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CIRCULATORY CLEARANCE OF MUSCLE ENZYMES IN NORMAL AND DYSTROPHIC CHICKENS

By

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ABSTRACT

CIRCULATORY CLEARANCE OF MUSCLE ENZYMES
IN NORMAL AND DYSTROPHIC CHICKENS

By

Harold David Husic

The most widely used test for the early diagnosis of Duchenne muscular dystrophy in humans, and for the detection of the X-linked trait in female carriers, is the determination of the levels of some muscle enzymes in the circulation. The activities of creatine kinase and muscle pyruvate kinase are also markedly elevated in the circulation of dystrophic chickens compared to normal chickens. However, the activities of some other abundant muscle proteins including AMP aminohydrolase (AMPAH) and adenylate kinase are not elevated. Some of the factors which determine the serum levels of these muscle enzymes in dystrophic chickens were investigated.

The circulatory clearance rates of several muscle enzymes were measured in normal and dystrophic chickens after the intravenous injection of the purified enzymes. AMPAH and adenylate kinase are cleared rapidly with half-lives of only a few minutes. However, the circulatory half-lives of creatine kinase and pyruvate kinase are several hours. Thus, those enzymes that are rapidly cleared are not elevated in the circulation of dystrophic chickens. However, based on the activities

of these enzymes in muscle press juices, AMPAH is extensively associated with intracellular structures and may not be released into the circulation.

The rapid circulatory clearance of AMPAH was studied in detail. AMPAH is cleared primarily by the spleen and the parenchymal cells of the liver, where the enzyme is internalized and degraded in lysosomes. The rapid clearance is inhibited by the intravenous injection of heparin and other sulfated polysaccharides.

AMPAH binds to hepatocyte monolayers *in vitro*. This binding to the cell surface of hepatocytes is inhibited by effectors of AMPAH activity, heparin, and other sulfated polysaccharides. The bound enzyme is internalized and degraded. AMPAH also binds to heparin. This may explain the heparin-induced release of AMPAH bound to hepatocyte monolayers.

These experiments describe the circulatory clearance rates of muscle proteins. The effect of clearance rates on the observed levels of these proteins in the circulation of dystrophic chickens are discussed. The *in vivo* and *in vitro* characteristics of the mechanism for the rapid circulatory clearance of AMPAH are also described.

to

my Mom and Dad

for their love and encouragement

and

to all of those with whom I have ever shared a tune

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LIST OF ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
AK	Adenylate kinase
AMP	Adenosine 5'-monophosphate
AMPAH	Adenosine 5'-monophosphate aminohydrolase (AMP deaminase)
ATP	Adenosine 5'-triphosphate
ATPase	Adenosine 5'-triphosphatase
BSA	Bovine serum albumin
Ci	Curies
cpm	Counts per minute
CTP	Cytosine 5'-triphosphate
DMD	Duchenne muscular dystrophy
EDTA	Ethylenediaminetetraacetic acid
Fru-1,6-P_2	Fructose-1,6-bisphosphate
GTP	guanosine 5'-triphosphate
HEPES	N-2-hydroxyethylpiperizine-N'-2-ethanesulfonic acid
IMP	Inosine 5'-monophosphate
ITP	Inosine 5'-triphosphate
MES	2(N-morpholino)ethanesulfonic acid
MIT	monoiodotyrosine
NADH	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate

NPC	Nonparenchymal cells
PBS	Phosphate-buffered saline
PC	Parenchymal cells
P_i	Orthophosphate
PP_i	Pyrophosphate
RNA	Ribonucleic acid
$t_{1/2}$	Half-life
TRIS	Tris(hydroxymethyl)aminomethane

CHAPTER I

LITERATURE REVIEW

A. DUCHENNE MUSCULAR DYSTROPHY

Muscular dystrophies are disorders that result in the dysfunction and degeneration of muscle. There are numerous muscular disorders that differ markedly in their clinical characteristics including degree of severity, age of onset, affected muscles, and role of inheritance in the transmission of the disorder. The primary causes for only a few muscular diseases are known. Myasthenia gravis which results in muscle weakness and fatigue, is an autoimmune disorder. These patients produce antibodies which bind to the acetylcholine receptor at the neuromuscular junction (1). Myopathies are associated with deficiencies of enzymes which metabolize glycogen (2), phosphofructokinase (3), AMP aminohydrolase (4), carnitine palmitoyl transferase (5), and carnitine (6). However, for other common muscular disorders the primary causes are not known and effective modes of treatment are not available.

