulted in a rapid increase in the enzyme which peaked 6 hours after the start of the experiment (Fig. 6). This rise was delayed several hours by the

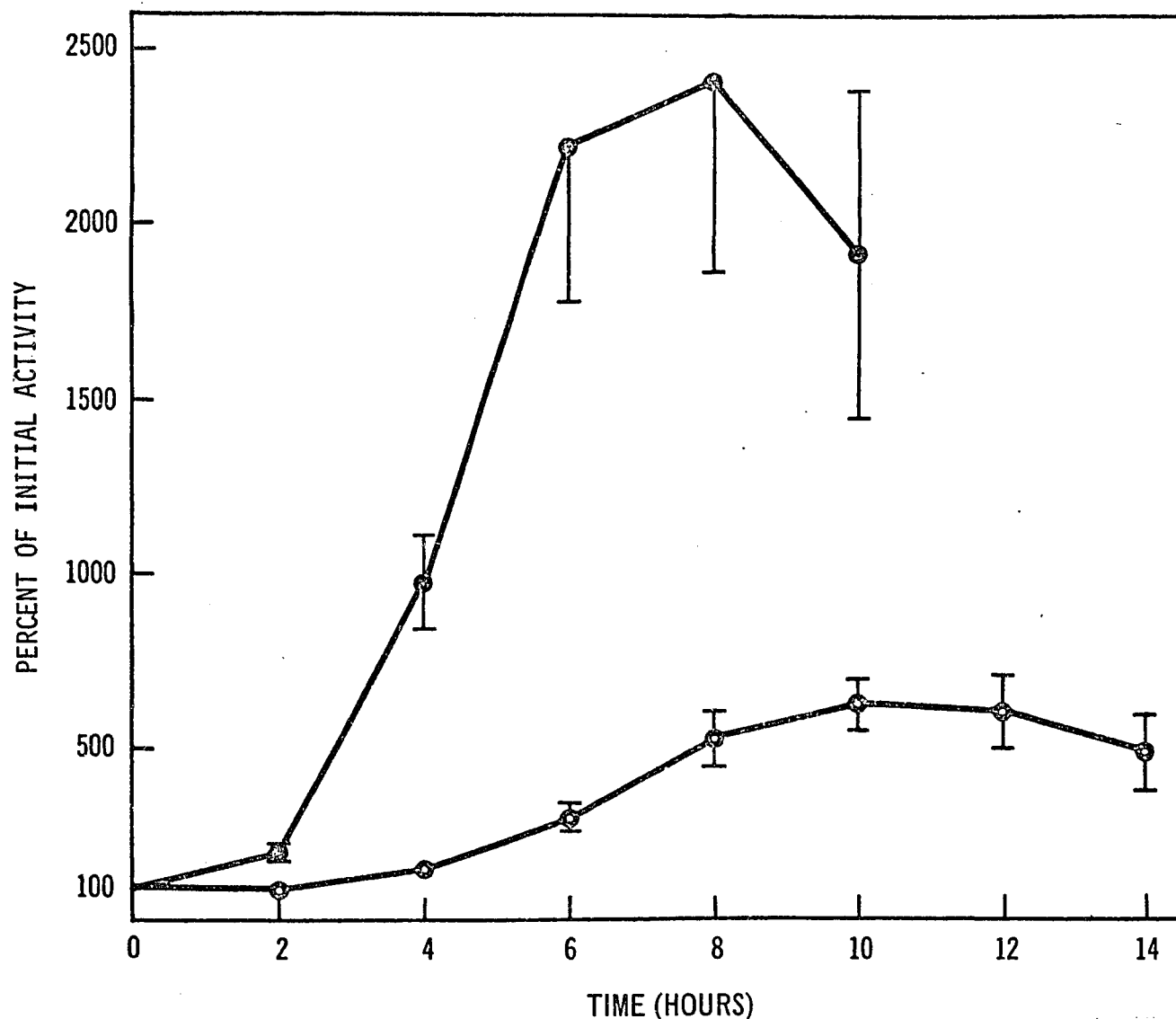


Fig. 4. Effect of cycloheximide on the cortisol stimulation of hepatic TAT in anesthetized adrenalectomized rats. Rats received (i.p.) either 30 mg cortisol/kg body weight (●) or 30 mg cortisol plus 1 mg cycloheximide/kg body weight (○). Points plotted represent means  $\pm$  s.e.m. (Appendix, Table 1).

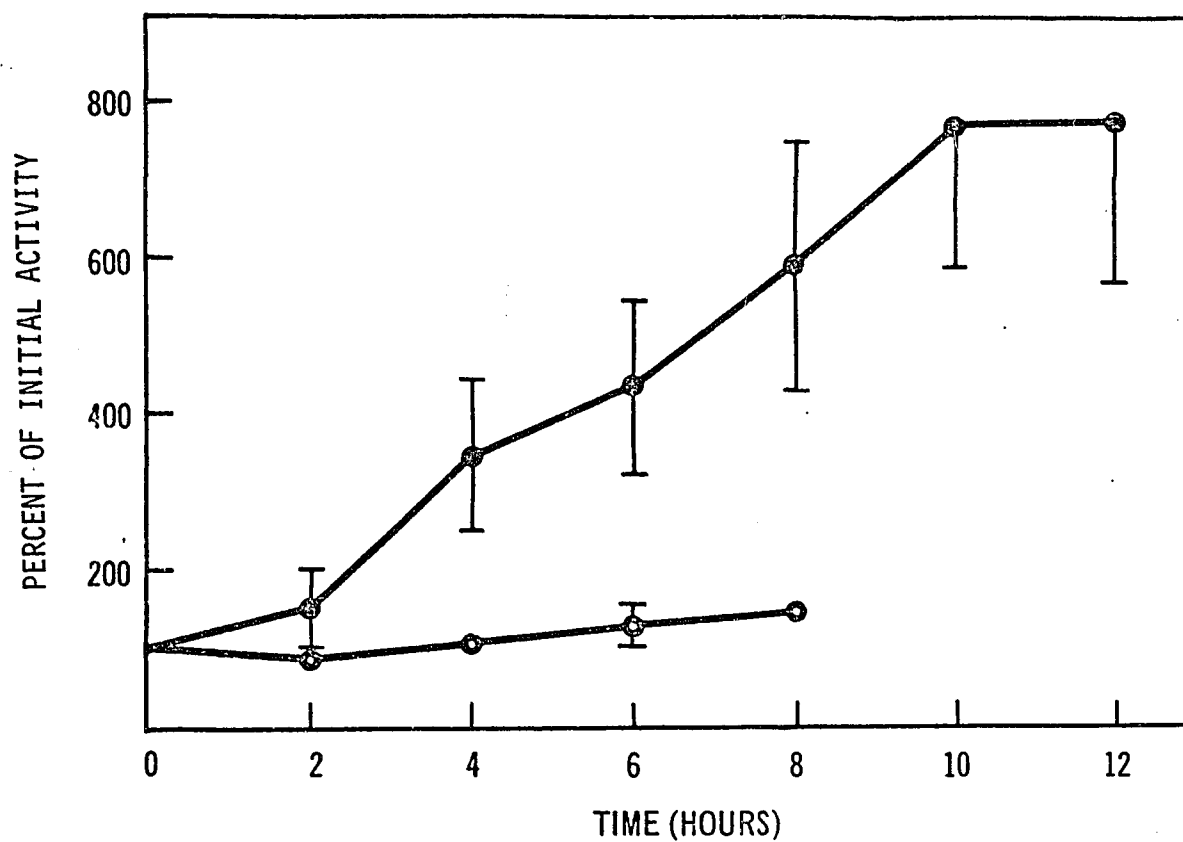


Fig. 5. Effect of cycloheximide (1 mg/kg body weight, i.p.) on the stress-mediated elevation of hepatic TAT in anesthetized intact rats. Control (●). Cycloheximide-treated (○). Points plotted represent means  $\pm$  s.e.m. (Appendix, Table 2).

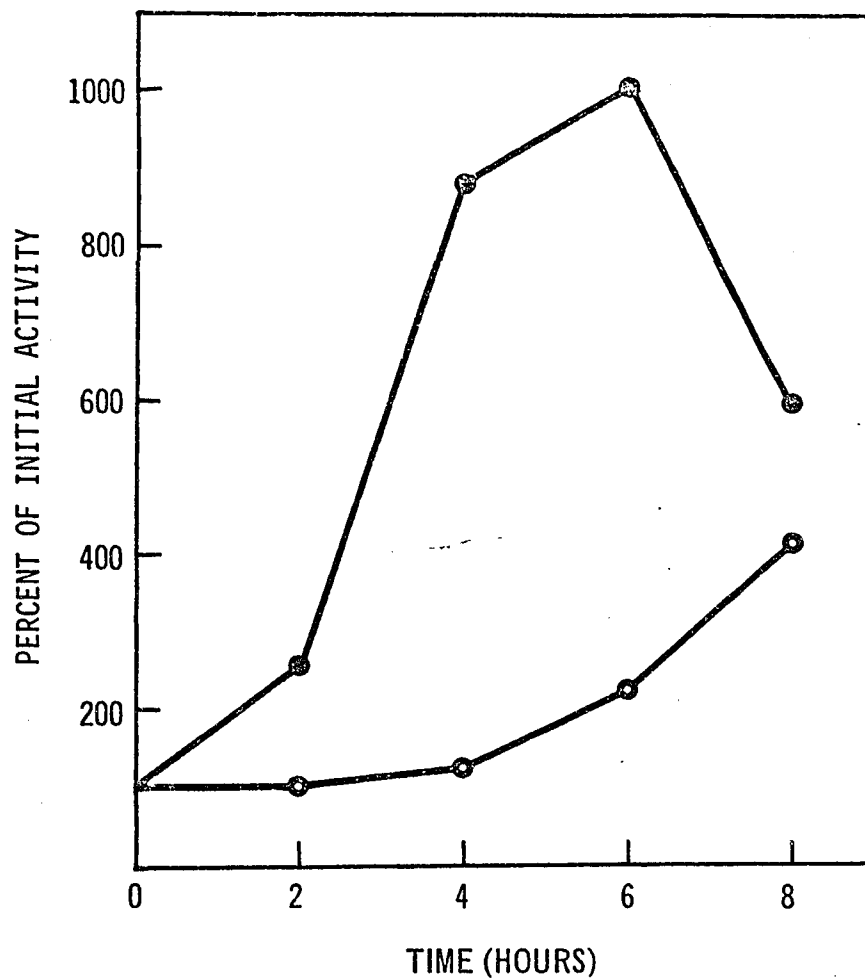


Fig. 6. Effect of cycloheximide on the cortisol stimulation of hepatic TAT in anesthetized intact rats. Rats were administered (i.p.) either 30 mg cortisol/kg body weight (o) or 30 mg cortisol plus 1 mg cycloheximide/kg body weight (o). Only one control rat and one cycloheximide-treated rat were used in this experiment. Points plotted represent means (Appendix, Table 3).

administration of cycloheximide (1 mg per kg body weight, i.p.).

### DISCUSSION

Cycloheximide was found to be very toxic to the rats as approximately half of the animals died before the experiment could be completed. The mortality rate was as high for anesthetized as for unanesthetized rats.

The results of the experiments with cycloheximide in unanesthetized adrenalectomized rats (Table I) suggest that, at best, the increase in TAT activity in response to cycloheximide was erratic and limited. The significance of the elevation in TAT noted in some animals (Table I, experiment III) is unclear since it occurred in only one of the four experiments. Since the enzyme is characterized by a large biological variation (Lin and Knox, 1957), as evidenced by the values reported for the saline controls in the four experiments (Table I) as well as the large standard errors within each group, this biological variation rather than an effect due to cycloheximide might be responsible, at least in part, for differences observed in experiment III (Table I).

The elevation in the enzyme activity may also be explained on the basis of the toxicity of the cycloheximide. Indeed Holten et al. (1967) have shown that toxicity due to

pyridoxine injections increases the hepatic amino acid pool. Such an increase could be responsible for the slight elevation in the enzyme activity observed in one experiment. In the biopsy technique described by Grossman and Mavrides (1967) each rat served as its own control and biological variation could not interfere with the evaluation of data. The results of Table II and Fig. 5 clearly indicate that cycloheximide had no stimulatory effect on TAT in anesthetized adrenalectomized or intact rat. Moreover, cycloheximide inhibited the cortisol-mediated stimulation in these animals (Figs. 4 and 6).

In agreement with the results of Lin and Knox (1957) and Rosen et al. (1963), the cortisol-mediated elevation of TAT was greater in adrenalectomized (Fig. 4) than intact rats (Fig. 6). The peak in the enzyme stimulation occurred between the 6th and 8th hour after the administration of cortisol which agreed with the findings of Grossman and Mavrides (1967). The peak occurred 1-2 hours later than reported by most other workers (Lin and Knox, 1958; Garren et al., 1964a) who followed enzyme activity in groups of rats killed at several time intervals following administration of the hormone.

Several possibilities exist which might explain this delayed peak in the enzyme activity.

1) Pentobarbital, or one of its metabolites, might have affected the enzyme directly. However, if this were

correct, the anesthesia should have stimulated TAT in the adrenalectomized rats which received only saline (Table II), which was not the case. Thus one can only conclude that the anesthetic had no direct effect on the enzyme and can not be responsible for the slightly prolonged enzyme elevation.

2) The anesthetic may have stimulated the formation of an unusual metabolic product of cortisol which in turn might have been responsible for the prolonged effect. Although cortisol is normally metabolized by the reduction of ring A to the tetrahydrocortisol derivative (Tomkins, 1959), some cortisol can be hydroxylated to the 6 $\beta$ -hydroxycortisol derivative. Both phenobarbital and pentobarbital have been reported to increase the hydroxylation of cortisol (Burstein and Klaiber, 1965; Conney et al., 1965; Berman and Green, 1971), a metabolite which might still have some glucocorticoid activity. However, in view of the fact that only 2 to 7% of the cortisol in phenobarbital-treated rats is metabolized to 6 $\beta$ -hydroxycortisol (Burstein and Bhavnani, 1967) consideration of an altered cortisol metabolism is not warranted. Thus it is unlikely that a metabolic product of cortisol was responsible for the prolonged effect on TAT.

3) The anesthetic might have prolonged the action of the steroid by inhibiting its degradation and allowing it a

longer time to act. Since some steroids are metabolized by the same type of microsomal enzymes as barbiturates, the administration of the latter could alter the metabolism of these steroids (Sharples, 1970). However Remmer and Mecker (1965) reported that phenobarbital administration has very little effect on cortisol  $\Delta^4$ -3-ketoreductase, indicating that the metabolism of the steroid remains unaffected by the barbiturate.

Thus it appears unlikely that the anesthetic played a role in delaying the peak in TAT activity. Indeed not all workers are in agreement as to the time of the peak, some even reporting it at 2.5 hours following administration of 25 mg cortisol per kg body weight (Csányi et al., 1967; Csányi and Greengard, 1968).

The six-fold elevation in TAT which occurred in the anesthetized adrenalectomized rats treated with both cortisol and cycloheximide is interesting (Fig. 4). The enzyme did not start to rise till the 4th hour and peaked between the 10th to 12th hour. One explanation for the elevation in the enzyme following the lag period might be the accumulation of TAT messenger RNA, as proposed by Kenney et al. (1968). Indeed Sisler and Siegel (1967) have reported that cycloheximide has no effect on RNA synthesis although Muramatsu et al. (1970) have suggested that approximately 70% of RNA synthesis is inhibited after 60 minutes, presumably

resulting from an inhibition of the DNA-dependent RNA polymerase synthesis. If one were to assume that the rats were able to remove or metabolize the antibiotic within 3 to 4 hours following its administration, then the pre-formed TAT messenger RNA would be free to be translated resulting in the retarded cortisol stimulation of the enzyme. The much lower height in the TAT peak observed for these animals as compared to their cortisol controls might be the result of the partial inhibition of the RNA synthesis by the antibiotic resulting in only a fraction of the TAT messenger RNA being available for translation as compared to the cortisol control.

Another possibility stems from the work of Jondor et al. (1966) who reported that cycloheximide selectively inhibited some microsomal drug-metabolizing enzymes in rat liver. It is conceivable that the metabolism of cortisol might also be affected. As the antibiotic was removed from the system, the cortisol not yet metabolized would then be free to act resulting in the delayed elevation of TAT.

Essentially the same explanations might also explain the delayed elevation in TAT observed in the intact anesthetized rats treated with cortisol and cycloheximide (Fig. 6).

The increase in TAT activity in intact anesthetized rats (Fig. 5) is also of interest. The pentobarbital anesthesia could not have been responsible for the stimulation

since it has been shown that this barbiturate increases plasma growth hormone concentration while decreasing plasma corticosterone concentration (Takakashi et al., 1971). In addition a direct effect of the barbiturate is ruled out by virtue of the fact that no stimulation was observed in adrenalectomized rats (Table II). Thus the effect must have been due to the stress of surgery. Other stresses such as tyrosine or celite injections (Lin and Knox, 1957; Kenney and Flora, 1961), immobilization (Hanninen and Hortiala, 1967) or shaking rats in reciprocal shakers (Schapiro et al., 1966) have been shown to stimulate TAT. In addition laparotomy has been shown to increase serum corticoids (Geller et al., 1969). Thus endogenous glucocorticoids, released by the adrenal gland in response to the stress of laparotomy, was probably responsible for the stimulation of the enzyme. This stress-mediated elevation of TAT by laparotomy was later confirmed by others (Tsukada et al., 1968; Geller et al., 1969).

In conclusion, the results of these experiments were not in agreement with those reported by Fiala and Fiala (1966). An increase in TAT following cycloheximide administration could not be consistently demonstrated. This failure could not be attributed to biological variation as indicated by the experiment with anesthetized adrenalectomized rats and anesthetized intact rats. In the report of these

workers (Fiala and Fiala, 1966) the results from 3 cycloheximide-treated adrenalectomized rats were compared with the results from only one control. The inclusion of more animals in their experiment might have eliminated the large discrepancy between their results and those reported in this thesis. This inability of cycloheximide to stimulate TAT has been confirmed by many workers (Kenney, 1967b; Peterkofsky and Tomkins, 1968; Jervell and Seglen, 1969; Gelehrter and Tomkins, 1970; Barker et al., 1971; Liberti et al., 1971).

Thus the hypothesis proposed in the introduction that cycloheximide might be selectively inhibiting the formation of a specific TAT repressor must be abandoned since the stimulation of the hepatic enzyme by this antibiotic could not be verified.

This part of the thesis has been published (Mavrides and Lane, 1967).

PART II

STUDIES ON THE DIURNAL RHYTHM OF HEPATIC TYROSINE  
AMINOTRANSFERASE

## INTRODUCTION

A diurnal rhythm in hepatic TAT has been reported to exist in adult rats (Potter et al., 1966; Civen et al., 1967b; Shambaugh et al., 1967; Wurtman and Axelrod, 1967). The enzyme peaks shortly after the beginning of the dark period and reaches a nadir shortly after the beginning of the light period. Since rats eat during the dark periods and rest during the light periods (Richter, 1965), the hepatic TAT rhythm has been suggested to be closely associated with their cyclic feeding habits (Wurtman and Axelrod, 1967; Wurtman et al., 1968a).

Adrenal corticoids stimulate TAT in rat liver (Lin and Knox, 1958). In addition, plasma corticosterone, the natural glucocorticoid of the rat (Bush, 1955), displays a diurnal rhythm with a peak which precedes the peak in hepatic TAT by just a few hours (Guillemin et al., 1959b; McCarthy et al., 1960; Civen et al., 1967b; Wurtman and Axelrod, 1967). These reports suggested a possible involvement of the adrenal cortical hormone in the operation of the enzyme rhythm. Indeed the experiments of Shambaugh et al. (1967) indicated a significant reduction in the amplitude of the enzyme rhythm following adrenalectomy or hypophysectomy. However, other workers reported that adrenalectomy (Civen et al., 1967b; Black and Axelrod, 1968b) or hypophysectomy (Wurtman and Axelrod, 1967) had no effect on the amplitude of the enzyme

rhythm. Experiments were designed to study the development of the enzyme rhythm in newborn rats and to establish whether the adrenal gland was at least partly responsible for the development and operation of the diurnal rhythm. In addition, other vertebrates were examined to establish whether they possessed a similar rhythm and whether a relationship existed between the stimulation of the enzyme by exogenous agents and an exhibition of a daily rhythm in the livers of these vertebrates.

### MATERIALS AND METHODS

#### 1. Chemical sources.

Corticosterone was obtained from Sigma Chemical Company (St. Louis, Missouri). Crystalline glucagon was obtained from Eli Lilly and Company (Indianapolis, Indiana). Propylene glycol, cupric sulfate, sodium potassium tartarate and sodium hydroxide were obtained from Fisher Scientific Company (St. Louis, Missouri). Zephiran chloride (benzalkonium chloride solution) was purchased from Frank O'Meara Limited (Ottawa, Ontario).

#### 2. Animals.

##### a) Rats.

For experiments concerned with the development of the rhythm, rats (Sprague-Dawley strain), on their 14th day of pregnancy, were received from the supplier (Canadian Breeding Laboratories, St. Constant, Quebec) and were

immediately placed in individual cages under cyclic lighting conditions (lights on from 0600 hours to 1800 hours). Rats designated as 1-day-old were actually 24-36 hours of age at the start of the experiment. The young rats were separated from their mother only at the time of killing, in groups of five to six from four to five litters regardless of sex. The stomachs of the young rats of up to 15 days of age were at all times engorged with milk and free of solid food on macroscopic examination. A mixed milk-solid food content was always found in the stomachs of the 21-day-old rats under all conditions. The pregnant rats whose litters were intended for constant darkness or constant light experiments were subjected to the respective conditions. In constant-darkness experiments the animals were actually exposed to light every 2nd day for about 10 minutes around noon time for the necessary changes of water and food.

For experiments concerned with the role of the adrenal gland in the development of the rhythm several litters of rats, born on the same day and maintained in constant light for their first 21 days, were pooled and divided into 2 groups regardless of sex. One group was adrenalectomized while the other group underwent a sham-operation. The adrenalectomized rats received 0.9% sodium chloride as drinking water.

For experiments concerned with the role of the adrenal gland in the adult rhythm, 21-day-old male rats were received from the same supplier and were immediately placed in cages in groups of 6 to 8 under the cyclic lighting conditions.

All animals were given food (Master Feeds, Toronto, Ontario) and water ad libitum.

b) Tadpoles.

Tadpoles (Rana catesbeiana) in the premetamorphic stage ranging in weight from 3 to 9 grams were obtained from North Carolina Biological Supply Company (North Carolina) and were immediately placed in groups of 10 to 12 in rectangular glass containers containing tap water. The containers were immersed in large water baths thermostatically maintained at 21°C. Two light-proof systems were constructed, one with constant light, the other with an automatically controlled cyclic lighting schedule (lights on from 0600 to 1800 hours). The light was provided by cool white fluorescent tubes. Their food consisted of canned spinach and the water in the glass containers was changed every 3 days.

Prior to the intraperitoneal injections the tadpoles were anesthetized by immersion for 1-2 minutes in ice-cold 50% aqueous ethanol.

c) Frogs.

Frogs (Rana catesbeiana) of an average weight of

200 grams were supplied by Seamac Specimens Limited (Oak Ridges, Ontario) and were immediately placed in a light-proof room equipped with cool white fluorescent tubes which were automatically turned on at 0600 and off at 1800 hours every day. A specially constructed platform was provided above a pool of continuously renewed water maintained in a temperature range of 20 to 25°C. An enclosure was made by a screen top and sides. The frogs were initially force-fed raw minced beef meat. Then flies were used, obtained in the pupae stage from the Canadian Department of Agriculture (Entomology Division, Ottawa, Ontario) and allowed to hatch in jars left in the enclosure. One week before the experiment live minnows and small tadpoles were obtained locally and were liberally added to the pool.

d) Pigeons.

Pigeons (Columbia livia) were collected locally and were placed in groups of 3 to 4 in large cages in a room provided with cyclic lighting (lights on from 0600 to 1800 hours). The pigeons were allowed food (Purina Pigeon Chow Checkers) and water ad libitum.

3. Adrenalectomy and sham operations.

The rats were anesthetized with sodium pentobarbital and the hair from the posterior lateral sides of their lumbar region was shaved. An incision was made in each side in this region just below the last rib. With the aid of

curved forceps the fatty tissue between the adrenal gland and the kidney was grasped and the adrenal gland was pulled away from the kidney and removed from the animal. No cutting was necessary and little bleeding occurred. For the rats undergoing the sham operation the adrenal gland was grasped by the forceps and then released leaving the organ intact. The muscular walls of the animals were then sewn with nylon thread and the skin was stapled. All instruments used were sterilized with Zephiran Chloride. Only a small percentage of the animals failed to survive and no animals showed signs of infection. The adrenalectomized rats were given 0.9% sodium chloride as drinking water.

All animals were sacrificed by decapitation. Tadpoles and newborn rats were decapitated with scissors while pigeons, frogs and older rats were decapitated in a hand-operated guillotine. The livers of all animals were then quickly removed, placed in a beaker and chilled in ice. All livers were dried, weighed and homogenized within 15 minutes after excision.

#### 4. Substances administered.

Corticosterone was dissolved in a 40% aqueous solution of propylene glycol. Crystalline glucagon and cortisol were administered as a saline solution.

##### 5. Calculation of food consumption.

The food was heated to 50°C for 3 hours to remove moisture before weighing. Exactly 200 grams of food was placed in each of 10 clean cages with only wood shavings at the bottom to absorb the moisture from the excrements and urine. At 0600 hours the rats were transferred to these cages and left undisturbed for the next 12 hours. At 1800 hours the process was repeated again. The food left in each cage at the end of both 12 hour periods was collected, dried at 50°C for 3 hours and weighed. The difference was assumed to be the food eaten by these rats during the respective time periods.

##### 6. Protein analysis.

An automated modification (Lane and Mavrides, 1969) of the biuret method (Gornall et al., 1949) was used on a Technicon Model I Autoanalyzer. The biuret reagent consisted of cupric sulfate (3 g ) and sodium potassium tartarate (12 g ) per liter of 3% sodium hydroxide. Fraction V bovine serum albumin was used as a protein standard.

##### 7. Preparation of tissue samples, assay of TAT, calculations and statistical analysis.

These were as described in Part I, Material and Methods.

## RESULTS

### 1. Development of the diurnal rhythm in hepatic TAT.

Groups of six rats, randomly collected at the time of killing from four to five litters irrespective of sex, were killed by decapitation at the indicated times and liver TAT was immediately assayed. These rats had been on a cyclic lighting schedule (lights on from 0600 to 1800 hours) since birth. The 1-day and 6-day-old rats (Figs. 7A and B) show no statistically significant variations over the 24-hour period. The first signs of a developing rhythmicity are seen in the 10-day and 15-day-old rats (Figs. 7C and D) in which statistically significant differences between the lowest and highest enzyme levels are present. By the 21st day (Fig. 8A) a full-fledged rhythm has developed. In the 21-day-old rats born and maintained in constant darkness, the range of hepatic TAT was considerably reduced while in the 21-day-old rats born and maintained in constant light the rhythm failed to develop altogether (Fig. 8B).

### 2. Role of the adrenal gland.

Following their first 21 days of life in constant light one group of rats was adrenalectomized while the other was sham-operated. Both groups were then placed on a cyclic lighting schedule (lights on from 0600 to 1800 hours) for the next 7 days. The results from this experiment are

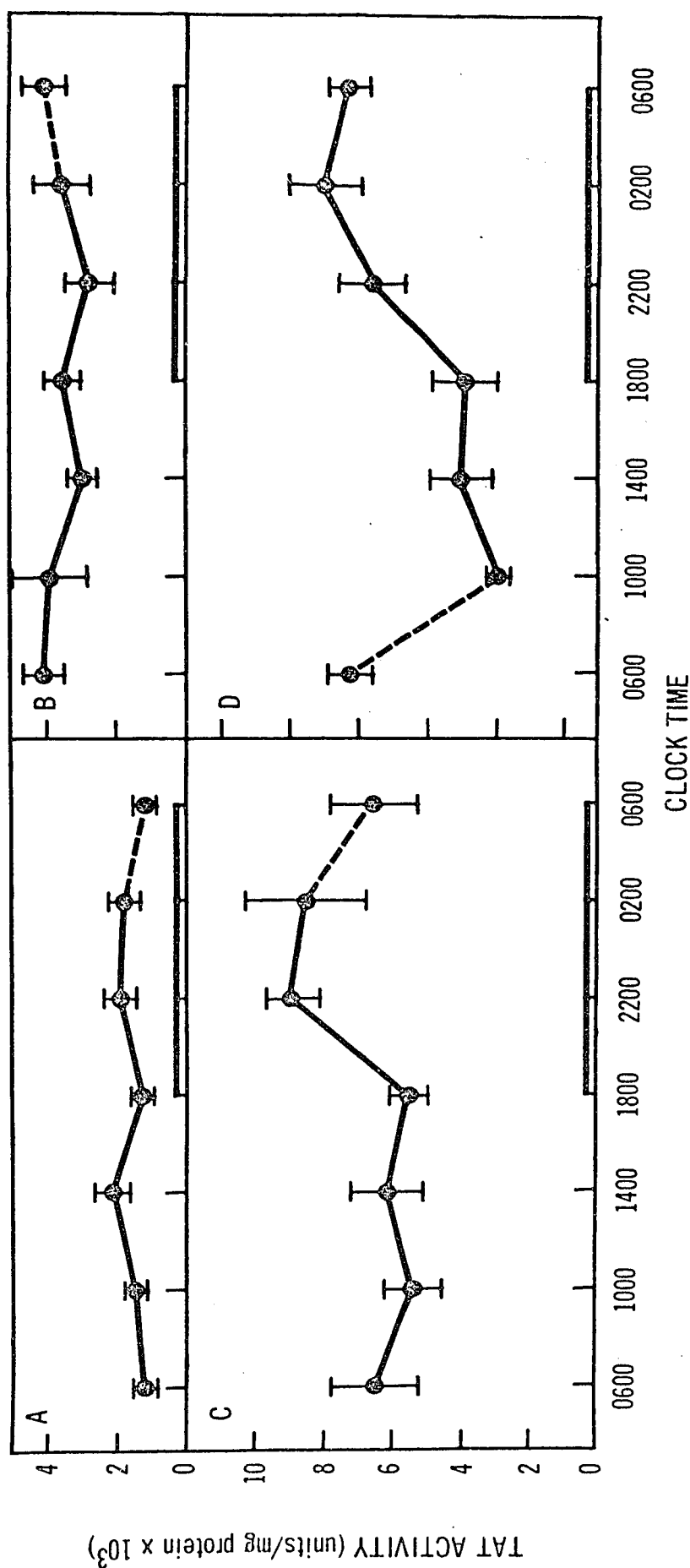


Fig. 7. Influence of age on the diurnal rhythm of hepatic TAT. Studies included 1-day-(A), 6-day-(B), 10-day-(C) and 15-day-old rats (D). Dark bars indicate the hours of darkness. The 24-hour cycle is completed with dashed lines. Points plotted represent means  $\pm$  s.e.m. (Appendix, Table 4).

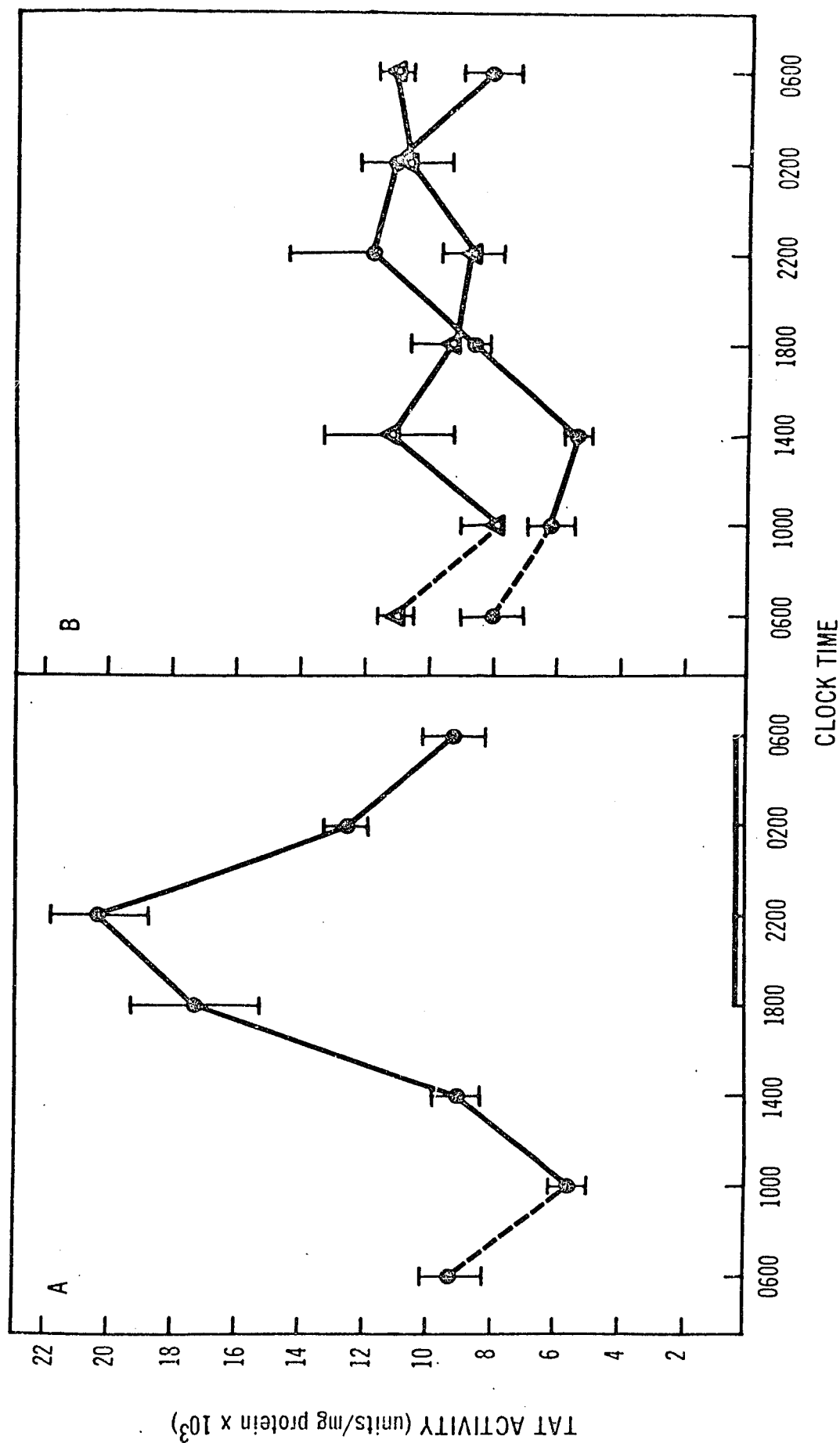


Fig. 8. Diurnal rhythm of hepatic TAT in 21-day-old rats. (A) Rats were maintained on a cyclic lighting schedule since birth. (B) Rats were housed in a constant light ( $\Delta$ ) or constant darkness ( $\bullet$ ) environment since birth. In A the dark bar indicates the hours of darkness. The 24-hour cycle is completed with dashed lines. Points plotted represent means  $\pm$  s.e.m. (Appendix, Table 4 and 5).

shown in Fig. 9 and indicate that a rhythm in TAT developed in only the sham-operated rats. The low values at 1000 and 1400 hours are significantly different from the high values at 1800, 2200 and 0200 hours. The values obtained for the adrenalectomized rats show no definite trend toward a rhythm.

In another study to determine whether the adrenal gland was necessary for the maintenance of the rhythm a group of 110 male rats averaging 75 g. in weight were subjected immediately upon arrival to a cyclic lighting schedule (lights on from 0600 to 1800 hours) for 1 week. A preliminary experiment had shown that 1 week on this schedule was sufficient for the establishment of the rhythm (Fig. 10A). The rats were subsequently transferred and kept in a room with constant light for 3 weeks. At the end of this period, groups of six animals randomly collected were killed by decapitation at the indicated times, and liver TAT was immediately assayed (Fig. 10A). Two distinct rises occurred in these animals, the first at 1000 hours and the second at 2200 hours. The reason is unknown, but in constant light the animals were highly excitable and the disturbance due to the removal of rats, which started at 1000 hours, may be responsible. However, the normal rhythm as such was abolished. It was recently shown that 1 week only of constant light severely represses the rhythm (Black and Axelrod, 1968). In two successive days following

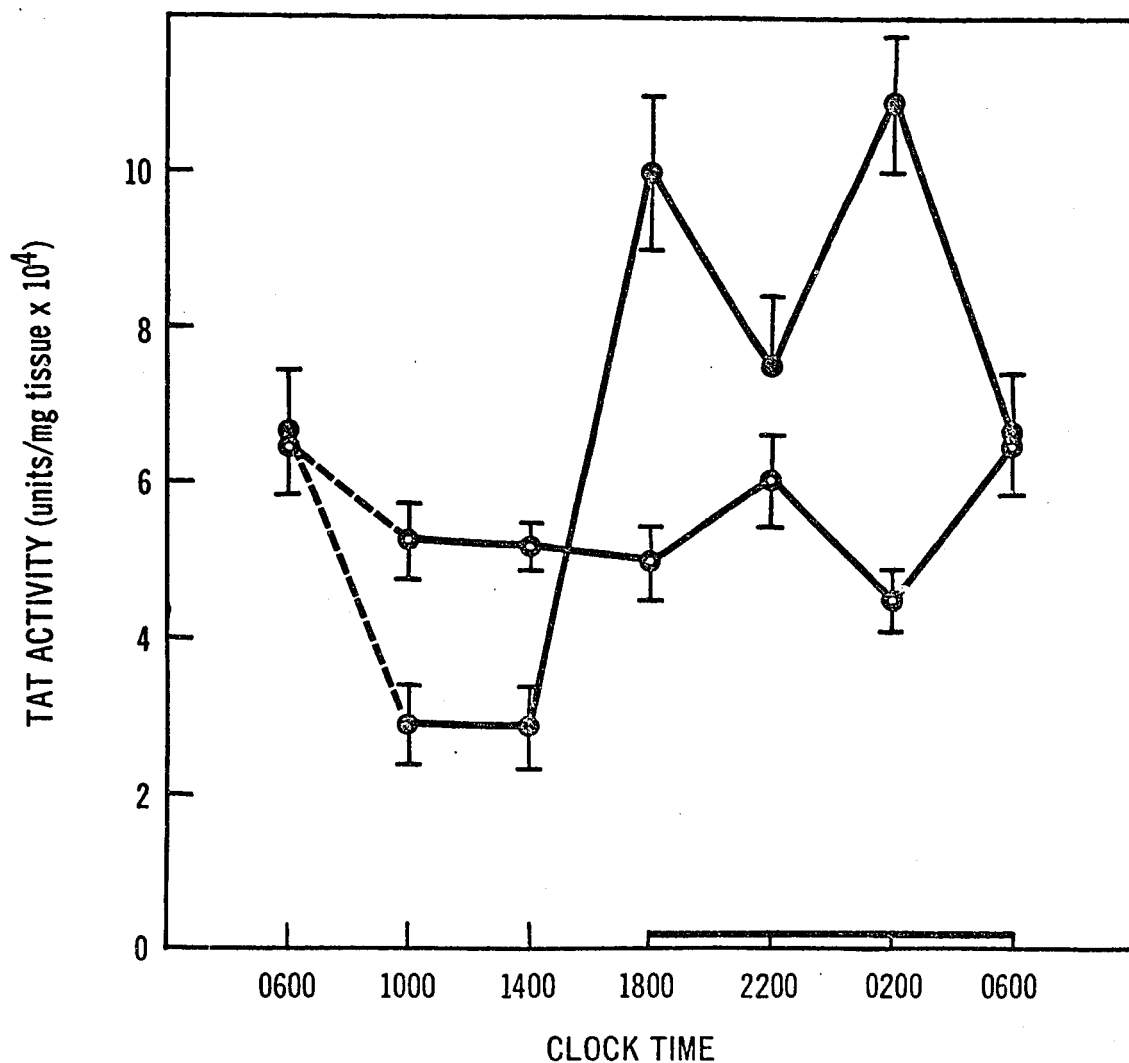


Fig. 9. Diurnal rhythm of hepatic TAT in 28-day-old adrenalectomized (o) and sham-operated rats (●). Rats were maintained in constant light for first 21 days of life. After operation they were subjected to cyclic lighting schedule for 7 days. Dark bar indicates hours of darkness. The 24-hour cycle is completed with dashed lines. Points plotted represent means  $\pm$  s.e.m. (Appendix, Table 6).

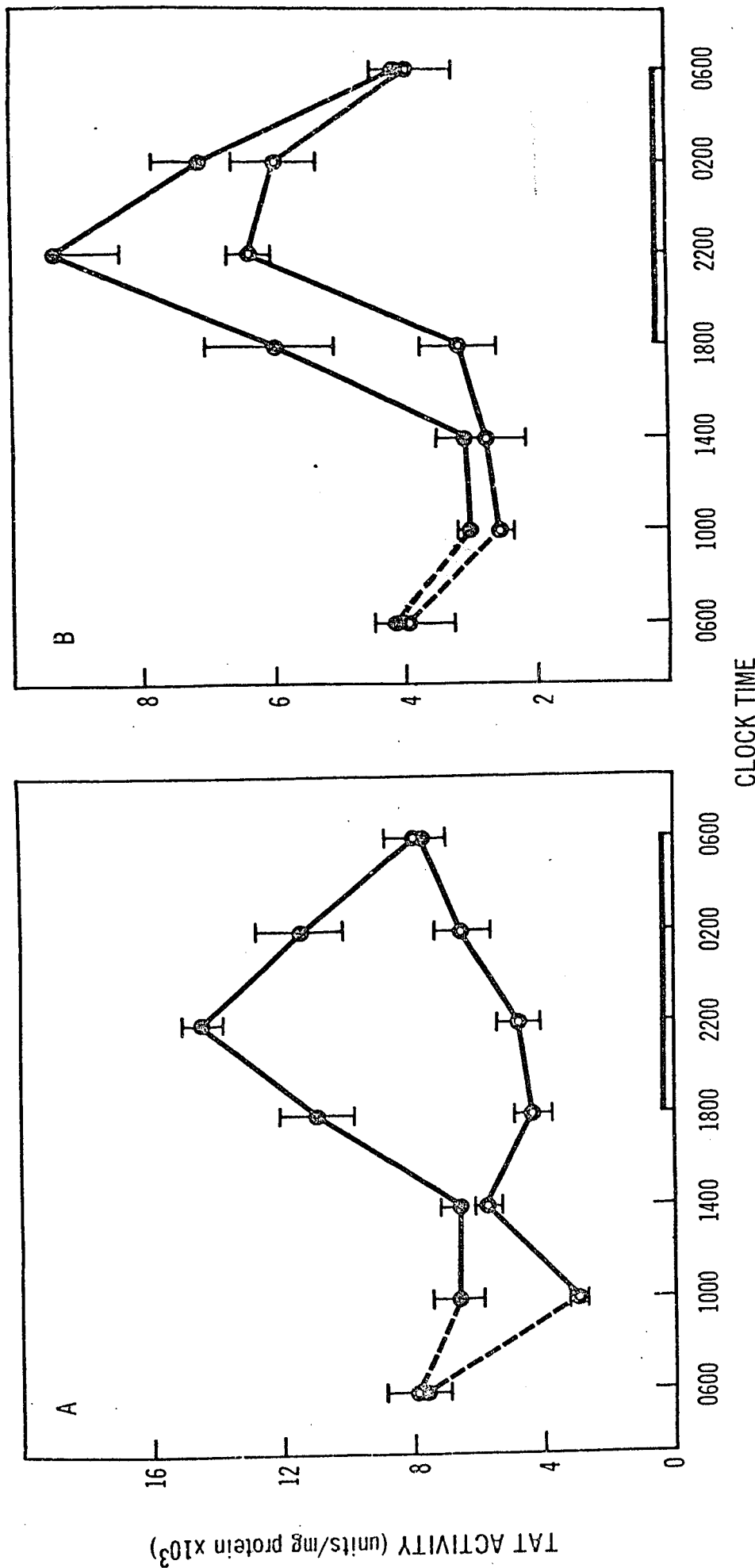


Fig. 10. Diurnal rhythm of hepatic TAT in adult rats. (A) Rats were maintained for 1 week on a cyclic lighting schedule (●) or 3 weeks in constant light (○). (B) After 3 weeks in constant light rats were either adrenalectomized (○) or sham-operated (●) and returned to a cyclic lighting environment for 10-11 days. Whenever a cyclic lighting schedule was used the dark bar indicates the hours of darkness. The 24-hour cycle is completed with dashed lines. Points plotted represent means  $\pm$  s.e.m. (Appendix, Table 7).

this experiment the remaining 74 rats were either bilaterally adrenalectomized or sham-operated so that half the number of animals in each group were operated on one day and the other half on the following day. Immediately after surgery the animals were placed back in the cyclic lighting schedule and mixed within each group irrespective of the day of surgery. Ten days later (11 days for half the animals), five to six rats from each group were killed at the indicated times and liver TAT was immediately assayed (Fig. 10B). The rhythm was profoundly modified as compared to that of the sham-operated controls. The levels of the enzyme activity from 0600 to 1400 hours were practically identical in the two groups of animals. But an early rise in activity after 1400 hours in the sham-operated rats with no parallel rise in the adrenalectomized rats resulted in significantly different levels for the two groups at 1800 ( $P < 0.05$ ) and 2200 hours ( $P < 0.05$ ). The subsequent fall in activity led to approximately equal activities at 0600 hours. The overall effect of adrenalectomy was a delayed rise and a halving in the range of activities. The overall effect is unlikely to originate from dietary differences (Table III). The consumption of food measured separately for the light and dark periods of the day were not statistically different for the two groups of rats. Moreover, the rate of growth for the two groups after surgery was not different (Table IV). The two groups were made up by random collection from the pool of rats averaging 197 gm in weight at the end of the 3-week step (Fig. 10A).

Table III

Food consumption of adrenalectomized and  
sham-operated rats on cyclic lighting schedule.

| OPERATION<br>PERFORMED | NUMBER<br>OF<br>RATS | FOOD CONSUMED*  |                 |
|------------------------|----------------------|-----------------|-----------------|
|                        |                      | LIGHT PERIOD    | DARK PERIOD     |
| Adrenalectomy          | 31                   | $0.29 \pm 0.07$ | $1.17 \pm 0.11$ |
| Sham-operation         | 34                   | $0.33 \pm 0.03$ | $1.36 \pm 0.06$ |

\* : g. food eaten/rat/hour  $\pm$  s.e.m.