The most common and widely studied of the neuromuscular diseases is Duchenne muscular dystrophy (DMD). In contrast to many other myopathies, the clinical characteristics are predictable and largely invariant from one patient to the next (7). The disease is genetically transmitted, follows a preditable mode of X-linked inheritance, and on the average affects one out of every 4800 male children (8). Symptoms usually occur

before three years of age and include initial weakness of the legs followed by weakness of the arms, and muscle hypertrophy early in the disease followed by atrophy at later stages. By adolescence, patients are wheelchair bound, and death from complications generally occurs by 25 years of age.

Clinical diagnosis of DMD is generally based on the examination of the serum levels of muscle enzymes and histochemical examination of muscle biopsies. Elevated levels of some muscle enzymes are invariably observed in DMD patients. This will be discussed in detail later in this review. Histochemical examination of DMD muscle biopsies shows: 1) large hyalinized muscle fibers; 2) small groups of fibers at the same stage of regeneration or degeneration; 3) proliferation of endomysial and perimysial connective tissue; 4) increase in intracellular and extracellular lipid; 5) fiber splitting; 6) heterogeneity of fiber diameter; 7) centrally placed nuclei; 8) nuclear proliferation; and 9) myofibrillar necrosis (7). Histochemical stains for enzyme activities reflect the pattern of enzyme activity differences in muscle homogenates discussed below.

Despite considerable research, the molecular basis for the primary defect in DMD continues to elude investigators. As with any genetically transmitted disorder the defect must be due to the absence, reduced amounts, or reduced functional activity of a protein that is necessary for normal cellular function.

If the disorder in DMD is due to the reduced activity of an enzyme, the defect could be detected by noting markedly reduced levels of the enzyme activity or the abnormal levels of a metabolite. Altered levels of many enzyme activities are observed in biopsies of DMD muscle,

however, the differences are not extreme and probably are secondary effects to the primary lesion. The levels of creatine kinase (9), adenylate kinase (9), AMP aminohydrolase (10,11), and the enzymes of glycogenolysis and glycolysis (9,12-14) are decreased in muscle biopsies from DMD patients compared to normal patients. On the other hand there are increased levels of enzymes of the citric acid cycle (9,12) and lysosomal enzymes (15-18). Differences in the levels of several metabolites are observed in the muscle of DMD patients compared to normal patients. Increased levels of muscle lipids are observed (19-21); ATP levels in the muscle of DMD patients are either not reduced (22) or slightly reduced (23,24); phosphorylcreatine and glycogen levels are slightly decreased (22). Because ATP and phosphorylcreatine levels are not markedly reduced in DMD muscle, it is clear that the decreased levels of glycolytic and glycogenolytic enzymes do not compromise the ability of DMD muscle to produce ATP and phosphorylcreatine. Therefore, the primary defect in DMD is not likely a defect in energy metabolism. Furthermore, the symptoms of DMD are more severe than those disorders in which there is an impaired ability to utilize carbohydrates for energy production as in the glycogen storage diseases (2) and phosphofructokinase deficiency (3).

The relative levels of enzymes involved in glycolytic and oxidative metabolism in muscle from DMD patients are similar to those observed in embryonic muscle tissue; it has been suggested that DMD may be a developmental disorder that results in the incomplete differentiation of muscle tissue (25,26). This suggestion is further supported by studies which show that adult muscle from DMD patients contains isoenzymes not normally found in muscle after embryonic development. The isoenzyme patterns of lactate dehydrogenase (27,28), creatine kinase (26,27,29), aldolase (30),

acetylcholinesterase (31), and hexokinase (32) in DMD muscle show measurable levels of isoenzymes characteristic of embryonic muscle, though the normal adult isoenzyme predominates in all cases. The presence of embryonic isoenzymes may be due to the high number of regenerating muscle fibers that are observed in DMD muscle tissue (33).

Several investigators have examined the properties of structural proteins of the contractile apparatus and other proteins in search for the primary lesion in DMD. Biochemical studies of myoglobin (34), myosin (35), actomyosin (36), and tropomyosin (36) show no evidence for alterations in the properties of these proteins isolated from DMD muscle.

Others have suggested that the defect in DMD results in the dysfunction of muscle membranes (36-38). Electron-microscopic investigations of DMD muscle biopsies show focal lesions of the sarcolemma (39-41) and an abnormal distribution and increased number of intramembranous particles (42,43). Muscle damage may be due to high intracellular concentrations of calcium caused by either an increased influx of calcium at the plasma membrane or impaired uptake of calcium by the sarcoplasmic reticulum (44). High intracellular concentrations of calcium could activate a neutral calcium-activated proteinase in skeletal muscle (45) and result in degradation of muscle tissue. Decreased levels of Mg-ATPase, Na/K-ATPase, and Ca-ATPase were observed in isolated sarcolemma from DMD muscle in one report (46), but not in another (47). Compared to normal muscle, the membrane bound enzyme adenylate cyclase is relatively unresponsive to stimulation by epinephrine or sodium fluoride in DMD sarcolemma (38,48). One report showed an altered distribution of concanavalin A binding sites in sarcolemma from DMD patients (49). A difference in the types of phospholipids associated with DMD membranes is observed (21).