Table IV

Effect of adrenalectomy or sham-operation on  
the growth of the rats.

| WEIGHT<br>BEFORE<br>OPERATION* | WEIGHT 10-11 DAYS AFTER OPERATION* |                      |
|--------------------------------|------------------------------------|----------------------|
|                                | ADRENALECTOMY                      | SHAM-OPERATION       |
| 197.7 $\pm$ 3.7 (36)**         | 239.9 $\pm$ 4.4 (31)               | 234.4 $\pm$ 4.5 (34) |

\* : g.  $\pm$  s.e.m.

\*\* : numbers in parentheses indicate the number of rats.

3. Comparative study of the hepatic TAT diurnal rhythm in other vertebrates.

a) Tadpoles.

A shipment of tadpoles was divided into two groups. One group was subjected to a cyclic lighting schedule (lights on from 0600 to 1800 hours) while the other group was maintained in constant light. The tadpoles were maintained in this environment for one month at the end of which groups were killed at the indicated times and their hepatic TAT was assayed. A rhythm was found in the tadpoles on the cyclic lighting schedule (Fig. 11A). Although the amplitude of the rhythm was not striking, the difference between the enzyme activity at 1400 hours and 0200 hours was significantly different ( $P < 0.01$ ). The tadpoles maintained in constant light displayed no such enzyme rhythm and no significant differences could be demonstrated for any pair of mean enzyme activities.

b) Frogs.

Hepatic TAT in frogs maintained for two months in a cyclic lighting environment (lights on from 0600 to 1800 hours) also displayed a diurnal rhythm (Fig. 11B). The peak in the enzyme occurred at 0200 hours and the low values obtained at 1000, 1400 and 1800 hours were significantly different from the high values at 0200 and 0600 hours. The amplitude of the rhythm in the frog was also much greater than in the tadpoles.

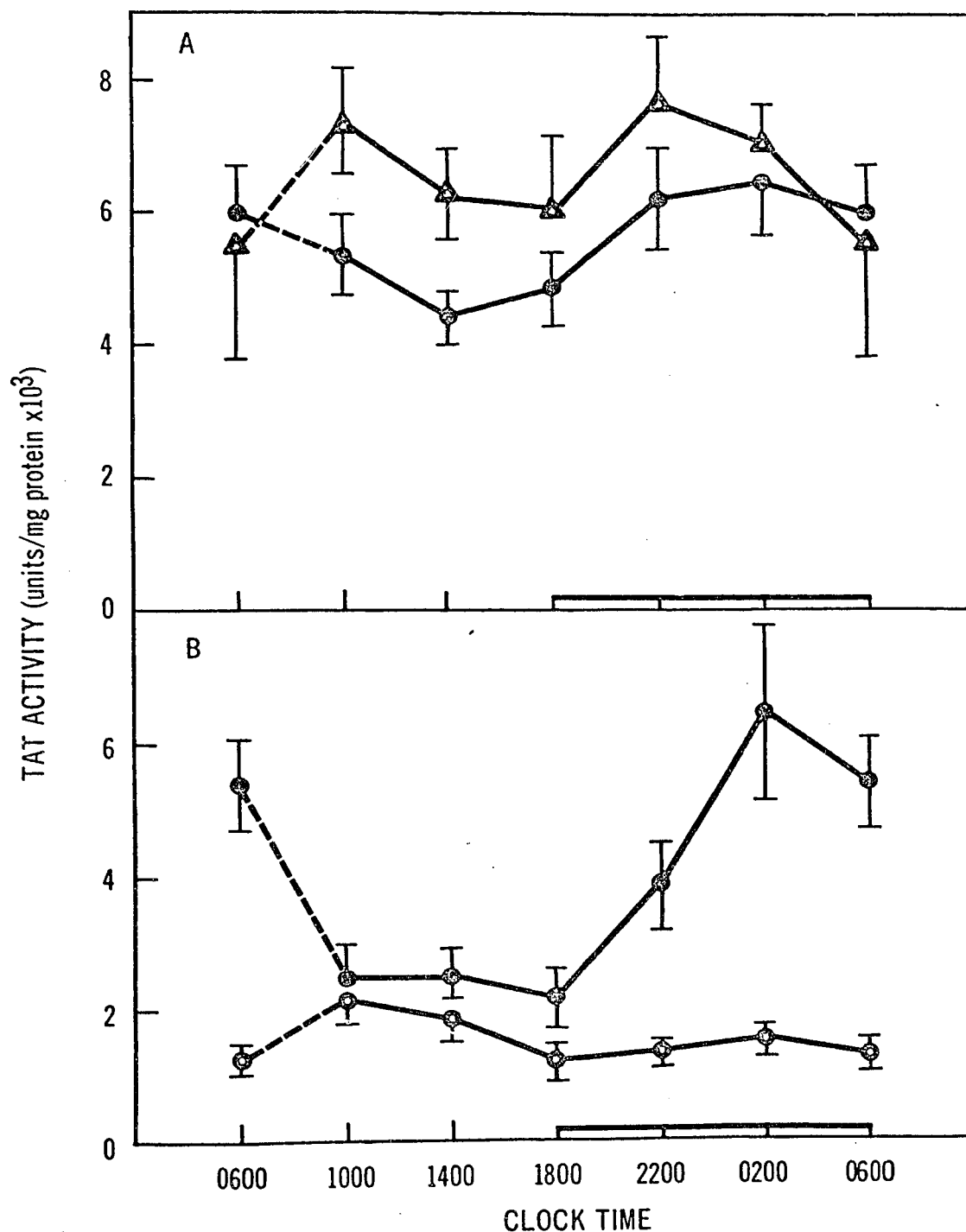


Fig. 11. Diurnal rhythm of hepatic TAT in tadpoles, frogs and pigeons. (A) Tadpoles were maintained in a cyclic lighting (●) or a constant light environment (Δ) for 4 weeks. (B) Frogs (●) and pigeons (○) were subjected to a cyclic lighting environment for 2 months. Whenever a cyclic lighting schedule was used the dark bar indicates the hours of darkness. The 24-hour cycle is completed with dashed lines. Points plotted represent means  $\pm$  s.e.m. (Appendix, Table 8).

c) Pigeons.

Pigeons were maintained in a cyclic lighting environment (lights on from 0600 to 1800 hours) for one month. A small rise in TAT in the morning hours (1000) was present which was significantly different from the later afternoon or evening values (Fig. 11B). When results were expressed in terms of unit tissue weight this small rise was not statistically significant. On the basis of these results a very weak diurnal rhythm appears to be present in pigeons.

4. Investigation of the responses of tadpole hepatic TAT to agents known to induce the enzyme in the rat.

Since pharmacological doses of cortisol have been shown to be ineffective on the bullfrog liver enzyme (Chan and Cohen, 1964), a similar study was conducted on tadpoles. The intraperitoneal administration of 200  $\mu$ g of cortisol was found to have no effect on hepatic TAT after 5 hours (Table V). Equally ineffective was corticosterone. Since glucagon was effective in amphibia (Rana catesbeiana) in raising blood glucose (Wright, 1959) this hormone was also included in the study. However, 50  $\mu$ g of glucagon was also ineffective as was a combined treatment of glucagon and cortisol.

Table V

TAT activity in tadpoles 5 hours after administration  
of cortisol, corticosterone and glucagon.

| SUBSTANCES<br>ADMINISTERED | AMOUNT<br>( $\mu$ g) | NUMBER<br>OF<br>TADPOLES | NUMBER OF<br>HOURS AFTER<br>INJECTION | TAT<br>ACTIVITY* |
|----------------------------|----------------------|--------------------------|---------------------------------------|------------------|
| None                       |                      | 6                        | 0                                     | $5.51 \pm 1.11$  |
| Saline                     |                      | 6                        | 5                                     | $6.76 \pm 1.05$  |
| Glucagon                   | 50                   | 6                        | 5                                     | $5.66 \pm 0.31$  |
| Cortisol                   | 200                  | 6                        | 5                                     | $7.14 \pm 1.09$  |
| Glucagon + Cortisol        | 50 + 200             | 5                        | 5                                     | $5.78 \pm 0.92$  |
| None                       |                      | 5                        | 0                                     | $8.54 \pm 2.05$  |
| Propylene glycol           | 40                   | 5                        | 5                                     | $7.79 \pm 1.60$  |
| Corticosterone             | 200                  | 5                        | 5                                     | $8.93 \pm 1.72$  |

\* : units/mg protein  $\times 10^3 \pm$  s.e.m.

## DISCUSSION

Since the adrenalectomies performed by other workers were on mature rats with an already entrained rhythm, a new approach was used by abolishing the rhythm with constant light followed by adrenalectomy. This approach was used to study the role of the adrenals in both the development of the rhythm in newborn rats and its operation in the adult rats.

A stimulation in hepatic TAT was reported to occur shortly after birth, with a peak in the enzyme appearing near the 12th hour (Sereni et al., 1959). The enzyme activity fell to the adult level over the next 36 hours. Reynolds and Potter (1970), however, showed that this rise was actually due to hypoglycemia caused by the removal of the rats from their mother at the time of birth. They showed that if the rats were left with their mother, no "explosive phase" could be observed. The results shown in Figure 7A for the one-day-old rats, taken from 24 to 36 hours after birth, show no indication that the activity is returning to a normal level after an "explosive phase". Thus our results tend to support the claim of Reynolds and Potter (1970) that the "explosive phase" reported by the other workers was, in fact, artifactual in nature.

The diurnal rhythm in TAT appears to begin to develop around the 10th day of age (Fig. 7C). The enzyme activity

at 2200 hours is significantly greater than the activities found during the light hours. In agreement with results for adult rats, the peak in activity occurs during the dark hours. This may possibly reflect the gradual development of a cyclic feeding habit (Siegel and Stackey, 1947) from the more diffuse one of the newborn (Gustaffson, 1948).

By the 15th day of age (Fig. 7D), the rhythm appears to be better defined since all of the activities obtained during the dark hours (2200, 0200, 0600) are significantly greater than those obtained during the light hours. However, not until the 21st day of age does the enzyme rhythm fully develop (Fig. 8A). The enzyme activities found at 1800, 2200 and 0200 hours are significantly greater than those found at 0600, 1000 and 1400 hours. The enzyme displays a four-fold change in activity with a peak occurring at 2200 hours in agreement with what has been reported for the adult rat (Civen et al., 1967b; Shambaugh et al., 1967; Wurtman and Axelrod, 1967).

The increase in the amplitude of the rhythm which develops sometime between the 15th and 21st day might be due to two factors. First, the 21-day-old rats had begun to eat the pelleted food, as evidenced by the fact that rats of this age will grow normally if removed from their mothers at this time. In addition gross examination of the stomachs of 21-day-old rats revealed some solid food present. Since the rat pellet diet is of higher protein content (30%) than

the mother's milk (10%) the increased amplitude of the enzyme rhythm could be explained on the basis of increased dietary protein intake. This is in agreement with the work of Potter et al. (1966) who reported a greater amplitude in the diurnal rhythm of hepatic TAT with a diet containing 30%-protein than with a diet containing 12%-protein.

Secondly, a rhythm in plasma corticosterone has been shown to develop about this time (Fiske and Leeman, 1964; Ulrich and Yuwiler, 1971) which might contribute to the development of the TAT diurnal rhythm. This is supported by the findings that an endogenous stimulation of TAT by a stress-mediated secretion of corticosterone can occur after the 2nd week of age (Franz and Knox, 1967). Further evidence for the involvement of plasma corticosterone in the development of the diurnal rhythm is provided by comparison of Figure 8A with Figures 7C and D. The enzyme activity in the 21-day-old rats started to rise well before the onset of darkness, the time when the plasma corticosterone rhythm rises, whereas in the younger rats (Figs. 7C and D) the rise only occurred after darkness.

That the enzyme rhythm is driven by the corticosterone rhythm is difficult to prove experimentally. If light was the synchronizer in the development of the various rhythms (cyclic feeding habits, plasma corticosterone rhythm and hepatic TAT rhythm), then in the absence of light no enzyme rhythm should develop. However, a rhythm with a reduced

amplitude did appear in this environment (Fig. 8B). This might have been due to the fact that a constant-darkness environment was impossible to maintain since the rats were exposed to light every second day around noon for the necessary changes of water and food.

In constant light the diurnal rhythm in hepatic TAT failed to develop (Fig. 8B). A correlation between the rhythm in plasma corticosterone and hepatic TAT is possible since constant light has been reported to abolish the corticosterone rhythm as well (Fiske and Leeman, 1964; Cheifetz et al., 1968). However this correlation might be coincidental since constant light may have also obliterated the cyclic feeding schedule of the rats. Indeed Cohn et al. (1970) have shown that feeding rats at hourly intervals significantly reduces the rhythm in intact rats and abolishes it in adrenalectomized rats.

Thus any attempt to consider the coupling of the hepatic TAT diurnal rhythm to the plasma corticosterone rhythm must depend upon evidence derived from other independent studies. For this reason studies were undertaken to examine the effect of adrenalectomy on the rats maintained for their first 21 days of life in a constant light environment. Adrenalectomy of these animals prevented the development of the enzyme rhythm as compared to their sham-operated controls (Fig. 9). This suggests that the cyclic secretion of glucocorticoids from the adrenal cortex may be one of the

factors necessary for the development of the TAT diurnal rhythm.

In another experiment, 3 weeks in constant light resulted in the abolition of the normal rhythm (Fig. 10A). Following adrenalectomy the rhythm reappeared upon transfer of the animals to a cyclic lighting schedule but with an amplitude only one half of that found in the sham-operated controls (Fig. 10B). This supports the premise that the adrenal gland, by virtue of the rhythmic secretion of corticosterone, might be one of the factors responsible for the generation of the diurnal rhythm in TAT.

The data of Table III on food consumption and of Table IV on growth rate rule out the possibility that the difference between the adrenalectomized and sham-operated rats was due to dietary factors.

Since the enzyme rhythm develops in adrenalectomized rats this suggests that the rhythmic secretion of corticosterone from the adrenal glands is not solely responsible for the enzyme rhythm. Black and Axelrod (1968a) suggested that the diurnal depletion and repletion of rat brain norepinephrine might be partly responsible for the diurnal rhythm in TAT. However evidence for the diurnal rhythm for this catecholamine in brain is conflicting. Walker (1967) demonstrated a diurnal rhythm for brain norepinephrine, peaking in the early morning hours and low in the evening. Scheving et al. (1968), on the other hand, found irregular fluctuations of the

catecholamine during the entire day with no tendency for a diurnal rhythm. In addition, Wurtman et al. (1968b) have shown that the diurnal rhythm in TAT still persisted after pretreating rats for 3 days with reserpine, which depletes the brain of norepinephrine. Thus it is unlikely, even if a diurnal rhythm exists for brain norepinephrine, that this catecholamine has any influence on the hepatic TAT diurnal rhythm.

The factor more likely to be responsible for the diurnal enzyme rhythm in adrenalectomized rats is the cyclic feeding habit of the rats (Siegel and Stackey, 1947; Zigmond et al., 1969). The importance of the rhythmic ingestion of food for the hepatic TAT diurnal rhythm was exemplified in the work of Cohn et al. (1970) who reported that the enzyme rhythm could be abolished by feeding adrenalectomized rats at hourly intervals instead of ad libitum. Thus the cyclic feeding habit of the rats appears to contribute to the hepatic TAT diurnal rhythm and may be responsible for the enzyme rhythm which develops in adrenalectomized rats. In addition a delayed rise and reduced amplitude in the enzyme activity was observed in these rats (Fig. 10B). This may have been due to the absence of corticosterone. The permissive action of this hormone in facilitating the entry of amino acids into the liver (Riggs, 1964) has been suggested to be one of the possible mechanisms whereby glucocorticoids might stimulate

certain hepatic enzymes (Schayer, 1967). Indeed Korner and Labrie (1967), Labrie and Korner (1968a) and Rosen and Milholland (1968) have reported a synergistic effect on hepatic TAT between amino acids and cortisol. Mavrides and Lane (1970) demonstrated the permissive role of cortisol in the stimulation of TAT by casein hydrolysate. Thus if the rats begin to eat toward the onset of darkness (Zigmond et al., 1969), the lack of glucocorticoids in the adrenalectomized rats would preclude any such permissive action of the hormone and the delayed entry of the nutrients into the cell would in turn delay the elevation in the enzyme. Even if the rats do not eat until after dark, the rising level of the glucocorticoids in the intact rats before darkness might mobilize some amino acids from the periphery (Friedberg and Greenberg, 1947; Clark, 1953; Silber and Porter, 1953; Aschkenasy and Wellers, 1959; Kaplan and Shimizu, 1963; Smith and Long, 1967), and the permissive action of the hormone would facilitate their entry into the liver, again explaining why the rise in TAT occurs sooner in intact than adrenalectomized rats.

Since a rhythmic feeding pattern in conjunction with the presence of an endogenous corticosterone rhythm could account in part for the rhythm in hepatic TAT in rats, the investigation was extended to other vertebrates such as amphibia and birds. A diurnal rhythm in hepatic TAT occurs in tadpoles with a peak occurring during the dark hours, and this rhythm can be desynchronized by constant light (Fig. 11A).

A similar rhythm occurs in the livers of frogs, while pigeons display a weak rhythm with the peak occurring during the early light hours (Fig. 11B).

Civen and co-workers (1967b) have hypothesized that a relationship may exist between an enzyme's response to glucocorticoids and its diurnal rhythmicity. Since corticosterone is the main glucocorticoid produced in pigeons (De Roos, 1960) and bullfrogs (Cartensen et al., 1961), demonstration that hepatic TAT in these vertebrates could be stimulated by this glucocorticoid would support the likelihood of such a relationship. Cortisol has been shown to stimulate hepatic TAT in pigeons (Chan and Cohen, 1964) suggesting a relationship between the enzyme rhythm in that species and the stimulation of TAT by glucocorticoids. However cortisol failed to stimulate hepatic TAT in bullfrogs (Chan and Cohen, 1964) and neither cortisol nor corticosterone has any effect on the enzyme in tadpoles (Table V). Thus these data do not support a correlation between the diurnal rhythm in hepatic TAT in amphibia (tadpoles and frogs) and the stimulation of the enzyme by pharmacological doses of the steroid.

The results thus support the conclusion that the adrenal gland is at least partly responsible for the development and operation of the diurnal rhythm of hepatic TAT in rats. This relationship may also be extended to birds but it is doubtful whether the correlation exists for

amphibia. Thus two factors responsible for the diurnal rhythm of hepatic TAT in rats are the cyclic feeding habits and the cyclic adrenal cortical secretion (Mavrides and Lane, 1970). Support of this has appeared in the literature (Cohn et al., 1970). The work involving the tadpoles, frogs and pigeons has also been published (Lane et al., 1970).

PART III

HORMONAL-DIETARY INTERACTIONS AND THE POSSIBLE ROLE OF  
GLUCONEOGENESIS IN THE REGULATION OF TYROSINE AMINOTRANSFERASE

## INTRODUCTION

Glucocorticoids, glucagon, high-protein diets, starvation and alloxan diabetes have been reported to increase the rate of gluconeogenesis (Kvam and Parks, 1960; Weber et al., 1961; Shrago et al., 1963; Young et al., 1964; Friedmann et al., 1965; Gracia et al., 1966; Sokal, 1966; Friedmann et al., 1967; Eisenstein and Strack, 1968; Freedland, 1968; Hurvitz and Freedland, 1968) as well as to stimulate the activity of hepatic TAT (Lin and Knox, 1957, 1958; Kenney, 1962c; Rosen et al., 1963; Goswami and Chatagner, 1966; Civen et al., 1967a; Csányi et al., 1967; Holten and Kenney, 1967; Yuwiler et al., 1969). Hence both hormonal and dietary means are characterized by their common action in stimulating both TAT and gluconeogenesis. If stimulation of the latter process by whatever means is responsible for the stimulation in TAT activity, then the magnitude of the enzyme response to hormonal administration would be subject to modification by changes in the dietary status of the animals (hormonal-dietary interactions).

In the following experiments the effect of altering the dietary status of the rats to the capacity of cortisol to stimulate TAT activity was studied. Diets with a varied protein content were used to alter the extent of hepatic gluconeogenesis. Non-pharmacological amounts of cortisol were administered in all experiments. The key enzyme for gluconeogenesis, PEPCK, was also studied in the same context

of hormonal-dietary interactions and a diurnal rhythm practically coincident with the TAT rhythm was revealed for this enzyme as well.

## MATERIALS AND METHODS

### 1. Chemical and material sources.

Venocath-18 catheters were purchased from Abbott Laboratories Limited (Montreal, Quebec). Portable infusion-withdrawal pumps (model 1100) were purchased from Harvard Apparatus Company (Dover, Massachusetts). Imidazole, PEP, IDP, GSH, NADH and malic acid dehydrogenase were products of Sigma Chemical Company (St. Louis, Missouri). Potassium bicarbonate, magnesium chloride and toluene were purchased from Fisher Scientific Company (St. Louis, Missouri). Sodium  $^{14}\text{C}$ -bicarbonate (4.7 mC/mmole) and 2,5-diphenyloxazole (PPO) were obtained from New England Nuclear (Boston, Massachusetts). Bio-Solv (formula BBS-3) was a product of Beckman Instruments Incorporated (Fullerton, California).

### 2. Animals.

Male rats (Sprague-Dawley strain, Canadian Breeding Laboratories, St. Constant, Quebec) weighing between 150 to 200 grams were placed immediately upon arrival in individual cages. Adrenalectomized rats, received within 2 to 3 days after the operation were maintained on 0.9% sodium chloride as drinking water and were used within 14 days of the

operation. Hypophysectomized rats were also received within 2 to 3 days after the operation and were given 10% dextrose as drinking water for 2 weeks. These rats were used between 1 and 2 months after the operation when their weights had stabilized. All animals were housed in a room with controlled lighting (lights on from 0600 to 1800 hours) and temperature ( $23 \pm 1^{\circ}\text{C}$ ). They were allowed food and water ad libitum. Except for the diurnal rhythm studies, all experiments were started at 0930 hours and terminated at 1530 hours.

For the diurnal rhythm studies male rats weighing approximately 60 grams were received and immediately divided into 2 groups. One group was placed in a room with the lights on from 0600 to 1800 hours, the other in a room with the lights on from 1800 to 0600 hours (reversed lighting schedule). The weight gain during the 3 week period until the day of the experiment was identical for both groups of rats. The normal changes of water and food for the rats on the reversed lighting schedule were conducted at night so as to insure that the treatment received by both groups of rats was as similar as possible. The animals were allowed a regular rat pellet diet ad libitum for 18 days at which time 1/3 were switched to a 0%-protein diet and 1/3 to a 60%-protein diet (3 days prior to the experimental day). Reversing the lighting schedule has been reported to also reverse the phases of the hepatic TAT rhythm (Black and

Axelrod, 1968) so that the peak in activity still occurred 4 hours into the dark period. This schedule thus allows for the study of the 24-hour rhythm within a 12-hour period. Rats from both groups were sacrificed at 1000, 1400 and 1800 hours. However for the sake of clarity in the figures, rats taken from the room in darkness at 1000, 1400 and 1800 hours will be assigned the clock time of 2200, 0200 and 0600 hours, respectively.

### 3. Diets.

Rats were normally fed a regular rat pellet diet (Master Feeds, Toronto, Ontario). This was a balanced diet containing approximately 22% protein and 8% amino acids. Control diets containing 0%-, 10%-, 30%- or 60%-protein were obtained from General Biochemicals (Chagrin Falls, Ohio). The composition of the protein-free diet was: 72% cane sugar, 15% corn starch, 8% corn oil, 4% salt mixture and 1% vitamins. Protein was added at the expense of cane sugar in the other 3 diets.

### 4. Anesthetized rats.

The technique used for anesthesia was the same as that described in Part I, Materials and Methods, with the following change. Before the first biopsy was taken the left or right femoral vein was exposed and catheterized. Blood was slowly withdrawn from the vein up to the end of the catheter and then returned into the vein with saline. The

catheter, now free of air, was connected to a 5 ml syringe attached to a infusion-withdrawal pump. The pump with a 3 r.p.m. motor and a 5-ml syringe will deliver 0.34 ml/hour. Before the infusion of the appropriate solution in isotonic saline was started, a zero time liver biopsy sample was excised.

#### 5. Substances administered intravenously.

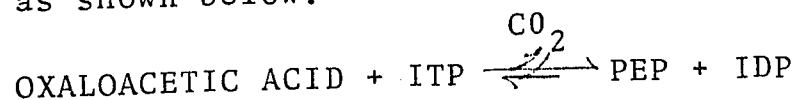
Cortisol and crystalline glucagon were made up to the correct concentration in sterilized isotonic saline solutions. All solutions were made up fresh before each experiment and the animals were numbered and weighed to correctly adjust the concentration of the infusion solutions before administration.

#### 6. Preparation of tissue samples, assay of TAT and calculations.

These were as described in Part I, Materials and Methods.

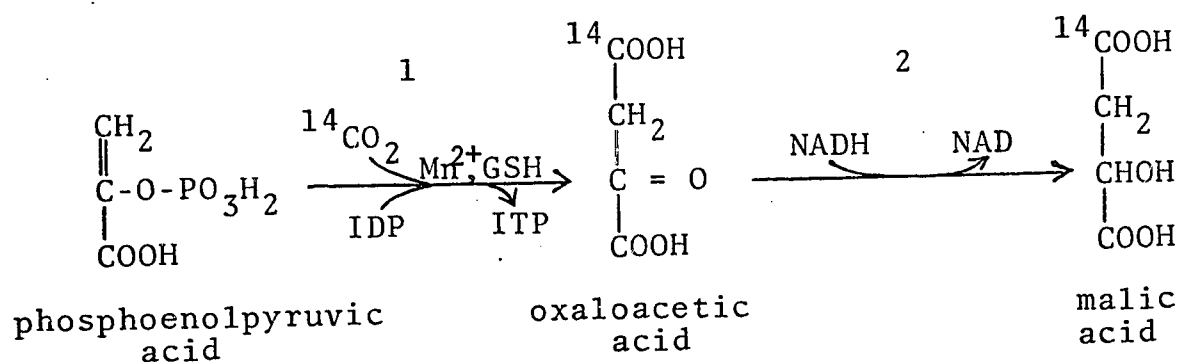
#### 7. Assay of phosphoenolpyruvate carboxykinase.

PEPCK catalyzes the conversion of oxaloacetic acid to PEP as shown below.



Chang and Lane (1966) recommended assaying the enzyme in the reverse direction by measuring the rate of incorporation

of radioactive carbon into oxaloacetic acid. Oxaloacetic acid is then converted to malic acid which is acid-stable. The entire reaction is shown below:



1...phosphoenolpyruvate carboxykinase

2...malic acid dehydrogenase

At the end of the incubation time any unreacted  $^{14}\text{C}$  is converted to  $^{14}\text{CO}_2$  by the addition of acid and driven off during the evaporation.

The preparation of the tissue samples for this assay was as described in Part I, Materials and Methods. PEP, IDP, GSH, NADH, malic acid dehydrogenase, potassium bicarbonate and magnesium chloride were made fresh each day by dissolving in distilled water. The scintillation mixture consisted of toluene containing 0.4% 2,5-diphenyloxazole and 10% Bio-Solv.

The assay mixture contained the following (in  $\mu$ moles unless otherwise expressed): imidazole buffer, pH 6.6, 100; PEP (neutralized before use), 1.25; IDP, 1.25; GSH, 2.0; NADH, 2.5; potassium bicarbonate, 50; sodium  $^{14}\text{C}$ -bicarbonate, 2  $\mu\text{C}$ ; magnesium chloride, 1.0; malic acid dehydrogenase, 1 unit; and the enzyme extract. The total volume of the assay was 1.0 ml. The mixture was preincubated for 15 minutes at  $30^{\circ}\text{C}$  and the reaction started by the addition of the enzyme preparation. The reaction was terminated after 15 minutes by the addition of 1 ml of 2N hydrochloric acid. Denatured protein was then removed by centrifugation (5,000 x g for 5 minutes). A 1.5 ml aliquot was pipetted into a liquid scintillation vial and the contents were evaporated to dryness under a stream of nitrogen in a water bath at  $75^{\circ}\text{C}$ . Then 0.5 ml of distilled water was added to the vial to dissolve the dry residue. This was followed by the addition of 10 ml of the liquid scintillation mixture and the contents were counted in a Beckman LS - 133 liquid scintillation counter.

a) Quench correction curve.

The quench correction curve was obtained using  $^{14}\text{C}$ -toluene of known radioactivity. A precise amount of  $^{14}\text{C}$ -toluene was pipetted into 16 vials containing the scintillation mixture. Increasing amounts of chloroform were added to each vial beginning with the second. The percent efficiency for each vial was calculated from their c.p.m./d.p.m. and this was

plotted versus the external standard ratio. A very slight curve was obtained (Fig. 12) which was considered for practical purposes to be a straight line. The equation for the line was calculated using the least squares formula described in Section 11.

b) Calculations.

The d.p.m. for a particular sample was calculated from the c.p.m. divided by the percent efficiency.

$$\text{d.p.m.} = \frac{\text{c.p.m.}}{\text{percent efficiency}}$$

After subtracting the d.p.m. of the blank from the d.p.m. of the sample, the  $\mu$ moles of product formed in the enzyme assay were calculated from the following equation:

$$\mu\text{moles of product formed} = \frac{\text{d.p.m. (product)} \times \mu\text{moles (substrate)}}{\text{d.p.m. (substrate)}}$$

Since the assay lasted 15 minutes and only 3/4 of the assay was evaporated to dryness in the vial, the following corrections were made to determine the actual units ( $\mu$ moles/minute) of enzyme present in the assay mixture.

$$\text{units of enzyme} = \mu\text{moles of product formed} \times 4/3 \times 1/15$$

c) Effect of pH and buffers.

Since the assay described by Chang and Lane (1966) was for pig liver mitochondria and not for rat liver cytoplasm, the pH optimum was checked using imidazole buffer as well as

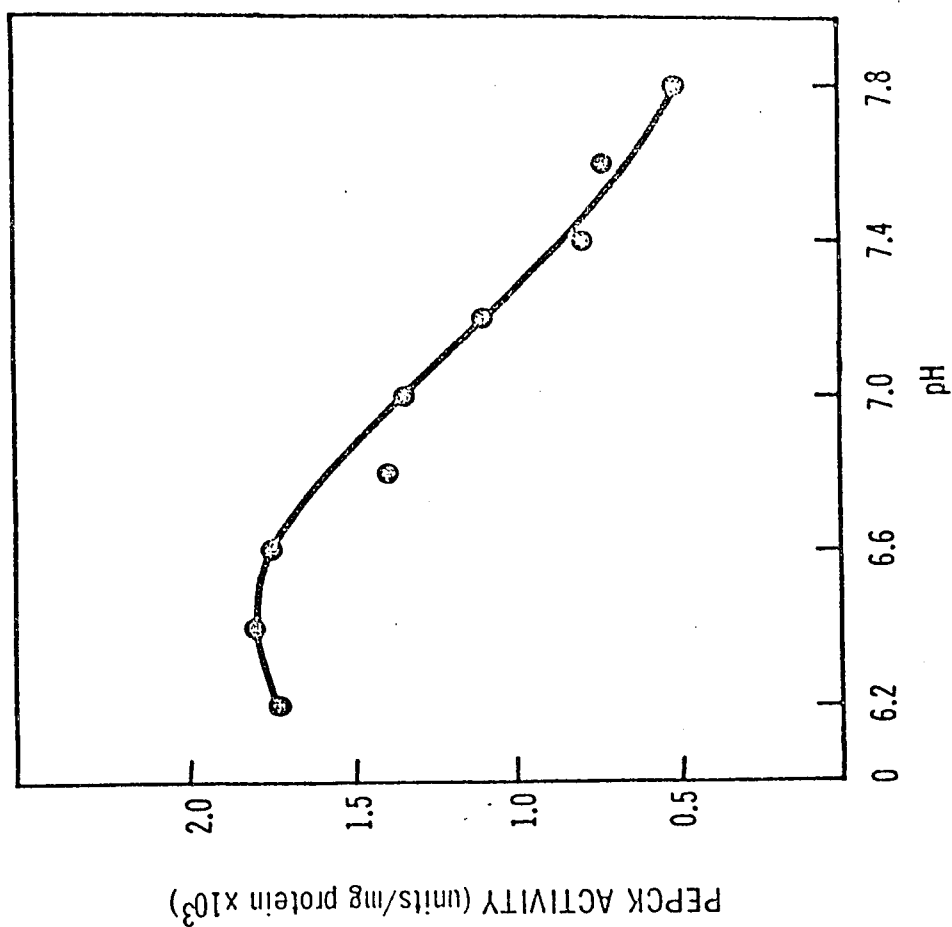


Fig. 13. PH profile of PEPCK using imidazole buffer.

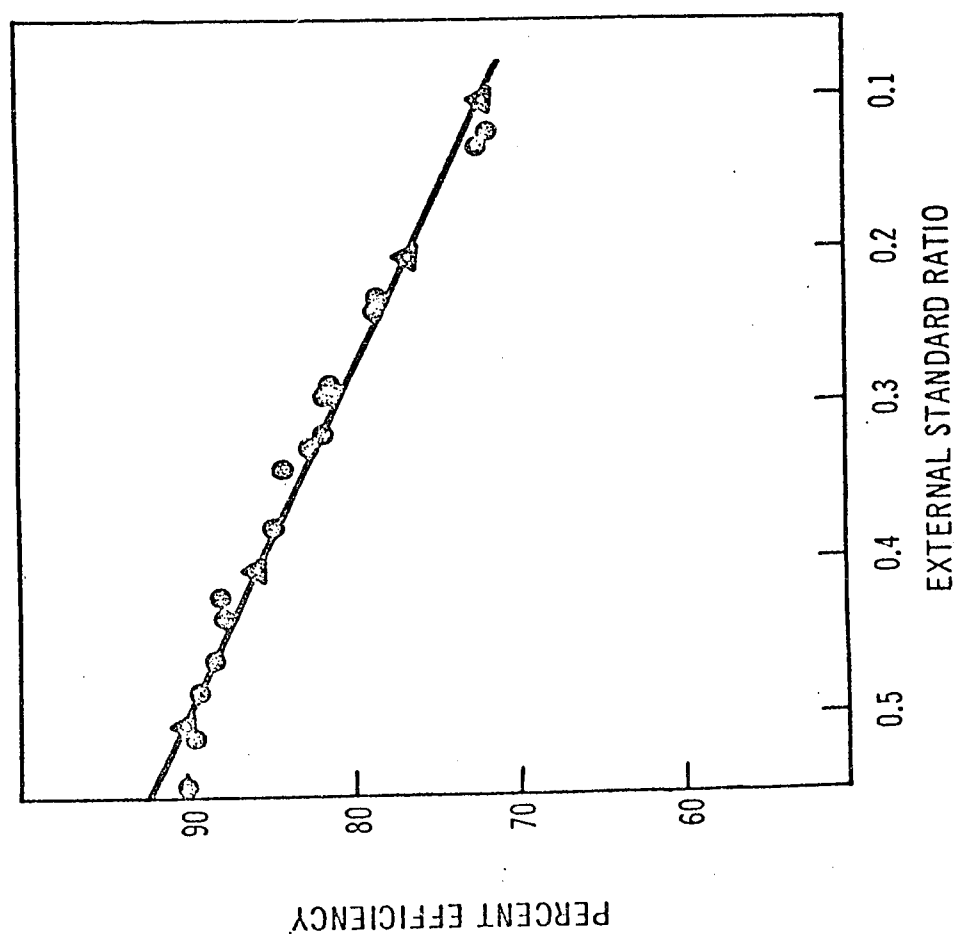


Fig. 12. Quench correction curve for PEPCK. Points calculated by method of least squares ( $\Delta$ ).

phosphate buffer. The optimum pH was found to be between 6.4 to 6.6 (Fig. 13), similar to pig liver mitochondria. Although phosphate buffer was found to be unsuitable as an assay buffer (1/3 the activity found with imidazole buffer at peak pH), the use of this buffer in the homogenizing medium did not interfere with the assay.

#### 8. Protein analysis.

This was as described in Part II, Materials and Methods.

#### 9. Glycogen analysis.

Liver glycogen was extracted by a method of Good et al. (1933) and estimated by the method of Morris (1948).

One hundred mg of liver was placed in a Pyrex test-tube containing 2 ml of 30% potassium hydroxide and the contents were heated for 20 minutes in a boiling water bath to dissolve the tissue. Following the addition of 2.5 ml of 95% alcohol and 0.2 ml saturated sodium sulfate solution, used to facilitate glycogen precipitation (Cook et al., 1968), the tubes were allowed to stand in ice for 20 minutes and were subsequently centrifuged at 3000 r.p.m. in a RC-2 Servall refrigerated centrifuge. Any excess alcohol remaining after decanting the supernatant was removed by returning the tube to the boiling water bath for a few seconds. The precipitate was then dissolved and made up to a known volume with water. A 2.5 ml aliquot was transferred to a Coleman tube (19 x 150 mm)

to which was added 5.0 ml of anthrone reagent (200 mg of anthrone in 100 ml of 95% sulfuric acid). The absorbance was read after 20 minutes at 620 nm in a Coleman Junior II Spectrophotometer.

a) Standard curve.

A stock solution of glycogen was diluted to give solutions of concentrations ranging from 10 - 60  $\mu$ g glycogen/ml. These solutions were used to construct the standard curve shown in Figure 14. A straight line was derived using the least squares formula as described in Section 11.

10. Plasma cortisol analysis.

Plasma cortisol was measured by the fluorometric method of Guillemin et al. (1959a) with the aid of an Aminco-Bowman Spectrophotometer.

a) Preparation of plasma.

At the end of the 2 hour infusion rats were killed by decapitation and trunk blood from each rat was collected into heparinized tubes. The tubes were then centrifuged in an International Clinical Centrifuge to separate the plasma from the red blood cells. The top plasma layers were then transferred to other tubes which were kept on the ice until used for analysis.

b) Analysis.

Duplicate analyses were performed on each sample to

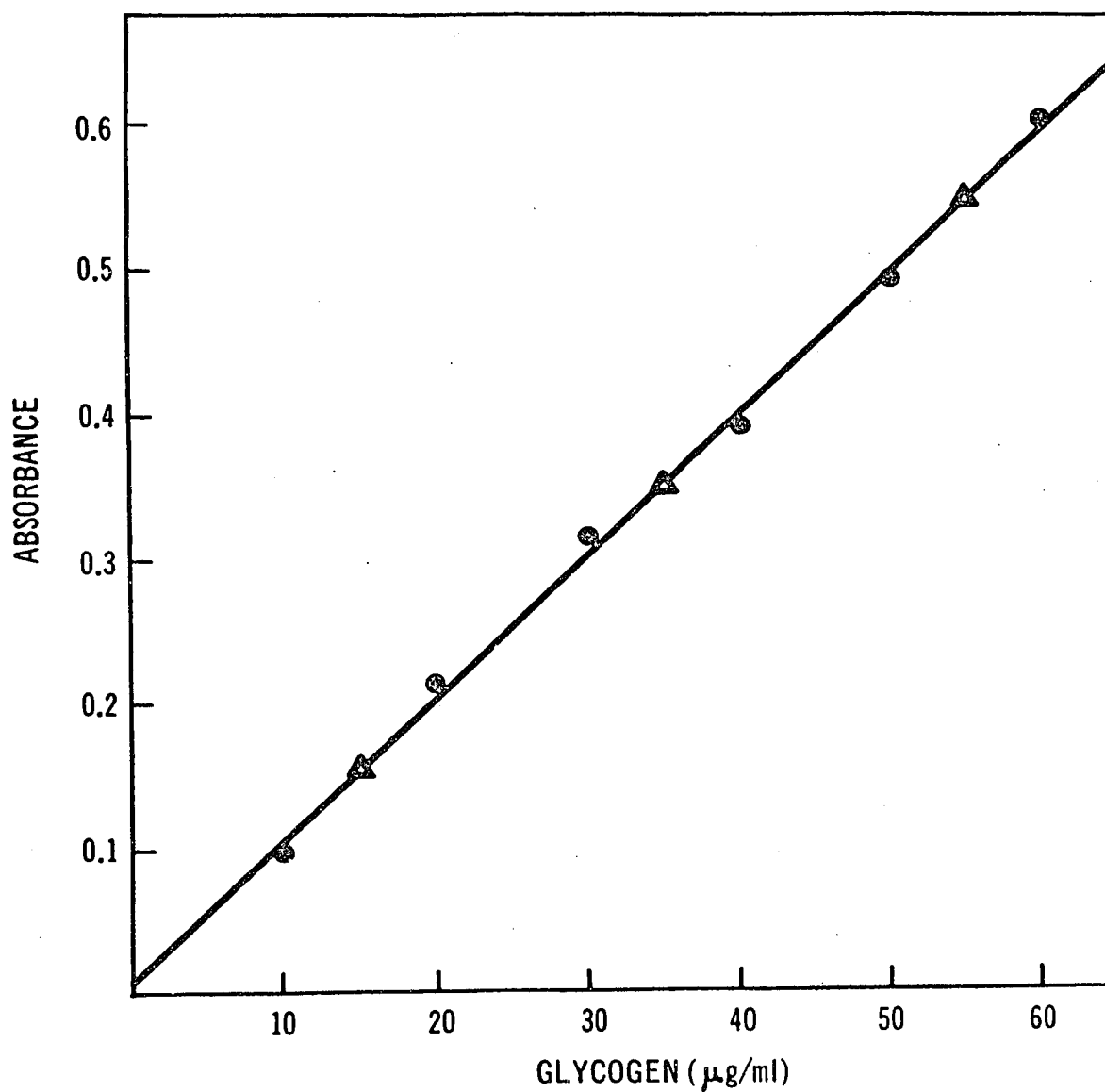


Fig. 14. Glycogen standard curve. Points calculated by method of least squares ( $\Delta$ ).

assure a greater accuracy. The plasma (0.5 ml) was pipetted into a 15.0 ml centrifuge tube and made to 2.0 ml with distilled water. The tube was shaken with 0.4 ml of iso-octane to remove the lipids. After centrifugation the upper iso-octane layer was removed by aspiration. To the bottom layer was added 2.0 ml of distilled water and the mixture was shaken for 30 seconds with 5.0 ml of chloroform. The aqueous layer was discarded and the chloroform layer was shaken as previously with 0.5 ml of 0.1N NaOH to remove estrogens and other phenolic substances. After centrifugation, the sodium hydroxide layer was removed. Part of the chloroform extract (4 ml) was transferred to another 15 ml centrifuge tube containing 1.2 ml of 30N sulfuric acid. The tube was stoppered, shaken vigorously for 30 seconds and centrifuged as before. After a 30 minute period 1.0 ml of the sulfuric acid extract was transferred to a round quartz cuvette (1.0 ml capacity) and the fluorescence was measured. The activation wavelength was 480 nm and the emission wavelength was 545 nm.

c) Standard curve.

The amount of cortisol in the plasma samples was proportional to the fluorescence measured and was expressed as  $\mu\text{g}$  cortisol/100 ml plasma with the aid of standard curve (Fig. 15). The equation for the line was determined by the least squares formula as described in Section 11.

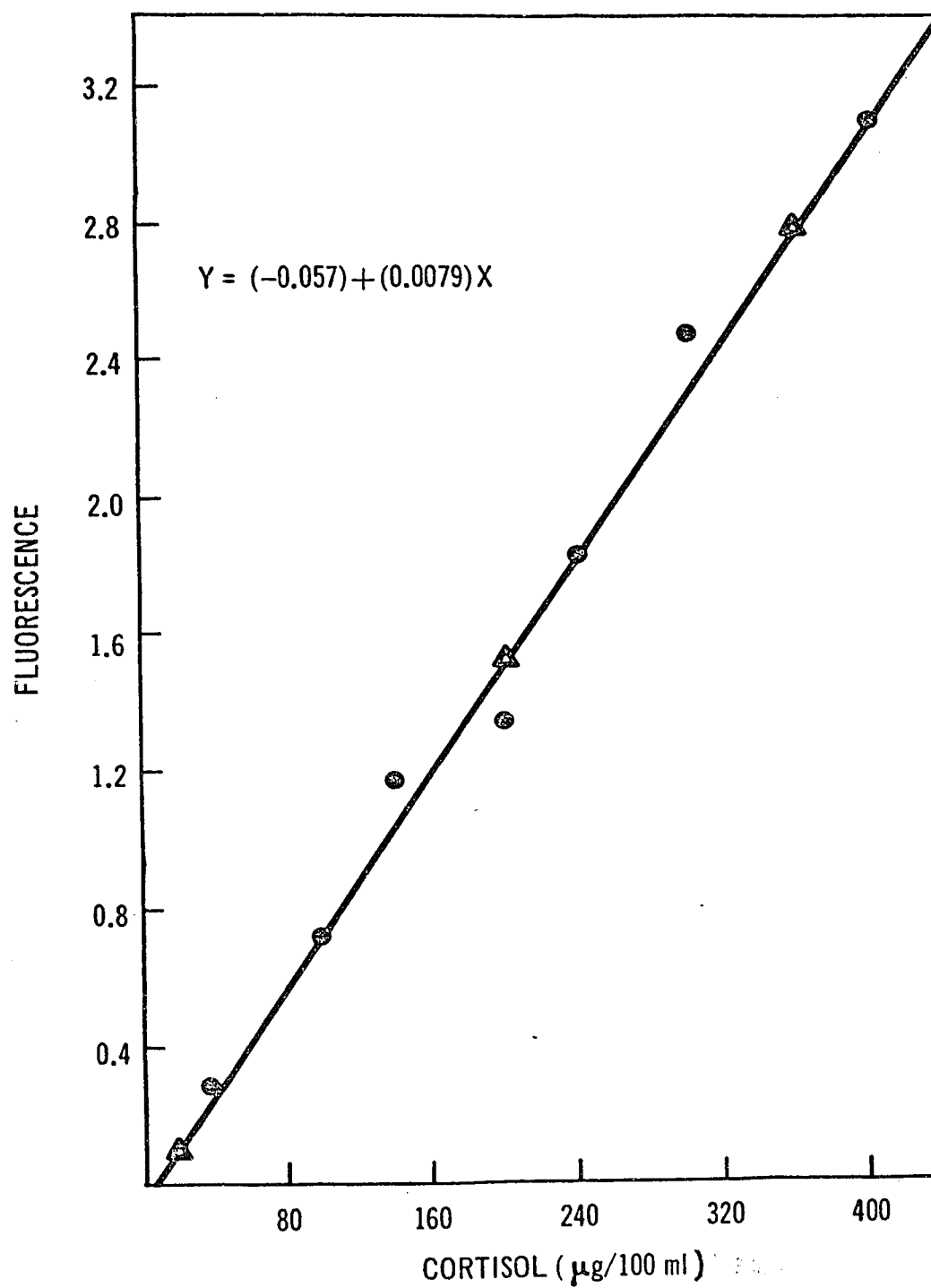


Fig. 15. Plasma cortisol standard curve.  
Points calculated by method of least squares ( $\Delta$ ).

d) Selection of activation and emission wavelengths.