Tissues other than muscle from DMD patients have also been studied extensively. Numerous reports show alterations in the characteristics of erythrocyte membranes from DMD patients; however, as reviewed by Rowland (38) most of the observed abnormalities are either contested or unconfirmed. Studies of the capping characteristics of DMD lymphocytes (50-52) and the growth patterns of cultured skin fibroblasts from DMD patients (53-55) have also produced controversial results.

The first observations that implicated defective muscle membranes in DMD were those which showed marked increases in the levels of some muscle enzymes in the serum of DMD patients. The early observation that aldolase activity is elevated in the serum of boys with DMD (56,57) was followed by reports of increased serum levels of creatine kinase (58), pyruvate kinase (58-60), glutamate-oxaloacetate transaminase (61), phosphoglucomutase (62), aspartate aminotransferase (62), alanine aminotransferase (62), glucosephosphate isomerase (62), triosephosphate isomerase (62), malate dehydrogenase (62), and myoblobin (63-66). Slightly increased levels of adenylate kinase (67,68) are reported in some patients. Of the serum enzymes studied, creatine kinase activity is the most widely used diagnostic test of muscular disease because the activity is more dramatically elevated than other muscle enzymes in the serum of DMD patients. Creatine kinase activity is often elevated at birth before the onset of muscular weakness in DMD patients (69-71), and is elevated in as many as 80% of the female carriers of the X-linked trait (72). The activity of creatine kinase is elevated in the blood of some affected fetuses, though assay of creatine kinase in fetal blood is not sufficiently reliable to be used for prenatal diagnosis of DMD (73-75). Increased serum levels of muscle enzymes are also observed in

patients with other myopathies though the elevation is usually not as dramatic as in DMD (76).

It is apparent that the enzymes which are elevated in the blood of DMD patients are from muscle tissue. The most convincing evidence is that the isoenzyme patterns of the enzymes found in the blood are similar to those in muscle. Aldolase activity in the serum of DMD patients is the muscle isoenzyme (77,78). Furthermore, arteriovenous differences in the activity of aldolase across the forearm of a DMD patient indicate release from muscle tissue (79). There are several isoenzymes of pyruvate kinase in human tissues and the increased serum activity in DMD patients is the muscle isoenzyme (59). The isoenzyme patterns of lactate dehydrogenase (27,80-83) and creatine kinase (27,83-86) also reflect the isoenzyme patterns observed in DMD muscle tissue. The levels of adenylate kinase are slightly increased in the serum of some DMD patients (67,68) and the serum enzyme is reported to be an "aberrant" form of the muscle isoenzyme (68). Also, in DMD patients the serum activity of muscle enzymes decreases and approaches normal levels at late stages of the disease when muscle mass is greatly reduced (87-89).

Several studies show that those enzymes which are elevated in the serum of DMD patients are often present in decreased levels in muscle tissue; it has been suggested that the decreased muscle activity of these enzymes is due to leakage from the muscle to the circulation (38,88,90). However, based on the rates of circulatory clearance of creatine kinase, and the muscle and plasma levels of creatine kinase, Pennington (76) estimated that less than 1% of the total muscle creatine kinase would need to be released from the muscle daily to the circulation to maintain the observed serum levels of the enzyme. This estimate is based on the

rate of clearance of creatine kinase after myocardial infarction. However, the major creatine kinase isoenzyme in the heart is the MB isoenzyme (91), and may be cleared at a different rate than the MM isoenzyme which predominates in muscle (26). Furthermore, whether the release of enzymes from the heart after myocardial infarction stops gradually or abruptly is not known. If the release stops gradually the actual rates of clearance of the enzymes may be more rapid than measured.

There are two theories which attempt to explain the pathological release of muscle enzymes into the circulation of DMD patients (38,76). The first is that the sarcolemma contains large physical interuptions that occur before actual necrosis of the muscle cell. Presumably, lesions of this type would render the sarcolemma permeable to all soluble sarcoplasmic constituents and extensive cellular degeneration and necrosis would soon follow. The second theory is that the sarcolemma of DMD patients is abnormally permeable to some macromolecules, and some or all soluble muscle enzymes are released from the sarcoplasm of viable muscle cells into the circulation. The evidence for these theories is discussed in an excellent review by Rowland (38).

The evidence that the release of muscle enzymes is through large physical interuptions in the sarcolemma is based primarily on electron microscopic studies. There is evidence of physical interuptions in the sarcolemma from DMD muscle that precedes muscle cell necrosis (39,41). The release of mitochondrial enzymes into the serum of DMD patients (83,92) is consistent with degenerate mitochondria at the site of the lesions in the sarcolemma (39). It seems likely that the release of muscle enzymes into the serum of DMD patients is at least in part due to physical interuptions in the sarcolemma.