The activation and emission wavelengths used by Guillemin et al. (1959a) in a Farrand Fluorometer were 436 and 530 nm respectively while Glick et al. (1964) in an Aminco-Bowman Spectrophotometer used 462 and 518 nm respectively. Using a standard solution of cortisol the activating wavelength was kept constant at 462 nm while the emission wavelength was varied until a peak in fluorescence was noted. The peak occurred at 545 nm (Fig. 16A). Then while keeping the emission wavelength constant at 545 nm, the activating wavelength was varied until a peak at 480 nm was noted (Fig. 16B). When the same procedure was applied to the blank, little change in the fluorescence was observed (Figs 16A and B).

11. Statistical analysis.

This was as described in Part I, Materials and Methods, but includes the least squares formulae reported by Croxton (1953). The formula for calculating the equation of a line with linear co-ordinates was:

$$Y = a + bX$$

where X is the abscissa and Y is the ordinate, a is the Y intercept and b is the slope of the line. To determine the value for a and b the following 2 equations were used:

$$\Sigma Y = Na + b\Sigma X$$

$$\Sigma XY = a\Sigma X + b\Sigma X^2$$

where N is the number of points in the line. Solving for

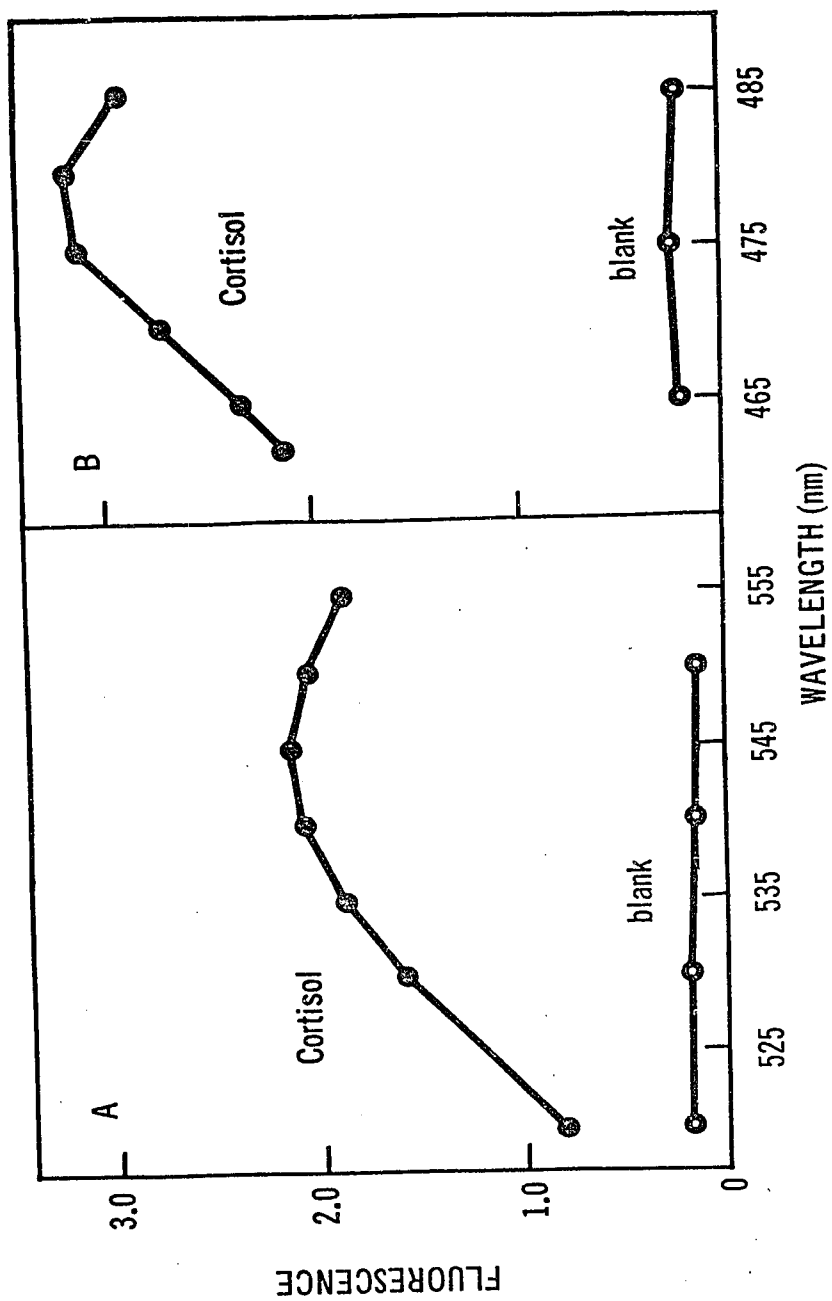


Fig. 16. Selection of emission (A) and activation (B) wavelengths for plasma cortisol analysis.

a and b the following 2 equations were obtained:

$$a = \frac{\Sigma X^2 \Sigma Y - \Sigma XY \Sigma X}{N \Sigma X^2 - (\Sigma X)^2}$$

$$b = \frac{N \Sigma XY - \Sigma X \Sigma Y}{N \Sigma X^2 - (\Sigma X)^2}$$

Where logarithmic co-ordinates were used the equation was altered to:

$$\log Y = \log a + b \log X$$

the value for a and b was calculated from the following 2 equations:

$$\Sigma \log Y = N \log a + b \Sigma \log X$$

$$\Sigma (\log X \log Y) = \log a \Sigma \log X + b \Sigma (\log X)^2$$

Solving for a and b:

$$\log a = \frac{\Sigma \log Y \Sigma (\log X)^2 - \Sigma \log X \Sigma (\log X \log Y)}{N \Sigma (\log X)^2 - (\Sigma \log X)^2}$$

$$b = \frac{N \Sigma (\log X \log Y) - \Sigma \log X \Sigma \log Y}{N \Sigma (\log X)^2 - (\Sigma \log X)^2}$$

## RESULTS

### 1. The effect of cortisol infusion on hepatic TAT activity in rats fed controlled protein diets.

Adrenalectomized and hypophysectomized rats were fed ad libitum a 0%-, 10%-, 30%- or 60%-protein diet for 5 days. On the morning of the experiment the rats were anesthetized and infused with 35 µg of cortisol/200 g body weight over

a 2-hour period. Liver biopsies were taken at the start and again at the 2nd, 4th, 5th, and 6th hours. The initial TAT activities in both groups of rats were greatest in the rats fed the 60%-protein diet (Table VI). As the protein content of the diet decreased the initial enzyme activities also decreased. For each diet except the 60%-protein diet the enzyme activities between the two groups of rats did not differ significantly. However the enzyme activity in the hypophysectomized rats fed the 60%-protein diet was more than twice that found in the adrenalectomized rats fed the same diet. The percent stimulation over the initial TAT activity following the cortisol infusion was greatest in the rats fed the protein-free diet independent of whether the rats were adrenalectomized or hypophysectomized (Fig. 17). As the content of dietary protein increased, the percent stimulation in both groups decreased. Although saline infusion alone had no significant effect on the enzyme activity in adrenalectomized rats, hypophysectomized rats showed a graded response which was dependent upon the protein content of the diet (Fig. 18). However, even allowing for the effect of saline in the hypophysectomized groups, the percent stimulation for any particular diet was still greater in this group.

## 2. Law of initial values.

Wilder formulated the "law of initial values" which states that the response to a given stimulus is inversely

Table VI

Effect of dietary protein on hepatic TAT in  
adrenalectomized and hypophysectomized rats fed  
controlled protein diets for 5 days.

| PERCENT<br>DIETARY<br>PROTEIN | TAT activity*            |                           | SIGNIFICANCE** |
|-------------------------------|--------------------------|---------------------------|----------------|
|                               | ADRENALECTOMIZED<br>RATS | HYPOPHYSECTOMIZED<br>RATS |                |
| 0                             | 2.07 ± 0.17 (13)***      | 1.85 ± 0.11 (14)          | P < 0.30       |
| 10                            | 2.43 ± 0.22 (19)         | 2.50 ± 0.24 (14)          | P < 0.90       |
| 30                            | 3.58 ± 0.43 (14)         | 4.70 ± 0.45 (14)          | P < 0.10       |
| 60                            | 4.85 ± 0.55 (12)         | 10.05 ± 1.31 (12)         | P < 0.005      |

\* : units/mg protein x 10<sup>3</sup> ± s.e.m.

\*\* : between groups fed same diet.

\*\*\* : numbers in parentheses indicate number of rats.

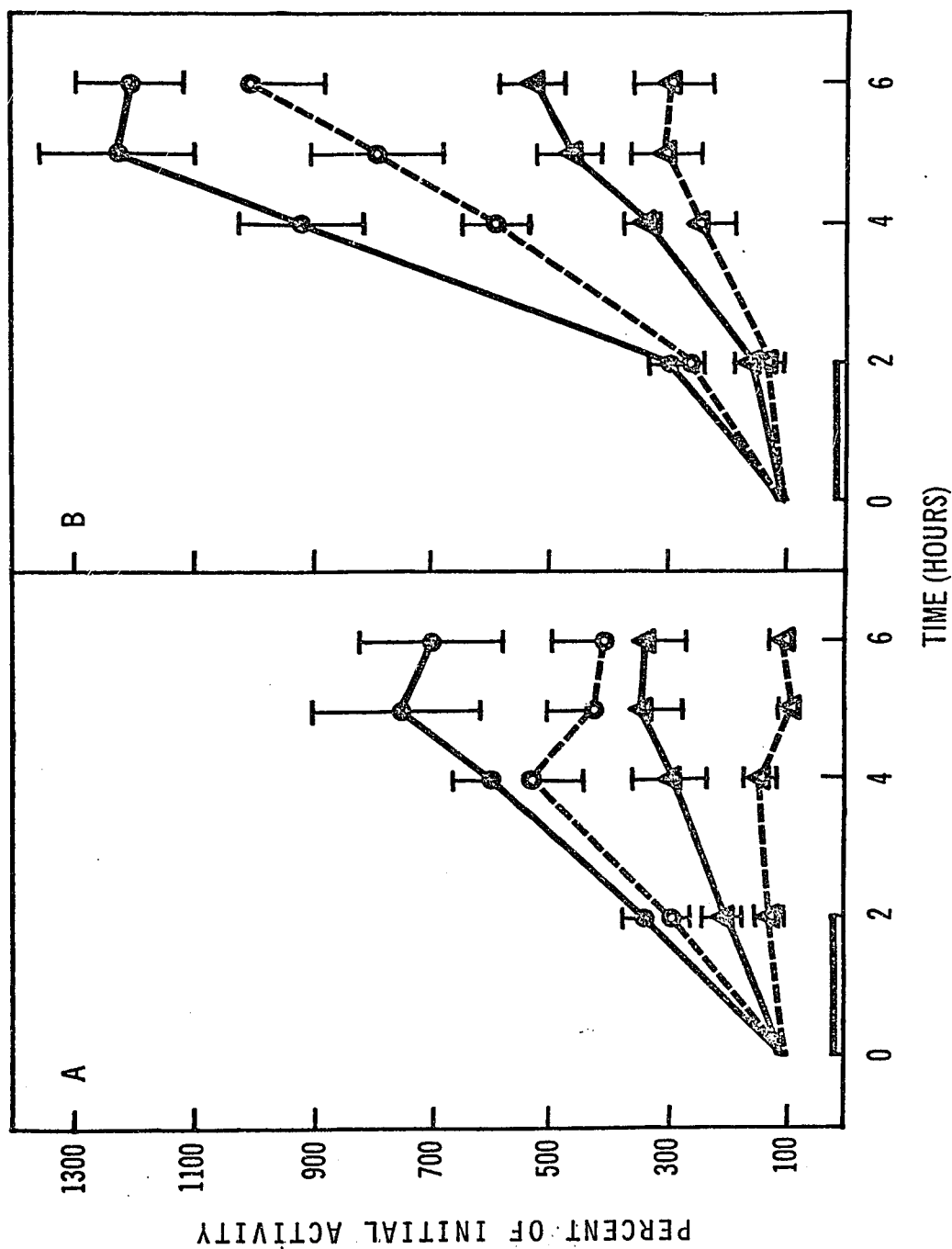


Fig. 17. Effect of infused cortisol (35 µg/200 g body weight) on hepatic TAT in anesthetized adrenalectomized (A) and hypophysectomized rats (B) fed a 0%-(o), 10%-(o), 30%-(Δ) or 60%-(Δ) protein diet for 5 days. Dark bar indicates time for cortisol infusion. Points plotted represent means  $\pm$  s.e.m. (Appendix, Tables 9-16).

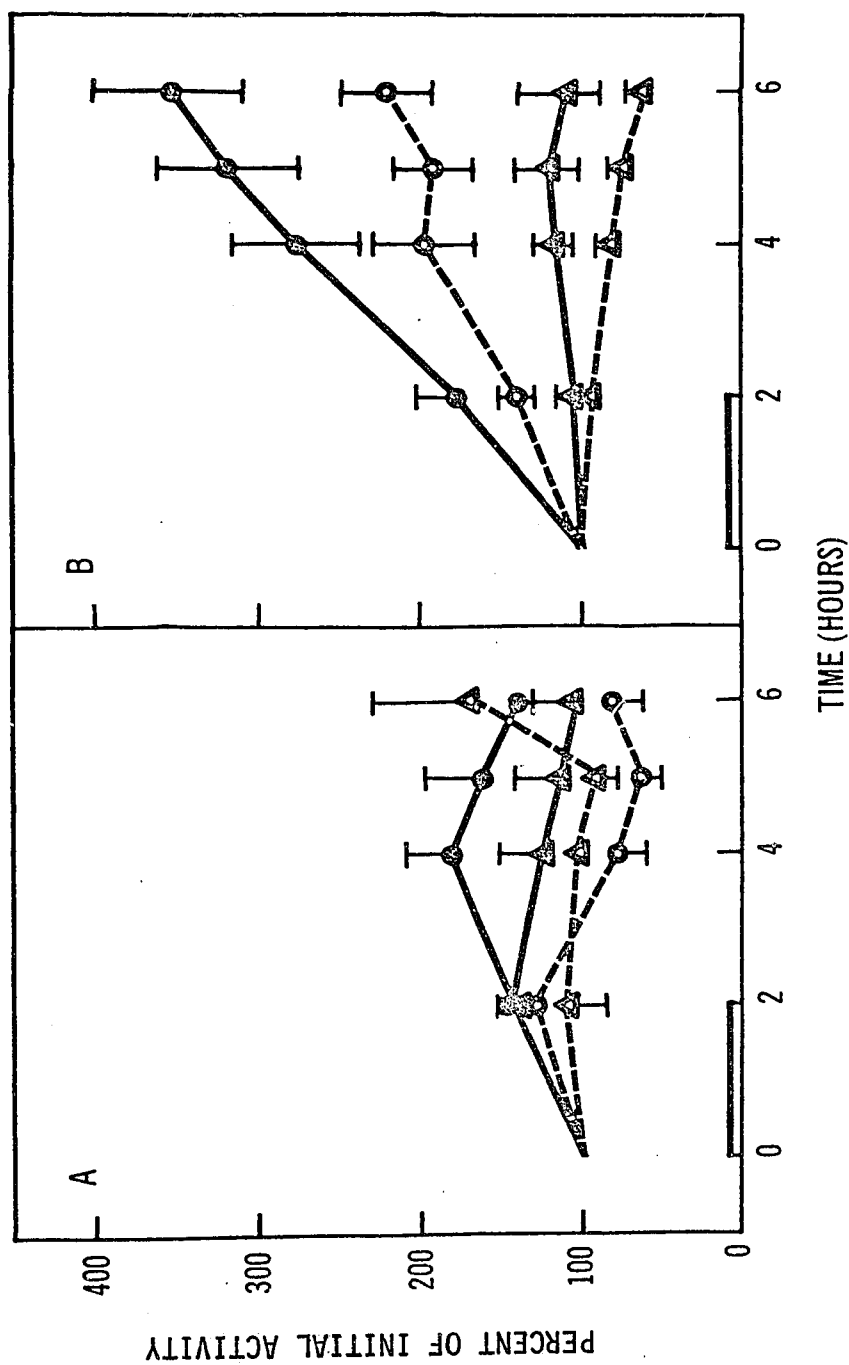


Fig. 18. Effect of infused saline (0.64 ml) on hepatic TAT in anesthetized adrenalectomized (A) and hypophysectomized rats (B) fed a 0%- (○), 10%- (◐), 30%- (Δ) or 60% protein diet for 5 days. Dark bar indicates time of saline infusion. Points plotted represent means  $\pm$  s.e.m. (Appendix, Table 9-16).

proportional to the initial value of the parameter being measured (Wilder, 1962). In the above experiments the stimulus was infused cortisol while the parameter was hepatic TAT activity. Thus if the law was to be obeyed the greatest response should have occurred in the rats with the lowest initial enzyme activities. Scatter plots, constructed on linear graph paper, revealed that the greatest percent stimulations did occur in the rats with the lowest initial enzyme activities (Fig. 19). These plots were made possible because of the design of the experiment in which each rat served as its own control. Since the points plotted resembled a hyperbola, the points were replotted on logarithmic graph paper where they appeared to have a fairly linear relationship (Fig. 20). Using the logarithmic coordinates lines of best fit for both groups of rats were derived from the method of least squares and these lines were drawn into figures 19 and 20.

### 3. Studies concerning the rates of metabolism of the infused cortisol.

Adrenalectomized and hypophysectomized rats, previously fed a controlled protein diet for 5 days, were infused with 200  $\mu$ g of cortisol/200 g body weight. At the end of the 2 hour infusion the rats were decapitated and trunk blood collected into heparinized tubes. No significant differences in the infused plasma cortisol concentration could be detected

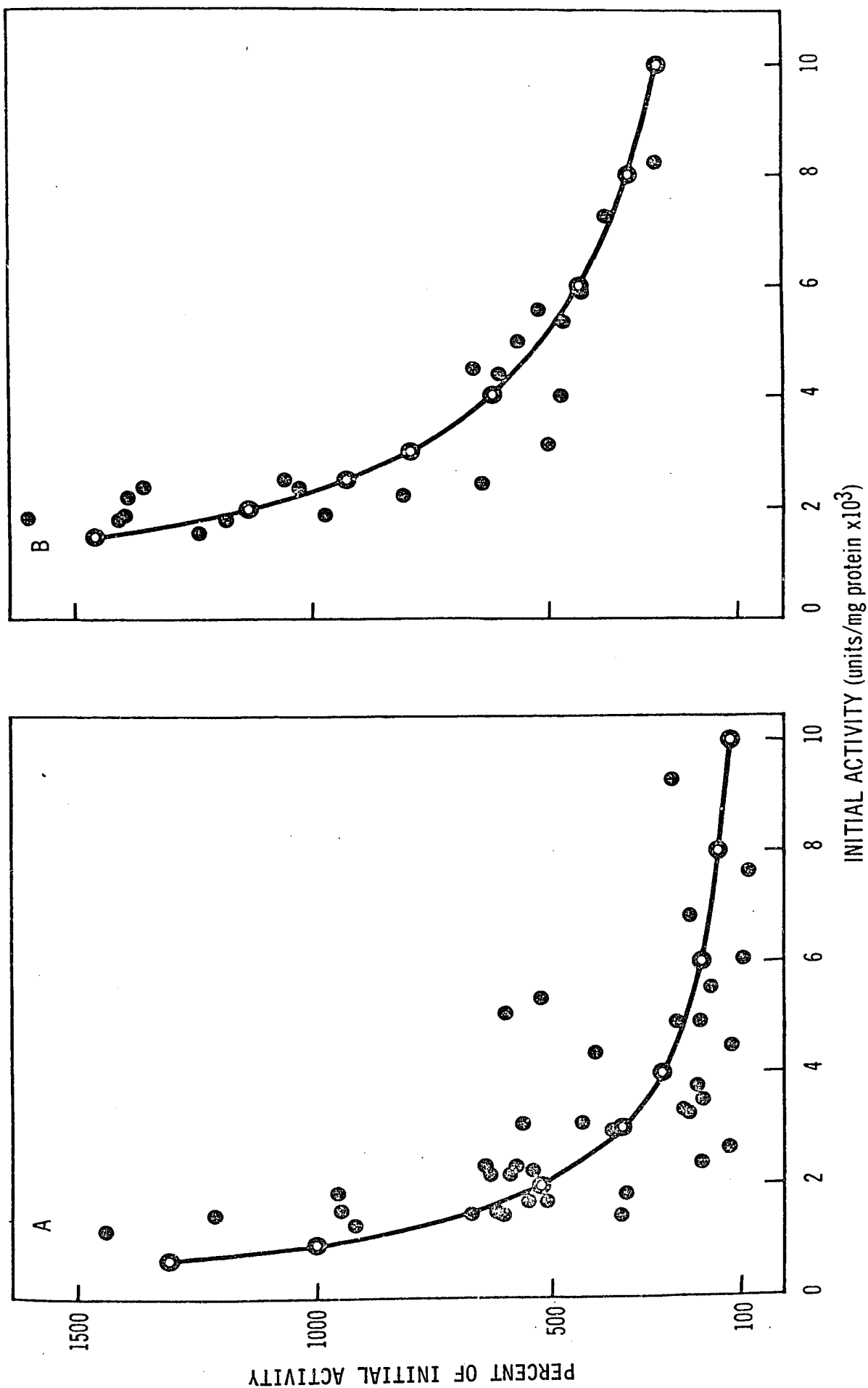


Fig. 19. Linear scatter plots of initial TAT activities versus maximum percent cortisol stimulation in adrenalectomized (A) and hypophysectomized rats (B) fed controlled protein diets for 5 days. Points calculated by method of least squares (o).

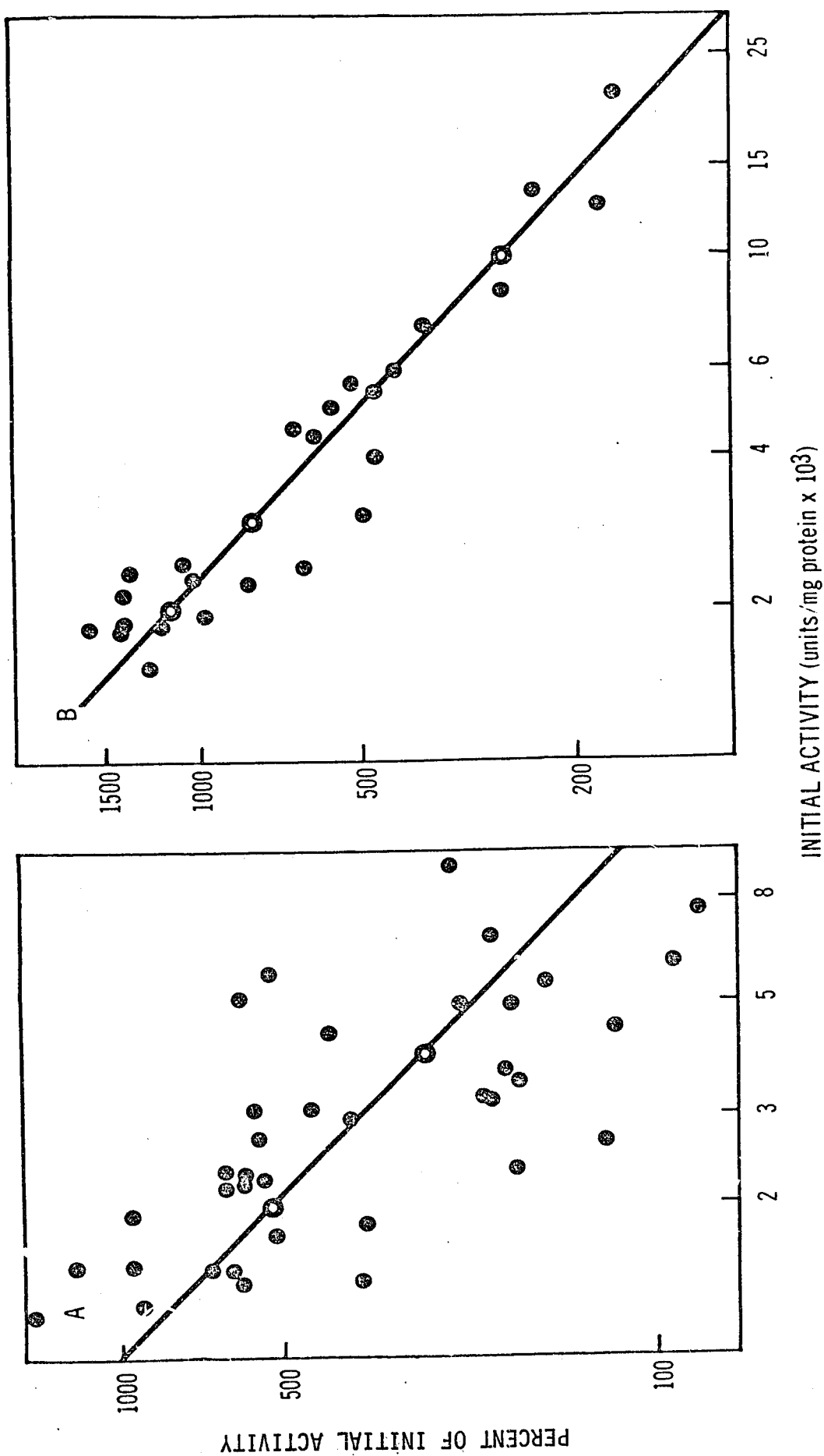


Fig. 20. Logarithmic scatter plots of initial TAT activities versus maximum percent cortisol stimulation in adrenalectomized (A) and hypophysectomized rats (B) fed controlled protein diets for 5 days. Points calculated by method of least squares (o).

between the adrenalectomized and hypophysectomized rats previously fed the same diet (Table VII). The plasma cortisol levels for the hypophysectomized rats did not even reflect a tendency to be higher than those found in the adrenalectomized rats. In fact, in three of the hypophysectomized groups (0%-, 30%- and 60%-protein), they were even slightly lower.

4. The effect of glucagon infusion on hepatic TAT activity in rats fed controlled protein diets.

Adrenalectomized and hypophysectomized rats, fed for 5 days a controlled diet consisting of 0%- or 60%-protein, were infused with 1 mg of glucagon/200 g body weight over a period of 2 hours. Liver biopsies were taken at the start of the infusion and again at the 2nd, 4th, 5th and 6th hours. Glucagon stimulation was found to obey the same dietary restrictions as the cortisol stimulation, that is, the effect on the enzyme was greater in the rats fed the protein-free diet than in the rats fed the 60%-protein diet (Fig. 21). In addition, similar to what was found with the cortisol stimulation, the percent stimulation due to glucagon infusion was lower in adrenalectomized than hypophysectomized rats fed the same diet.

5. The effect of cortisol infusion on hepatic TAT and PEPCK activities in hypophysectomized rats fed controlled protein diets.

Table VII

Plasma cortisol concentration in adrenalectomized and hypophysectomized rats after a 2 hour infusion of cortisol (200  $\mu$ g/200 g body weight).

| PERCENT<br>DIETARY<br>PROTEIN | PLASMA CORTISOL*         |                           | SIGNIFICANCE** |
|-------------------------------|--------------------------|---------------------------|----------------|
|                               | ADRENALECTOMIZED<br>RATS | HYPOPHYSECTOMIZED<br>RATS |                |
| 0                             | 121.5 $\pm$ 7.2 (6)***   | 131.8 $\pm$ 4.4 (5)       | P < 0.30       |
| 10                            | 154.7 $\pm$ 10.0 (6)     | 131.8 $\pm$ 5.7 (6)       | P < 0.10       |
| 30                            | 110.7 $\pm$ 6.6 (6)      | 100.8 $\pm$ 7.5 (6)       | P < 0.40       |
| 60                            | 113.7 $\pm$ 10.8 (6)     | 94.5 $\pm$ 5.3 (6)        | P < 0.30       |

\* :  $\mu$ g/100 ml plasma  $\pm$  s.e.m.

\*\* : between groups fed same diet.

\*\*\*: numbers in parentheses indicate the number of rats.

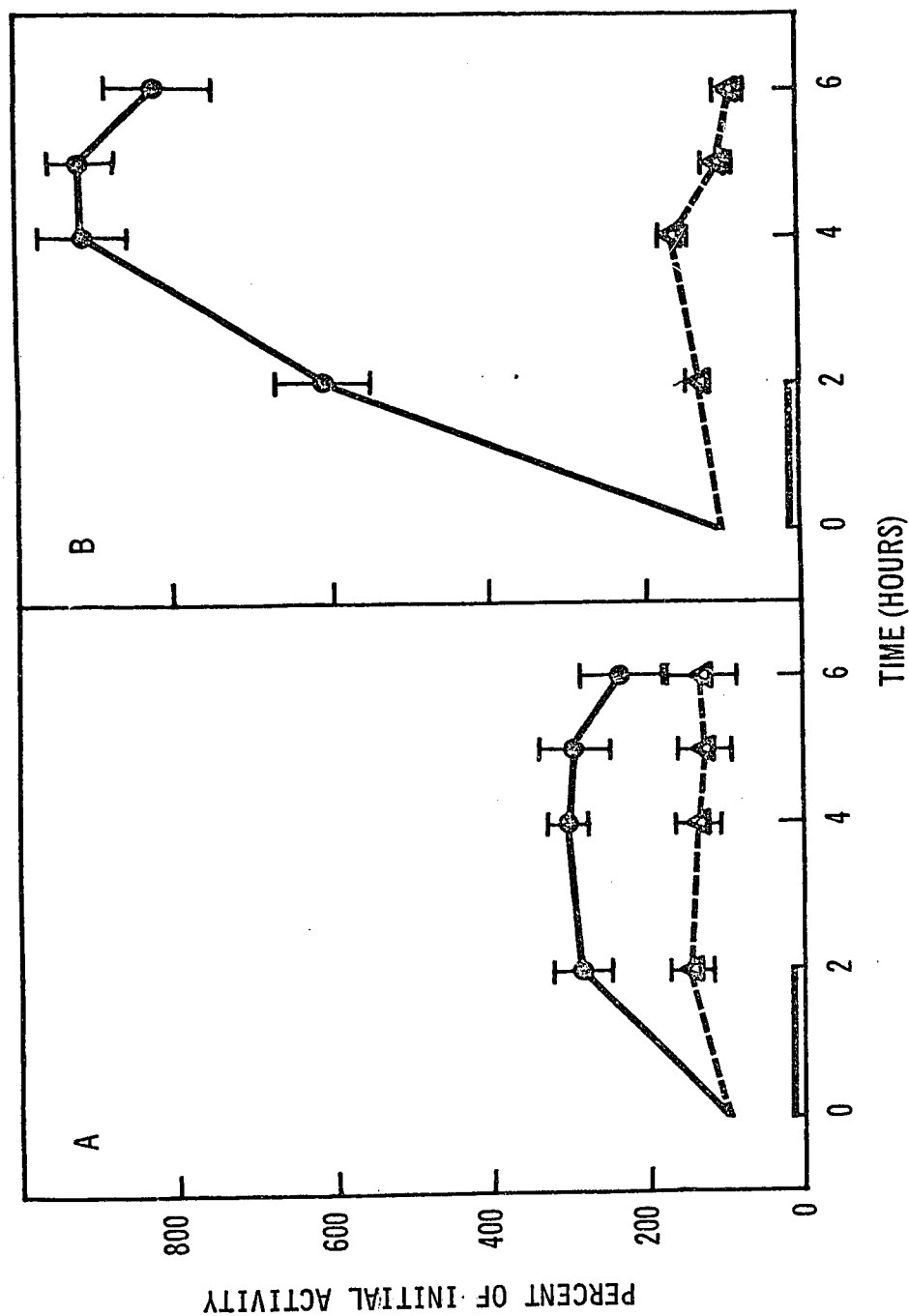


Fig. 21. Effect of infused glucagon (1 mg/200 g body weight) on hepatic TAT in anesthetized adrenalectomized (A) and hypophysectomized rats (B) fed a 0%- (●) or 60%- (△) protein diet for 5 days. Dark bar indicates time of glucagon infusion. Points plotted represents means  $\pm$  s.e.m. (Appendix, Tables 17 and 18).

Hypophysectomized rats, fed for 3 days a controlled diet consisting of 0%- or 60%-protein, were infused with 35  $\mu$ g of cortisol/200 g body weight. Liver biopsies were taken at the start of the infusion and again at the 1st, 2nd, 4th and 6th hours. Similar to TAT, the initial activity of PEPCK was found to be dependent upon the dietary status of the animal, being low in the rats fed the protein-free diet and high in the rats fed the 60%-protein diet (Table VIII). In addition the percent cortisol stimulation of hepatic PEPCK was found to be subject to the same dietary restrictions as the stimulation of TAT, that is, greater in rats fed the protein-free diet than in rats fed the 60%-protein diet (Fig. 22). The stimulation of PEPCK in the saline controls was almost negligible in the rats fed the 60%-protein diet and only slight in the rats fed the protein-free diet (Appendix, Tables 20 and 23). In addition to the two enzyme activities, hepatic glycogen content was also determined on pieces of liver biopsied at the previously stated time intervals. The liver glycogen content in the rats fed either diet was found to decrease during the course of the 6 hour experimental period (Fig. 22). Although the rate of glycogen depletion was similar for both groups of rats, the infused cortisol appeared to delay its depletion as compared to the saline controls (Appendix, Tables 21 and 24).

Table VIII

Effect of dietary protein on hepatic TAT and  
PEPCK in hypophysectomized rats fed  
controlled protein diets for 3 days.

| PERCENT<br>DIETARY<br>PROTEIN | number<br>of<br>rats | ENZYME ACTIVITY* |              |
|-------------------------------|----------------------|------------------|--------------|
|                               |                      | TAT              | PEPCK        |
| 0                             | 11                   | 2.05 ± 0.22      | 3.80 ± 0.46  |
| 60                            | 10                   | 12.23 ± 1.31     | 10.09 ± 1.00 |

\* : units/mg protein x 10<sup>3</sup> ± s.e.m.

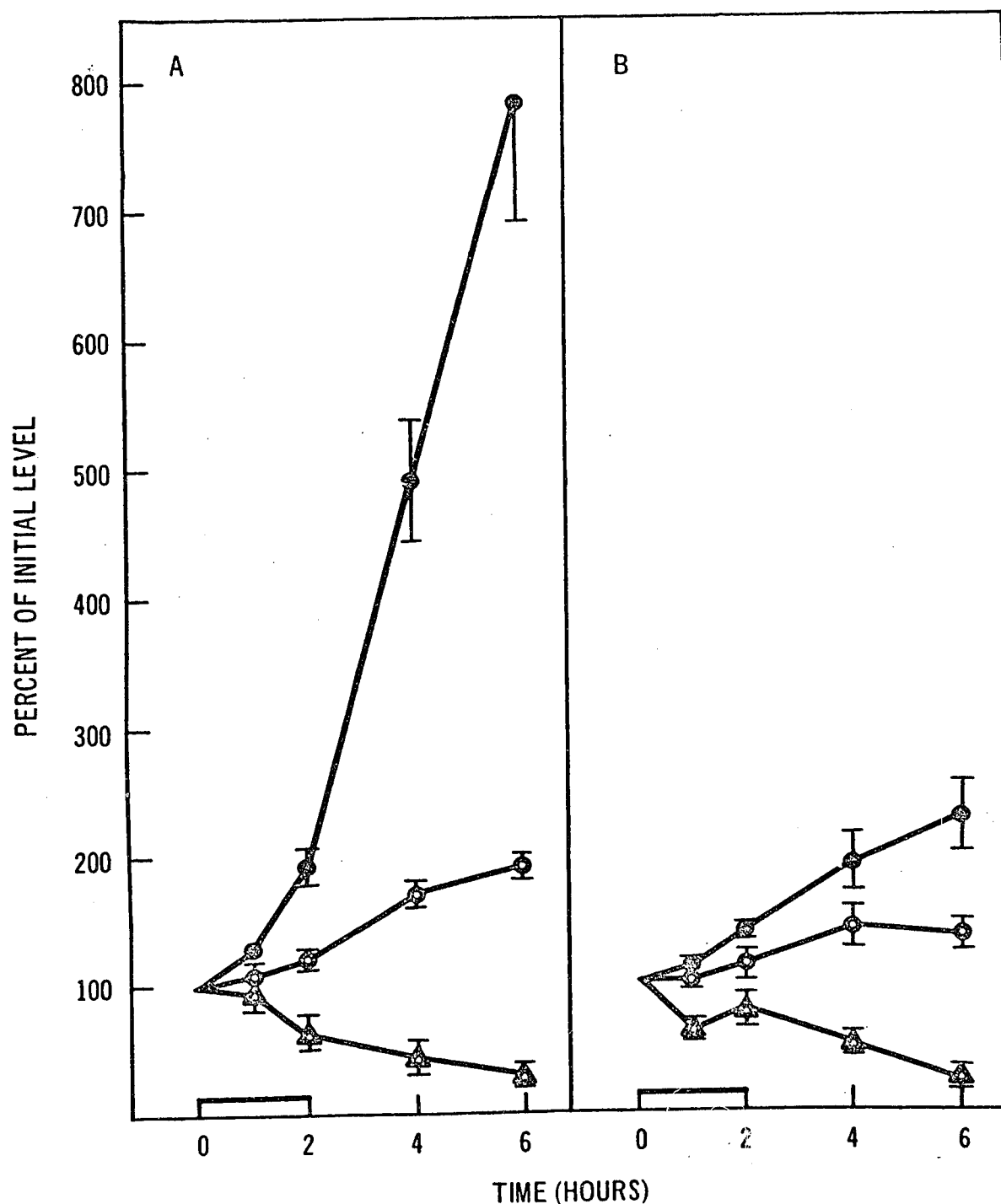


Fig. 22. Effect of cortisol infusion (35  $\mu$ g/200 g. body weight) on hepatic TAT (●), PEPCK (○) and glycogen content (Δ) in hypophysectomized rats fed a 0%- (A) or 60%- (B) protein diet for 3 days. Dark bar indicates time of cortisol infusion. Points plotted represent means  $\pm$  s.e.m. (Appendix, Tables 19-24).

6. Studies concerning the diurnal rhythms of hepatic TAT and PEPCK in rats fed controlled diets.

Intact rats, fed for 3 days a protein-free, 60%-protein, or normal rat pellet diet, were killed at the time intervals as described in Materials and Methods. A significant diurnal rhythm in TAT and PEPCK was found in the 3 groups of rats studied (Figs. 23 A and B). For the rats fed the protein-free diet the activity of TAT at 1000 and 2200 hours was  $2.17 \times 10^{-3}$  and  $4.15 \times 10^{-3}$  units/mg protein respectively ( $P < 0.01$ ) while for PEPCK the activity at 0600 and 1800 hours was  $1.93 \times 10^{-3}$  and  $3.60 \times 10^{-3}$  units/mg protein respectively ( $P < 0.01$ ) (Appendix, Table 25). The rise in PEPCK activity in the 3 groups of rats preceded by just a few hours the rise in TAT activity. For both enzymes the range within which the fluctuations occurred increased as the protein content of the diet increased. The diurnal rhythm in hepatic glycogen content appeared to be inversely related to the enzyme rhythms (Fig. 23C).

DISCUSSION

To investigate whether the level of hepatic TAT and the rate of gluconeogenesis are related, the hormonal-dietary interactions on the enzyme were studied in adrenalectomized and hypophysectomized rats. Adrenalectomized rats were chosen for the study because their TAT activity

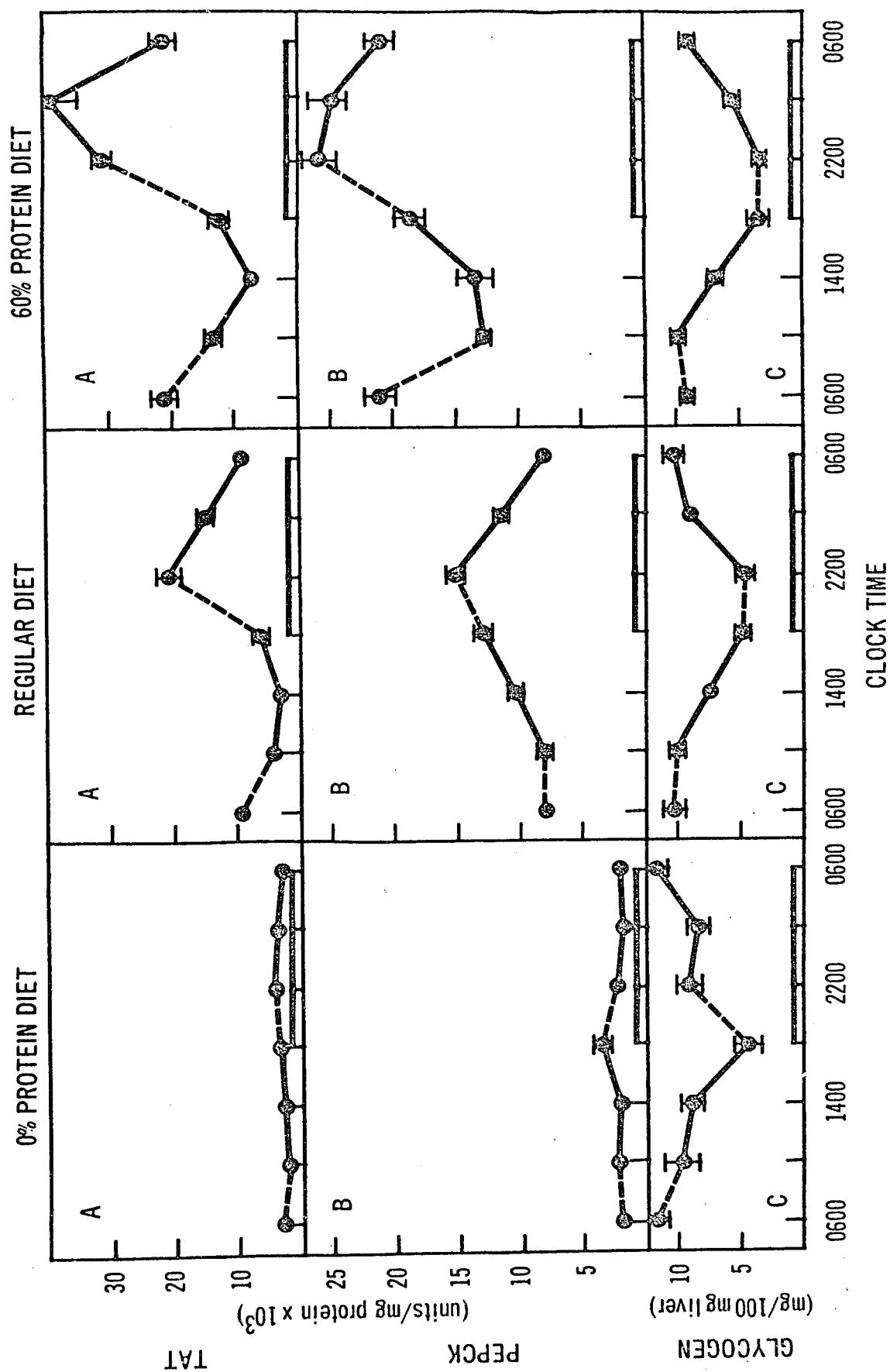


Fig. 23. Diurnal rhythm of hepatic TAT (A), PEPCK (B) and glycogen (C) in intact rats fed a controlled diet for 3 days. Dark bar indicates hours of darkness. The 24-hour cycle is completed with dashed lines. Points plotted represent means  $\pm$  s.e.m. (Appendix, Tables 25-27).

remained unchanged by the stresses of the anesthesia and surgery (Grossman and Mavrides, 1967; this thesis, Table II). Hypophysectomized rats were chosen because they lack not only endogenous glucocorticoids but also growth hormone which has been suggested to act as a "repressor" on the enzyme synthesis (Garren et al., 1964b; Kenney, 1967a).

The initial enzyme activities in both groups studied were greater in the rats fed the high-protein diets than in those fed the diets of low-protein content (Table VI). This is in agreement with the results of Rosen et al. (1963) who reported similar findings in intact rats. The greater enzyme activities in the rats fed the diets of high-protein content may be due to an elevated rate of gluconeogenesis in these rats. Indeed high-protein diets have been shown to stimulate key gluconeogenic enzymes such as PEPCK (Young et al., 1964; this thesis, Table VIII and Fig. 23B) and glucose-6-phosphatase (Freedland, 1968; Hurvitz and Freedland, 1968). Except for the rats fed the 60%-protein diet, the initial TAT activities between the two groups of rats fed the same diet were not significantly different. For the rats fed the 60%-protein diet the difference between the TAT activities may have been due to the absence of growth hormone in the hypophysectomized group.

Cortisol infusion results in the enzyme stimulation the percent magnitude of which depends upon the previous

dietary status of the rats (Fig. 17). In both groups fed the protein-free diet the percent stimulation of TAT following the cortisol infusion was greater than in rats fed diets containing protein. This effect is reflected in Fig. 19 in which the rats fed the protein-free diet are those with the lowest initial TAT activities and the greatest percent stimulation over their initial levels. As the protein content of the diet increases, paralleled by a rise in the initial TAT activity, the percent stimulation due to the infused cortisol decreases. This observation is compatible with a common mechanism responsible for the stimulation of TAT by both dietary protein and cortisol.

The finding that cortisol stimulates hepatic TAT to a greater extent in hypophysectomized than in adrenalectomized rats fed the same diet (Fig. 17) agrees with the results reported by Grossman and Mavrides (1967) and Csányi and Greengard (1968). This greater sensitivity may be due to the absence of growth hormone in the former group. The increased sensitivity is not due to a difference in the metabolism of cortisol since the level of this hormone immediately after the two hour infusion was not significantly different between the two groups of rats fed the same diet (Table VII).

Similarly a difference in the cortisol inactivation rates will not account for the graded response to the

hormone in the rats fed diets of varying protein content (Table VII). In the adrenalectomized group, the rats fed the 10%-protein diet had a plasma cortisol level significantly higher than the rats fed one of the other three diets. If the graded response were due to a prolonged inactivation time for cortisol, then these rats should have had the greatest percent stimulation, which was not the case (Fig. 17A). In the hypophysectomized group equal plasma cortisol levels were found in the animals fed the 0%- and 10%-protein diets. The cortisol levels were also equal in the rats fed the 30%- and 60%-protein diets but were lower than the cortisol levels in the two previous groups. However the significance of the difference in the plasma cortisol level is questionable since it did not parallel the graded response in TAT (Fig. 17B). Thus it appears safe to exclude differences in the cortisol inactivation rates as being responsible for the results illustrated in Fig. 17.

The graded cortisol stimulation of hepatic TAT in rats fed diets of varying protein content is not associated with the release of endogenous glucagon either. It had been hypothesized that the high-carbohydrate content of low-protein diets diminished the glucagon secretion in favor of its storage in the pancreatic  $\alpha$ -cells. The subsequent anesthetic and surgical stress might trigger, by an unknown mechanism, a glucagon release the magnitude

of which would be proportional to the carbohydrate content of the previously fed diet. Thus, if such a hypothesis were true, the administration of a large amount of glucagon should result in an enzyme stimulation, the extent of which should be independent of the previous dietary status of the animals. The increase, however, in the enzyme due to the glucagon infusion was similar to what had occurred following the cortisol infusion (Fig. 21). Thus, glucagon, possibly released by the anesthetic and surgical stress, can not be the unknown endogenous factor underlying the dietary modifications of the cortisol stimulation since its own action was subject to similar dietary modifications.

Since the rate of gluconeogenesis was known to be related to the dietary status of the rats (Shrago et al., 1963; Young et al., 1964; Foster et al., 1966) one of the key gluconeogenic enzymes was examined to assert whether changes in this enzyme paralleled the changes in hepatic TAT. In agreement with the work of Young et al. (1964) the initial activity of PEPCK, like TAT, was found to be greatest in the rats fed the high-protein diet (Table VIII). Following the cortisol infusion the percent stimulation in PEPCK, like the stimulation in TAT although not of the same magnitude, was greater in the rats fed the protein-free diet than in the rats fed the 60%-protein diet (Fig. 22). This indicated that the graded response in TAT to infused cortisol was accompanied by a similar response in

the rate of gluconeogenesis. The glycogen content of the liver declined during the 6 hour period studied but this decline might simply reflect the diurnal rhythm in the hepatic glycogen level (Fig. 23C).

If a relationship between the level of TAT and the rate of gluconeogenesis exists for rats infused with cortisol, it is conceivable that such a relationship also exists between the normal TAT rhythm and the diurnal changes in the rate of gluconeogenesis. The presence of a diurnal rhythm in the activity of the key gluconeogenic enzyme, PEPCK, would be in support of this notion.

Indeed for any particular diet both enzymes displayed a remarkable similarity in their diurnal variation (Figs. 23A and B). A small significant ( $P < 0.005$ ) diurnal rhythm in both TAT and PEPCK was present even in the rats fed the protein-free diet (Appendix, Table 25). As the protein content of the diet increased the range within which the fluctuations occurred also increased for both enzymes.

An inverse relationship was found between the two enzyme activities and the liver glycogen content under all dietary conditions (Fig. 23). The time courses of the glycogen levels are in general agreement with the data reported by Potter et al. (1966). Depletion of liver glycogen was counteracted by an increase in the enzyme activities whereas during glycogen repletion the two enzyme activities subsided. The inverse relationship

between PEPCK and glycogen has also been reported for normal rats in which renal loss of glucose was induced by floridzin with a resultant liver glycogen depletion (Foster et al., 1966).

Although the amplitudes of the PEPCK diurnal rhythms in the rats fed the normal and 60%-protein diet were less than the TAT diurnal rhythms, PEPCK activity nevertheless began to rise sooner (Figs. 23A and B). This correlated with the work of Lardy's group (Shrago et al., 1963; Foster et al., 1966; Shrago and Lardy, 1966) who reported that a very early increase in PEPCK activity occurred in the rats injected with pharmacological doses of cortisol. Foster et al. (1966) suggested that the adrenal cortical hormones affect the gluconeogenic enzymes by altering carbohydrate metabolism, rather than by a direct effect on messenger RNA species coding for these enzymes. Thus the earlier increase in the activity of PEPCK might mean that TAT activity is sufficiently high to cope with a modest early increase in gluconeogenic demand for precursors (Fig. 23). As the demand for gluconeogenic precursors increased, TAT activity rose to meet the need. Thus TAT activity would be stimulated by a requirement for gluconeogenic precursors rather than by a provision of substrate. Indeed, TAT is suppressed rather than stimulated by its substrate tyrosine, injected intraperitoneally into adrenalectomized rats (Rosen and

Milholland, 1963; Kenney and Albritton, 1965).

The relationship between the rate of gluconeogenesis and the level of hepatic TAT activity is supported by the works of Greengard and Dewey (1967) and Holt and Oliver (1968) who reported that the injection of glucose into newborn rats prevented the postpartum rise in TAT. Similar findings were reported for PEPCK (Yeung and Oliver, 1968). Civen et al. (1965) reported that large doses of glucose could prevent the cortisol stimulation of hepatic TAT. This is supported by the work of Pestana (1968) who reported that the intragastric administration of glucose suppressed the stimulation of this enzyme by either glucagon or cortisol. This correlates with the report that glucose administration significantly reduces the rate of gluconeogenesis in rat liver (Ruderman and Herrera, 1968; Felig et al., 1970) as well as the cortisol stimulation of hepatic PEPCK (Foster et al., 1966). Peraino et al. (1966) have reported that glucose significantly diminishes the stimulation of TAT by casein hydrolysate. Hanoune et al. (1971) have recently reported that a single, oral glucose load administered to normal, fasted rats can strongly depress the TAT activity for at least 16 hours. In addition, work with Tetrahymena pyriformis, a ciliated protozoan, has shown that the inclusion of glucose in the culture medium significantly represses the activities of TAT (Mavrides and D'Iorio, 1969) and PEPCK (Shrago and Shug, 1966) as

well as the rate of gluconeogenesis in these organisms (Levy, 1967). Similar findings for TAT in Reuber H-35 cells were reported by Mendelson et al. (1971). In addition, in support of Figure 17, the percent cortisol stimulation of TAT in these cells was greater when the medium contained glucose (Mendelson et al., 1971).

Thus, on the whole, the results of the experiments just discussed suggest a positive correlation between the rate of gluconeogenesis and the level of TAT activity in the liver.

This part of the thesis has been published (Mavrides and Lane, 1969; Lane and Mavrides, 1970).

GENERAL DISCUSSION

Hepatic TAT is the first enzyme in the catabolism of tyrosine, a pathway which yields both a ketogenic and a glucogenic precursor. Since the report that glucocorticoids stimulate the activity of TAT, an explanation for this rapid elevation in the enzyme activity has been sought. Although cortical and pancreatic hormones, cyclic AMP, alloxan diabetes, starvation and high-protein diets have been shown to stimulate TAT, it should be noted that the effects of these agents or conditions are not specific for this enzyme since they result in the stimulation of a multitude of hepatic enzymes including the key gluconeogenic enzymes (Weber et al., 1961; Shrago et al., 1963; Young et al., 1964; Hurvitz and Freedland, 1968). Since both TAT and the key gluconeogenic enzymes appear to be stimulated by the same agents, the level of TAT might be regulated by a cellular control mechanism responding to the varying demands of the gluconeogenic pathway. The role of the hormones or dietary conditions which stimulate hepatic TAT might be to affect the flow of metabolites into this pathway.

Studies concerning the hormonal-dietary interactions have revealed that when the rate of gluconeogenesis, as measured by the level of PEPCK activity, is increased by high-protein diets the percent stimulation of both TAT and PEPCK by cortisol and glucagon is reduced (Figs. 17, 21 and

22). These results indicate that the level of TAT correlates with the rate of gluconeogenesis and agrees with the findings that a diurnal rise in PEPCK activity closely parallels the rise in TAT activity. In addition high protein diets increase both the activity of PEPCK and TAT at all the times examined during the cycle (Fig. 23).

The peak in the plasma corticosterone rhythm has been reported to occur a few hours before the peak activity of hepatic TAT and PEPCK suggesting that the daily hormonal fluctuations might be one of the factors responsible for the rhythm in these enzymes. Adrenalectomy significantly reduced the amplitude of the TAT rhythm in adult rats and prevented the normal development of the rhythm in young rats (Figs. 9 and 10B). The afternoon rise in TAT coincided with an increase in the rate of gluconeogenesis, as evidenced by an increase in PEPCK activity, and a decrease in hepatic glycogen content (Fig. 23). This rise may have been initiated by glucocorticoids since the rise in TAT activity was delayed in the adult adrenalectomized rats (Fig. 10B).

The diurnal rhythm in TAT also depends upon the feeding habits of the animals since the rhythm in adult rats can be shifted by altering the feeding schedule (Fuller and Snood, 1968). In these animals the enzyme activity rose before the rats were allowed food, a time when hepatic

glycogen levels would be low and the rate of gluconeogenesis would be increasing. In young rats the development of the enzyme rhythm occurred at a time when the rats are developing a cyclic feeding pattern (Figs. 17 and 18A). At this time they are gradually being weaned onto the regular rat pellet diet which contains more protein than milk. Thus these rats may also have been developing a PEPCK rhythm but this was not examined.

The hypothesis that gluconeogenesis from tyrosine is significant under gluconeogenic conditions could perhaps be tested in the following way: Isotopically labelled tyrosine would be administered (preferably intravenously) to adrenalectomized rats previously treated with cortisol. The rate of incorporation of the amino acid into liver glucose would be compared to the rates of incorporation from other labelled amino acids which are ordinarily considered to be more glucogenic than tyrosine (for example alanine and aspartic acid). This type of experiment could be repeated after the administration of pancreatic hormones.

Similar experiments could be performed with the isolated perfused liver.

Studies concerning the rate of incorporation of the label from tyrosine at the peak and nadir times of the TAT diurnal rhythm might also be attempted since this would give some idea as to whether the increased rate of incorporation occurs under physiological conditions.

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### APPENDIX

The data shown in the tables are plotted  
in the corresponding figures.

Table 1 (Fig. 4)  
Effect of cycloheximide on the cortisol stimulation of hepatic TAT in anesthetized adrenalectomized rats.

|                 |  | CORTISOL             |                  |                                   |                       |                  |                                   |
|-----------------|--|----------------------|------------------|-----------------------------------|-----------------------|------------------|-----------------------------------|
| TIME<br>(hours) |  | CONTROL              |                  |                                   | CYCLOHEXIMIDE-TREATED |                  |                                   |
|                 |  | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity | number<br>of<br>rats  | TAT<br>activity* | percent<br>of initial<br>activity |
| 0               |  | 4                    | 2.85 ± 0.66      | 100                               | 9                     | 3.04 ± 0.17      | 100                               |
| 2               |  | 4                    | 5.61 ± 1.25      | 199 ± 23                          | 9                     | 2.75 ± 0.21      | 90 ± 3                            |
| 4               |  | 4                    | 25.96 ± 3.45     | 972 ± 130                         | 9                     | 4.56 ± 0.37      | 150 ± 10                          |
| 6               |  | 4                    | 56.40 ± 5.50     | 2212 ± 439                        | 8                     | 8.99 ± 1.38      | 294 ± 40                          |
| 8               |  | 4                    | 61.76 ± 9.50     | 2408 ± 543                        | 6                     | 15.44 ± 3.05     | 521 ± 78                          |
| 10              |  | 4                    | 49.93 ± 9.75     | 1912 ± 471                        | 5                     | 19.09 ± 3.28     | 620 ± 70                          |
| 12              |  |                      |                  |                                   | 4                     | 19.31 ± 4.10     | 596 ± 101                         |
| 14              |  |                      |                  |                                   | 4                     | 15.65 ± 4.34     | 478 ± 109                         |

\* : units/mg protein x 10<sup>3</sup> ± s.e.m.

Table 2 (Fig. 5)  
Effect of cycloheximide on the hepatic TAT in anesthetized intact rats.

| TIME<br>(hours) | SALINE               |                  |                                   |                      | CYCLOHEXIMIDE-TREATED |                                   |                      |                                   |
|-----------------|----------------------|------------------|-----------------------------------|----------------------|-----------------------|-----------------------------------|----------------------|-----------------------------------|
|                 | CONTROL              |                  |                                   |                      |                       |                                   |                      |                                   |
|                 | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity | number<br>of<br>rats | TAT<br>activity*      | percent<br>of initial<br>activity | number<br>of<br>rats | percent<br>of initial<br>activity |
| 0               | 5                    | 4.25 $\pm$ 0.73  | 100                               | 2                    | 4.89 $\pm$ 1.02       | 100                               | 2                    | 100                               |
| 2               | 5                    | 5.72 $\pm$ 1.30  | 150 $\pm$ 51                      | 2                    | 4.16 $\pm$ 0.78       | 86 $\pm$ 1                        | 2                    | 86 $\pm$ 1                        |
| 4               | 5                    | 12.68 $\pm$ 2.18 | 346 $\pm$ 95                      | 2                    | 5.26 $\pm$ 1.38       | 107 $\pm$ 6                       | 2                    | 107 $\pm$ 6                       |
| 6               | 5                    | 16.26 $\pm$ 2.71 | 433 $\pm$ 110                     | 2                    | 6.58 $\pm$ 2.57       | 129 $\pm$ 26                      | 2                    | 129 $\pm$ 26                      |
| 8               | 5                    | 21.88 $\pm$ 4.72 | 591 $\pm$ 158                     | 1                    | 5.59                  | 144                               | 1                    | 144                               |
| 10              | 5                    | 29.68 $\pm$ 6.26 | 770 $\pm$ 179                     |                      |                       |                                   |                      |                                   |
| 12              | 4                    | 24.80 $\pm$ 7.06 | 774 $\pm$ 204                     |                      |                       |                                   |                      |                                   |

\* : units/mg protein  $\times 10^3 \pm$  s.e.m.

Table 3 (Fig. 6).  
Effect of cycloheximide on the cortisol stimulation of  
hepatic TAT in anesthetized intact rats.

| CORTISOL        |                  |                                   |                       |                                   |  |
|-----------------|------------------|-----------------------------------|-----------------------|-----------------------------------|--|
| TIME<br>(hours) | CONTROL          |                                   | CYCLOHEXIMIDE-TREATED |                                   |  |
|                 | TAT<br>activity* | percent<br>of initial<br>activity | TAT<br>activity*      | percent<br>of initial<br>activity |  |
| 0               | 3.96             | 100                               | 3.04                  | 100                               |  |
| 2               | 10.00            | 253                               | 3.08                  | 101                               |  |
| 4               | 34.92            | 882                               | 3.77                  | 124                               |  |
| 6               | 39.96            | 1009                              | 6.82                  | 224                               |  |
| 8               | 23.37            | 590                               | 12.68                 | 417                               |  |

\* : units/mg protein x  $10^3 \pm$  s.e.m.

Table 4 (Figs. 7 and 8a)

Development of the diurnal rhythm of hepatic TAT in young rats.

| CLOCK | TAT activity* |             |               |               |                |
|-------|---------------|-------------|---------------|---------------|----------------|
|       | 1 DAY**       | 6 DAY       | 10 DAY        | 15 DAY        | 21 DAY         |
| 0600  | 1.16 ± 0.31   | 4.06 ± 0.63 | 6.55 ± 1.26   | 7.23 ± 0.60†† | 9.22 ± 0.93†   |
| 1000  | 1.14 ± 0.32   | 3.89 ± 1.07 | 5.39 ± 0.82†  | 2.93 ± 0.34†  | 5.61 ± 0.58†   |
| 1400  | 2.11 ± 0.48   | 2.93 ± 0.46 | 6.14 ± 1.03†  | 4.03 ± 0.93†  | 9.07 ± 0.78†   |
| 1800  | 1.22 ± 0.32   | 3.51 ± 0.51 | 5.50 ± 0.56†  | 3.90 ± 0.92†  | 17.29 ± 2.03†† |
| 2200  | 1.87 ± 0.49   | 2.75 ± 0.68 | 8.95 ± 0.77†† | 6.57 ± 0.99†† | 20.28 ± 1.53†† |
| 0200  | 1.78 ± 0.50   | 3.57 ± 0.79 | 8.58 ± 1.73   | 7.95 ± 1.04†† | 12.55 ± 0.68†† |

\* : units/mg protein x 10<sup>3</sup> ± s.e.m.

\*\* : age of rats

In each age group † is significantly different from †† (P&lt;0.05).

Table 5 (Fig. 8b)

Effect of constant light or darkness on the  
hepatic TAT diurnal rhythm.

| CLOCK<br>TIME | TAT activity*          |                      |
|---------------|------------------------|----------------------|
|               | CONSTANT LIGHT         | CONSTANT DARKNESS    |
| 0600          | 11.06 $\pm$ 0.54 (6)** | 8.04 $\pm$ 0.93 (6)  |
| 1000          | 7.90 $\pm$ 1.05 (5)    | 6.22 $\pm$ 0.71 (6)  |
| 1400          | 11.25 $\pm$ 2.05 (6)   | 5.35 $\pm$ 0.39 (6)  |
| 1800          | 9.24 $\pm$ 1.30 (6)    | 8.59 $\pm$ 0.48 (6)  |
| 2200          | 8.67 $\pm$ 0.90 (6)    | 11.78 $\pm$ 2.57 (6) |
| 0200          | 10.68 $\pm$ 1.33 (6)   | 11.04 $\pm$ 1.08 (6) |

\* : units/mg protein  $\times 10^3 \pm$  s.e.m.

\*\* : numbers in parentheses indicate number of rats.

Table 6 (Fig. 9)

Effect of adrenalectomy on the development  
of the hepatic TAT diurnal rhythm.

| CLOCK<br>TIME | TAT activity*         |                     |
|---------------|-----------------------|---------------------|
|               | SHAM-OPERATED         | ADRENALECTOMIZED    |
| 0600          | 6.62 $\pm$ 0.81 (5)** | 6.46 $\pm$ 0.60 (6) |
| 1000          | 2.88 $\pm$ 0.51 (4)   | 5.23 $\pm$ 0.46 (6) |
| 1400          | 2.85 $\pm$ 0.51 (4)   | 5.17 $\pm$ 0.30 (6) |
| 1800          | 9.97 $\pm$ 0.96 (5)   | 4.97 $\pm$ 0.47 (7) |
| 2200          | 7.44 $\pm$ 0.95 (5)   | 6.02 $\pm$ 0.41 (6) |
| 0200          | 10.87 $\pm$ 0.86 (5)  | 4.46 $\pm$ 0.41 (7) |

\* : units/mg tissue  $\times 10^4 \pm$  s.e.m.

\*\* : numbers in parentheses indicate number of rats.

Table 7 (Fig. 10)  
Effect of adrenalectomy on the hepatic TAT diurnal rhythm.

| CLOCK<br>TIME | TAT activity*                  |                   |                          |                  |
|---------------|--------------------------------|-------------------|--------------------------|------------------|
|               | CYCLIC<br>LIGHTING<br>SCHEDULE | CONSTANT<br>LIGHT | CYCLIC LIGHTING SCHEDULE |                  |
|               |                                |                   | sham-operated            | adrenalectomized |
| 0600          | 7.61 ± 0.66 (5)**              | 7.89 ± 0.96 (6)   | 4.14 ± 0.37 (5)          | 4.01 ± 0.71 (5)  |
| 1000          | 6.67 ± 0.77 (5)                | 2.87 ± 0.25 (6)   | 3.06 ± 0.17 (6)          | 2.61 ± 0.22 (5)  |
| 1400          | 6.53 ± 0.66 (5)                | 5.76 ± 0.39 (6)   | 3.11 ± 0.45 (5)          | 2.81 ± 0.62 (5)  |
| 1800          | 10.97 ± 1.12 (6)               | 4.34 ± 0.58 (6)   | 6.10 ± 0.99 (6)          | 3.22 ± 0.58 (5)  |
| 2200          | 14.48 ± 0.60 (6)               | 4.74 ± 0.64 (6)   | 9.35 ± 0.99 (6)          | 6.41 ± 0.32 (6)  |
| 0200          | 11.44 ± 1.31 (6)               | 6.46 ± 0.85 (6)   | 7.17 ± 0.71 (6)          | 6.01 ± 0.64 (5)  |

\* : units/mg protein x 10<sup>3</sup> ± s.e.m.

\*\* : numbers in parentheses indicate number of rats.



Table 9 (Figs. 17a and 18a)

Effect of cortisol infusion on hepatic TAT in adrenalectomized rats  
fed a protein-free diet for 5 days.

| TIME<br>(hours) | SALINE CONTROL       |                  |                                   | CORTISOL-TREATED     |                  |                                   |
|-----------------|----------------------|------------------|-----------------------------------|----------------------|------------------|-----------------------------------|
|                 | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity |
| 0               | 7                    | 2.17 ± 0.30      | 100                               | 6                    | 1.95 ± 0.14      | 100                               |
| 2               | 7                    | 3.08 ± 0.40      | 143 ± 7                           | 6                    | 6.49 ± 0.52      | 339 ± 35                          |
| 4               | 7                    | 3.95 ± 0.91      | 178 ± 30                          | 6                    | 11.24 ± 0.78     | 595 ± 67                          |
| 5               | 6                    | 3.45 ± 1.05      | 163 ± 34                          | 6                    | 13.79 ± 1.77     | 752 ± 138                         |
| 6               | 6                    | 2.94 ± 1.14      | 140 ± 42                          | 5                    | 13.63 ± 1.46     | 699 ± 122                         |

\* : units/mg protein x 10<sup>3</sup> ± s.e.m.

Table 10 (Figs. 17a and 18a)  
 Effect of cortisol infusion on hepatic TAT in adrenalectomized rats  
 fed a 10%-protein diet for 5 days.

| TIME<br>(hours) | SALINE CONTROL       |                  |                                   | CORTISOL-TREATED     |                  |                                   |
|-----------------|----------------------|------------------|-----------------------------------|----------------------|------------------|-----------------------------------|
|                 | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity |
| 0               | 6                    | 2.75 ± 0.58      | 100                               | 13                   | 2.29 ± 0.20      | 100                               |
| 2               | 6                    | 3.23 ± 0.32      | 132 ± 18                          | 13                   | 6.29 ± 0.62      | 294 ± 33                          |
| 4               | 6                    | 1.86 ± 0.28      | 77 ± 17                           | 13                   | 10.90 ± 1.16     | 531 ± 87                          |
| 5               | 6                    | 1.57 ± 0.22      | 64 ± 12                           | 13                   | 8.72 ± 1.16      | 425 ± 78                          |
| 6               | 6                    | 1.98 ± 0.33      | 81 ± 18                           | 13                   | 8.04 ± 1.08      | 405 ± 85                          |

\* : units/mg protein x 10<sup>3</sup> ± s.e.m.

Table 11 (Figs. 17a and 18a)

Effect of cortisol infusion on hepatic TAT in adrenalectomized rats  
fed a 30%-protein diet for 5 days.

| TIME<br>(hours) | SALINE CONTROL       |                  |                                   | CORTISOL-TREATED     |                  |                                   |
|-----------------|----------------------|------------------|-----------------------------------|----------------------|------------------|-----------------------------------|
|                 | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity |
| 0               | 4                    | 3.34 ± 0.37      | 100                               | 10                   | 3.68 ± 0.59      | 100                               |
| 2               | 4                    | 4.56 ± 0.18      | 142 ± 16                          | 10                   | 6.64 ± 0.93      | 207 ± 33                          |
| 4               | 4                    | 3.83 ± 0.48      | 124 ± 28                          | 10                   | 9.66 ± 2.18      | 297 ± 62                          |
| 5               | 4                    | 3.66 ± 0.61      | 116 ± 26                          | 10                   | 11.20 ± 2.77     | 347 ± 72                          |
| 6               | 4                    | 3.39 ± 0.44      | 109 ± 23                          | 9                    | 11.97 ± 3.34     | 342 ± 74                          |

\* : units/mg protein x 10<sup>3</sup> ± s.e.m.

Table 12 (Figs. 17a and 18a)  
 Effect of cortisol infusion on hepatic TAT in adrenalectomized rats  
 fed a 60%-protein diet for 5 days.

| TIME<br>(hours) | SALINE CONTROL       |                  |                                   | CORTISOL-TREATED     |                  |                                   |
|-----------------|----------------------|------------------|-----------------------------------|----------------------|------------------|-----------------------------------|
|                 | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity |
| 0               | 6                    | 4.50 $\pm$ 0.94  | 100                               | 6                    | 5.19 $\pm$ 0.65  | 100                               |
| 2               | 6                    | 3.93 $\pm$ 0.38  | 112 $\pm$ 26                      | 6                    | 6.10 $\pm$ 0.20  | 129 $\pm$ 20                      |
| 4               | 6                    | 3.48 $\pm$ 0.35  | 105 $\pm$ 33                      | 6                    | 6.83 $\pm$ 0.83  | 143 $\pm$ 23                      |
| 5               | 5                    | 3.87 $\pm$ 0.68  | 94 $\pm$ 17                       | 6                    | 4.42 $\pm$ 0.70  | 93 $\pm$ 16                       |
| 6               | 4                    | 5.35 $\pm$ 0.57  | 170 $\pm$ 59                      | 5                    | 4.90 $\pm$ 0.87  | 100 $\pm$ 19                      |

\* : units/mg protein  $\times 10^3 \pm$  s.e.m.

Table 13 (Figs. 17b and 18b)  
Effect of cortisol infusion on hepatic TAT in hypophysectomized rats  
fed a protein-free diet for 5 days.

| TIME<br>(hours) | SALINE CONTROL       |                  |                                   | CORTISOL-TREATED     |                  |                                   |
|-----------------|----------------------|------------------|-----------------------------------|----------------------|------------------|-----------------------------------|
|                 | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity |
| 0               | 9                    | 1.73 $\pm$ 0.14  | 100                               | 5                    | 2.06 $\pm$ 0.16  | 100                               |
| 2               | 9                    | 2.83 $\pm$ 0.17  | 177 $\pm$ 25                      | 5                    | 5.79 $\pm$ 0.68  | 288 $\pm$ 38                      |
| 4               | 9                    | 4.44 $\pm$ 0.37  | 276 $\pm$ 39                      | 5                    | 18.46 $\pm$ 1.68 | 916 $\pm$ 102                     |
| 5               | 9                    | 5.14 $\pm$ 0.31  | 318 $\pm$ 43                      | 5                    | 24.74 $\pm$ 2.17 | 1223 $\pm$ 128                    |
| 6               | 9                    | 5.76 $\pm$ 0.34  | 354 $\pm$ 45                      | 5                    | 24.54 $\pm$ 1.89 | 1203 $\pm$ 90                     |

\* : units/mg protein  $\times 10^3 \pm$  s.e.m.

Table 14 (Figs. 17b and 18b)  
 Effect of cortisol infusion on hepatic TAT in hypophysectomized rats  
 fed a 10%-protein diet for 5 days.

| TIME<br>(hours) | SALINE CONTROL       |                  |                                   | CORTISOL-TREATED     |                  |                                   |
|-----------------|----------------------|------------------|-----------------------------------|----------------------|------------------|-----------------------------------|
|                 | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity |
| 0               | 6                    | 2.88 ± 0.50      | 100                               | 8                    | 2.21 ± 0.15      | 100                               |
| 2               | 6                    | 3.87 ± 0.46      | 141 ± 11                          | 8                    | 5.84 ± 0.74      | 267 ± 32                          |
| 4               | 6                    | 5.21 ± 0.63      | 197 ± 32                          | 8                    | 12.57 ± 0.75     | 586 ± 54                          |
| 5               | 6                    | 5.44 ± 1.22      | 191 ± 24                          | 8                    | 16.51 ± 1.56     | 790 ± 113                         |
| 6               | 6                    | 5.77 ± 0.43      | 220 ± 28                          | 8                    | 21.29 ± 2.16     | 1003 ± 125                        |

\* : units/mg protein x 10<sup>3</sup> ± s.e.m.

Table 15 (Figs. 17b and 18b)

Effect of cortisol infusion on hepatic TAT in hypophysectomized rats  
fed a 30%-protein diet for 5 days.

| TIME<br>(hours) | SALINE CONTROL       |                  |                                   | CORTISOL-TREATED     |                  |                                   |
|-----------------|----------------------|------------------|-----------------------------------|----------------------|------------------|-----------------------------------|
|                 | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity |
| 0               | 9                    | 4.31 $\pm$ 0.61  | 100                               | 5                    | 5.41 $\pm$ 0.54  | 100                               |
| 2               | 9                    | 4.37 $\pm$ 0.49  | 106 $\pm$ 10                      | 5                    | 8.71 $\pm$ 1.82  | 158 $\pm$ 24                      |
| 4               | 9                    | 4.74 $\pm$ 0.49  | 119 $\pm$ 11                      | 5                    | 17.98 $\pm$ 0.81 | 342 $\pm$ 29                      |
| 5               | 9                    | 4.65 $\pm$ 0.50  | 122 $\pm$ 20                      | 5                    | 24.15 $\pm$ 1.46 | 464 $\pm$ 53                      |
| 6               | 9                    | 4.14 $\pm$ 0.69  | 114 $\pm$ 26                      | 5                    | 27.49 $\pm$ 0.81 | 528 $\pm$ 55                      |

\* : units/mg protein  $\times 10^3 \pm$  s.e.m.

Table 16 (Figs. 17b and 18b)  
 Effect of cortisol infusion on hepatic TAT in hypophysectomized rats  
 fed a 60%-protein diet for 5 days.

| TIME<br>(hours) | SALINE CONTROL       |                  |                                   | CORTISOL-TREATED     |                  |                                   |
|-----------------|----------------------|------------------|-----------------------------------|----------------------|------------------|-----------------------------------|
|                 | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity |
| 0               | 6                    | 9.11 $\pm$ 1.18  | 100                               | 6                    | 11.00 $\pm$ 2.41 | 100                               |
| 2               | 6                    | 8.69 $\pm$ 1.07  | 97 $\pm$ 6                        | 6                    | 14.30 $\pm$ 2.39 | 133 $\pm$ 21                      |
| 4               | 6                    | 7.48 $\pm$ 0.77  | 84 $\pm$ 5                        | 6                    | 21.62 $\pm$ 2.01 | 246 $\pm$ 59                      |
| 5               | 6                    | 6.94 $\pm$ 0.53  | 76 $\pm$ 8                        | 6                    | 27.16 $\pm$ 2.01 | 301 $\pm$ 60                      |
| 6               | 6                    | 6.10 $\pm$ 0.76  | 65 $\pm$ 6                        | 6                    | 25.56 $\pm$ 2.14 | 291 $\pm$ 66                      |

\* : units /mg protein  $\times 10^3 \pm$  s.e.m.

Table 17 (Fig. 21a)  
 Effect of glucagon infusion on hepatic TAT in adrenalectomized rats  
 fed a controlled protein diet for 5 days.

| TIME<br>(hours) | PROTEIN-FREE DIET    |                  |                                   | 60%-PROTEIN DIET     |                  |                                   |
|-----------------|----------------------|------------------|-----------------------------------|----------------------|------------------|-----------------------------------|
|                 | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity |
| 0               | 7                    | 2.72 ± 0.26      | 100                               | 6                    | 9.17 ± 1.43      | 100                               |
| 2               | 7                    | 7.62 ± 1.05      | 286 ± 36                          | 6                    | 11.49 ± 0.83     | 145 ± 27                          |
| 4               | 7                    | 7.90 ± 0.45      | 302 ± 25                          | 6                    | 11.10 ± 1.27     | 139 ± 26                          |
| 5               | 5                    | 7.64 ± 0.93      | 204 ± 45                          | 6                    | 9.92 ± 1.77      | 128 ± 33                          |
| 6               | 4                    | 6.03 ± 1.10      | 232 ± 52                          | 6                    | 9.89 ± 2.73      | 132 ± 44                          |

\* : units/mg protein x 10<sup>3</sup> ± s.e.m.

Table 18 (Fig. 21b)  
Effect of glucagon infusion on hepatic TAT in hypophysectomized rats  
fed a controlled protein diet for 5 days.

| TIME<br>(hours) | PROTEIN-FREE DIET    |                  |                                   | 60%-PROTEIN DIET     |                  |                                   |
|-----------------|----------------------|------------------|-----------------------------------|----------------------|------------------|-----------------------------------|
|                 | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity |
| 0               | 4                    | 1.92 $\pm$ 0.21  | 100                               | 4                    | 15.09 $\pm$ 2.36 | 100                               |
| 2               | 4                    | 11.36 $\pm$ 0.55 | 610 $\pm$ 61                      | 4                    | 19.05 $\pm$ 1.92 | 130 $\pm$ 9                       |
| 4               | 4                    | 17.34 $\pm$ 1.16 | 911 $\pm$ 54                      | 4                    | 23.77 $\pm$ 1.49 | 165 $\pm$ 16                      |
| 5               | 4                    | 17.58 $\pm$ 2.09 | 914 $\pm$ 39                      | 4                    | 15.14 $\pm$ 1.22 | 107 $\pm$ 17                      |
| 6               | 4                    | 15.79 $\pm$ 2.24 | 818 $\pm$ 66                      | 4                    | 13.27 $\pm$ 2.19 | 91 $\pm$ 18                       |

\* : units/mg protein  $\times 10^3 \pm$  s.e.m.

Table 19 (Fig. 22a)  
 Effect of cortisol infusion on hepatic TAT in hypophysectomized rats  
 fed a protein-free diet for 3 days.

| TIME<br>(hours) | SALINE CONTROL       |                  |                                   | CORTISOL-TREATED     |                  |                                   |
|-----------------|----------------------|------------------|-----------------------------------|----------------------|------------------|-----------------------------------|
|                 | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity |
| 0               | 6                    | 2.06 $\pm$ 0.38  | 100                               | 5                    | 2.05 $\pm$ 0.20  | 100                               |
| 1               | 6                    | 1.87 $\pm$ 0.19  | 96 $\pm$ 9                        | 5                    | 2.59 $\pm$ 0.26  | 126 $\pm$ 1                       |
| 2               | 6                    | 2.64 $\pm$ 0.17  | 143 $\pm$ 21                      | 5                    | 3.84 $\pm$ 0.22  | 193 $\pm$ 15                      |
| 4               | 6                    | 3.39 $\pm$ 0.40  | 175 $\pm$ 20                      | 5                    | 9.84 $\pm$ 1.00  | 491 $\pm$ 47                      |
| 6               | 6                    | 4.17 $\pm$ 0.74  | 231 $\pm$ 53                      | 5                    | 15.65 $\pm$ 1.84 | 781 $\pm$ 88                      |

\* : units/mg protein  $\times 10^3 \pm$  s.e.m.

Table 20 (Fig. 22a)  
Effect of cortisol infusion on hepatic PEPCK in hypophysectomized  
rats fed a protein-free diet for 3 days.

| TIME<br>(hours) | SALINE CONTROL       |                    |                                   | CORTISOL-TREATED     |                    |                                   |
|-----------------|----------------------|--------------------|-----------------------------------|----------------------|--------------------|-----------------------------------|
|                 | number<br>of<br>rats | PEPCK<br>activity* | percent<br>of initial<br>activity | number<br>of<br>rats | PEPCK<br>activity* | percent<br>of initial<br>activity |
| 0               | 6                    | 3.70 ± 0.56        | 100                               | 5                    | 3.92 ± 0.83        | 100                               |
| 1               | 6                    | 3.35 ± 0.51        | 91 ± 4                            | 5                    | 4.34 ± 1.11        | 108 ± 10                          |
| 2               | 6                    | 3.79 ± 0.56        | 104 ± 5                           | 5                    | 4.52 ± 0.83        | 120 ± 8                           |
| 4               | 6                    | 4.96 ± 0.57        | 140 ± 12                          | 5                    | 6.38 ± 1.00        | 170 ± 9                           |
| 6               | 6                    | 4.40 ± 0.45        | 127 ± 15                          | 5                    | 7.32 ± 1.32        | 192 ± 7                           |

\* : units/mg protein x 10<sup>3</sup> ± s.e.m.

Table 21 (Fig. 22a)  
 Effect of cortisol infusion on hepatic glycogen in hypophysectomized rats  
 fed a protein-free diet for 3 days.

| TIME<br>(hours) | SALINE CONTROL       |                      |                                | CORTISOL-TREATED     |                      |                                |
|-----------------|----------------------|----------------------|--------------------------------|----------------------|----------------------|--------------------------------|
|                 | number<br>of<br>rats | Glycogen<br>content* | percent of<br>initial<br>level | number<br>of<br>rats | Glycogen<br>content* | percent of<br>initial<br>level |
| 0               | 6                    | 5.69 $\pm$ 0.68      | 100                            | 5                    | 3.85 $\pm$ 1.23      | 100                            |
| 1               | 6                    | 5.80 $\pm$ 1.02      | 103 $\pm$ 13                   | 5                    | 3.30 $\pm$ 1.37      | 93 $\pm$ 11                    |
| 2               | 6                    | 5.01 $\pm$ 1.39      | 79 $\pm$ 17                    | 5                    | 2.85 $\pm$ 1.36      | 63 $\pm$ 15                    |
| 4               | 6                    | 2.26 $\pm$ 0.68      | 36 $\pm$ 9                     | 5                    | 1.92 $\pm$ 0.97      | 43 $\pm$ 12                    |
| 6               | 6                    | 0.59 $\pm$ 0.24      | 10 $\pm$ 4                     | 5                    | 1.17 $\pm$ 0.58      | 28 $\pm$ 8                     |

\* : mg/100 mg of liver  $\pm$  s.e.m.

Table 22 (Fig. 22b)

Effect of cortisol infusion on hepatic TAT in hypophysectomized rats  
fed a 60%-protein diet for 3 days.

| TIME<br>(hours) | SALINE CONTROL       |                  |                                   | CORTISOL-TREATED     |                  |                                   |
|-----------------|----------------------|------------------|-----------------------------------|----------------------|------------------|-----------------------------------|
|                 | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity | number<br>of<br>rats | TAT<br>activity* | percent<br>of initial<br>activity |
| 0               | 5                    | 11.83 $\pm$ 2.49 | 100                               | 5                    | 12.63 $\pm$ 1.21 | 100                               |
| 1               | 5                    | 10.51 $\pm$ 1.99 | 92 $\pm$ 7                        | 5                    | 13.93 $\pm$ 1.68 | 110 $\pm$ 7                       |
| 2               | 5                    | 10.02 $\pm$ 1.85 | 89 $\pm$ 7                        | 5                    | 17.48 $\pm$ 1.82 | 139 $\pm$ 6                       |
| 4               | 5                    | 7.37 $\pm$ 0.89  | 70 $\pm$ 12                       | 5                    | 23.54 $\pm$ 1.54 | 193 $\pm$ 22                      |
| 6               | 5                    | 8.72 $\pm$ 2.23  | 79 $\pm$ 18                       | 5                    | 27.98 $\pm$ 2.93 | 228 $\pm$ 27                      |

\* : units/mg protein  $\times 10^3 \pm$  s.e.m.

Table 23 (Fig. 22b)  
 Effect of cortisol infusion on hepatic PEPCK in hypophysectomized  
 rats fed a 60%-protein diet for 3 days.

| TIME<br>(hours) | SALINE CONTROL       |                    |                                   | CORTISOL-TREATED     |                    |                                   |
|-----------------|----------------------|--------------------|-----------------------------------|----------------------|--------------------|-----------------------------------|
|                 | number<br>of<br>rats | PEPCK<br>activity* | percent<br>of initial<br>activity | number<br>of<br>rats | PEPCK<br>activity* | percent<br>of initial<br>activity |
| 0               | 5                    | 9.98 $\pm$ 1.52    | 100                               | 5                    | 10.20 $\pm$ 1.48   | 100                               |
| 1               | 5                    | 8.30 $\pm$ 1.42    | 84 $\pm$ 9                        | 5                    | 10.10 $\pm$ 1.36   | 99 $\pm$ 2                        |
| 2               | 5                    | 9.95 $\pm$ 1.40    | 102 $\pm$ 12                      | 5                    | 10.96 $\pm$ 0.94   | 113 $\pm$ 12                      |
| 4               | 5                    | 10.36 $\pm$ 1.62   | 102 $\pm$ 13                      | 5                    | 13.68 $\pm$ 1.09   | 142 $\pm$ 16                      |
| 6               | 5                    | 11.99 $\pm$ 2.03   | 121 $\pm$ 15                      | 5                    | 13.30 $\pm$ 1.15   | 136 $\pm$ 11                      |

\* : units/mg protein  $\times 10^3 \pm$  s.e.m.

Table 24 (Fig. 22b)  
 Effect of cortisol infusion on hepatic glycogen in hypophysectomized  
 rats fed a 60%-protein diet for 3 days.

| TIME<br>(hours) | SALINE CONTROL       |                      |                                | CORTISOL-TREATED     |                      |                                |
|-----------------|----------------------|----------------------|--------------------------------|----------------------|----------------------|--------------------------------|
|                 | number<br>of<br>rats | Glycogen<br>content* | percent of<br>initial<br>level | number<br>of<br>rats | Glycogen<br>content* | percent of<br>initial<br>level |
| 0               | 5                    | 5.80 $\pm$ 0.64      | 100                            | 5                    | 4.47 $\pm$ 0.72      | 100                            |
| 1               | 4                    | 4.49 $\pm$ 0.92      | 77 $\pm$ 12                    | 5                    | 2.92 $\pm$ 0.64      | 63 $\pm$ 7                     |
| 2               | 5                    | 2.69 $\pm$ 0.85      | 45 $\pm$ 11                    | 5                    | 3.33 $\pm$ 0.59      | 77 $\pm$ 12                    |
| 4               | 5                    | 0.41 $\pm$ 0.19      | 7 $\pm$ 3                      | 5                    | 2.36 $\pm$ 0.64      | 51 $\pm$ 9                     |
| 6               | 5                    | 0.24 $\pm$ 0.15      | 5 $\pm$ 4                      | 5                    | 1.15 $\pm$ 0.90      | 24 $\pm$ 8                     |

\* : mg/100 mg of liver  $\pm$  s.e.m.

Table 25 (Fig. 23)

Diurnal rhythm in hepatic TAT, PEPCK and glycogen content in intact rats fed a protein-free diet for 3 days.

| Clock time | # of rats | TAT activity*   | PEPCK activity* | Glycogen content** |
|------------|-----------|-----------------|-----------------|--------------------|
| 1000       | 5         | 2.17 $\pm$ 0.44 | 2.34 $\pm$ 0.31 | 9.67 $\pm$ 1.41    |
| 1400       | 5         | 2.68 $\pm$ 0.47 | 1.91 $\pm$ 0.19 | 9.01 $\pm$ 0.76    |
| 1800       | 5         | 3.31 $\pm$ 0.50 | 3.60 $\pm$ 0.51 | 4.36 $\pm$ 1.19    |
| 2200       | 6         | 4.15 $\pm$ 0.28 | 2.43 $\pm$ 0.19 | 9.12 $\pm$ 0.95    |
| 0200       | 6         | 3.69 $\pm$ 0.37 | 1.69 $\pm$ 0.23 | 8.37 $\pm$ 0.78    |
| 0600       | 6         | 2.70 $\pm$ 0.16 | 1.93 $\pm$ 0.19 | 11.64 $\pm$ 0.68   |

\* : units/mg protein  $\times 10^3 \pm$  s.e.m.

\*\* : mg/100 mg liver  $\pm$  s.e.m.

Table 26 (Fig. 23)

Diurnal rhythm in hepatic TAT, PEPCK and glycogen content in intact rats fed a normal pellet diet.

| Clock time | # of rats | TAT activity*    | PEPCK activity*  | Glycogen content** |
|------------|-----------|------------------|------------------|--------------------|
| 1000       | 6         | 4.26 $\pm$ 0.60  | 8.18 $\pm$ 0.45  | 9.93 $\pm$ 0.48    |
| 1400       | 5         | 2.77 $\pm$ 0.46  | 10.33 $\pm$ 0.51 | 7.32 $\pm$ 0.23    |
| 1800       | 5         | 5.99 $\pm$ 1.09  | 12.99 $\pm$ 0.69 | 4.66 $\pm$ 0.34    |
| 2200       | 6         | 20.78 $\pm$ 1.87 | 15.10 $\pm$ 0.74 | 4.46 $\pm$ 0.52    |
| 0200       | 6         | 14.67 $\pm$ 1.10 | 11.47 $\pm$ 0.44 | 8.88 $\pm$ 0.27    |
| 0600       | 6         | 9.00 $\pm$ 0.61  | 7.98 $\pm$ 0.32  | 10.26 $\pm$ 0.76   |

\* : units/mg protein  $\times 10^3 \pm$  s.e.m.

\*\* : mg/100 mg liver  $\pm$  s.e.m.

Table 27 (Fig. 23)

Diurnal rhythm in hepatic TAT, PEPCK and glycogen content in intact rats fed a 60%-protein diet for 3 days.

| Clock time | # of rats | TAT activity*    | PEPCK activity*  | Glycogen content** |
|------------|-----------|------------------|------------------|--------------------|
| 1000       | 6         | 13.21 $\pm$ 1.15 | 12.64 $\pm$ 0.46 | 9.86 $\pm$ 0.37    |
| 1400       | 5         | 6.91 $\pm$ 0.60  | 13.39 $\pm$ 1.34 | 6.77 $\pm$ 0.54    |
| 1800       | 5         | 12.07 $\pm$ 1.62 | 18.60 $\pm$ 1.19 | 3.25 $\pm$ 0.61    |
| 2200       | 6         | 30.71 $\pm$ 1.50 | 25.89 $\pm$ 1.47 | 3.24 $\pm$ 0.33    |
| 0200       | 6         | 38.79 $\pm$ 4.21 | 24.98 $\pm$ 1.58 | 5.40 $\pm$ 0.32    |
| 0600       | 6         | 21.21 $\pm$ 2.07 | 20.92 $\pm$ 1.15 | 9.09 $\pm$ 0.45    |

\* : units/mg protein  $\times 10^3 \pm$  s.e.m.

\*\* : mg/100 mg liver  $\pm$  s.e.m.