Several lines of evidence suggest that the release of muscle enzymes into the circulation of DMD patients is due primarily to the increased permeability of the sarcolemma to muscle proteins. Elevated levels of serum enzymes are usually observed in the early stages of DMD and in female carriers of the X-linked trait, often when there is little or no evidence of sarcolemmal lesions or cell necrosis in muscle biopsies (93). A decrease in the serum levels of muscle enzymes are observed when DMD patients are treated over a period of time with drugs known to influence membrane properties. Prednisolone (94,95) and diethylstilbesterol (96-98) reduce the serum levels of creatine kinase in DMD patients. However, it can not be readily determined if the effect of these drugs is due to the alteration of membrane properties or due to the effect of these drugs on other cellular properties. The altered permeability of the sarcolemma may be in part determined by the metabolic state of the cell. For example, the release of muscle enzymes from normal human muscle after strenuous exercise (99,100) may be related to decreased ATP levels in the muscle (99).

If the release of muscle enzymes into the circulation of DMD patients is by passage through large lesions in the sarcolemma, it would be expected that all soluble muscle proteins would be released from muscle tissue at similar rates and that similar levels of increased activity of different muscle enzymes would be observed. However, the extent of elevation of different abundant muscle enzymes in the serum of DMD patients differs considerably (Table 1). Creatine kinase is the most markedly elevated enzyme in the serum of DMD patients; levels nearly 400 times that of the serum of normal controls are observed. Aldolase,

TABLE 1. Some Properties of Muscle Proteins and the Extent of Elevation in DMD. Data are compiled from the references given in parentheses.

Protein	Fold Elevation in DMD Serum	Molecular Weight (X 10^{-3} daltons)	Half-life for Clearance after Myocardial Infarction (118)	Evidence for Association with Intracellular Structures
Creatine Kinase	383 (62)	86 (103)	15 hr	yes (119)
Phosphoglucomutase	37 (62)	60 (109)	--	--
Pyruvate Kinase	27 (59)	230 (104)[b]	--	yes (120)
Aldolase	23 (62)	160 (105)[b]	21 hr	yes (120-122)
Myoglobin	12 (63)	17 (117)[b]	--	--
Lactate Dehydrogenase	6.3 (62)	142 (106)[b]	10 hr	yes (122)
Aspartate Aminotransferase	5 (62)	90 (107)[b]	--	yes (120)
Alanine Aminotransferase	5 (62)	115 (107)[b]	--	--
Glyceraldehyde-3-Phosphate Dehydrogenase	3.9 (62)	137 (108)[b]	--	no (120)
Glucosephosphate Isomerase	3.6 (62)	134 (110)	--	yes (120)
Malate Dehydrogenase	3.1 (62)	67 (111)[b]	16 hr	--
AMP Aminohydrolase	1.7 (62)	278 (112)[b]	--	yes (123,124)
Adenylate Kinase	1.5 (67,68)	21.5 (113)	--	no (120)
Phosphorylase	1.3 (101)	170-495 (114)[b]	--	--
Phosphofructokinase	nd[a] (59,102)	380 (115)[b]	--	yes (120)
Hexokinase	nd (59)	100 (116)[b]	--	no (120)

[a]nd = not detected.
[b]Molecular weights are for mammalian species other than humans.

phosphoglucomutase, pyruvate kinase, and myoglobin levels are increased 12-40 fold; malate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, glucosephosphate isomerase, alanine aminotransferase and lactate dehydrogenase levels are increased 3-6 fold; however, the levels of AMP aminohydrolase, adenylate kinase, and phosphorylase are only slightly increased; phosphofructokinase and hexokinase are not detected.

Differences in the relative levels of muscle enzymes in the blood of DMD patients may be due to different rates of clearance and/or inactivation after release into the circulation. Bar and Ohlendorf (118) determined the rates of loss of the activity of several heart enzymes from the circulation after myocardial infarction. The half-lives for the loss of lactate dehydrogenase M_4, aldolase, creatine kinase, glutamate-oxaloacetate transminase, and malate dehydrogenase activity are shown in Table 1. The rates of loss of these activities are similar, and there is no apparent correlation between the observed rate of loss of enzyme activity and the extent of elevation in the serum of DMD patients. However, as discussed above, with the exception of lactate dehydrogenase M_4, the isoenzymes released from DMD muscle may be different from the heart isoenzymes and lost from the circulation at a different rate. No studies are available which show the rates of loss of activity of those enzymes which are not elevated in the serum of DMD patients.

The release of muscle enzymes into the circulation of DMD patients could be reduced by the binding of muscle enzymes to intracellular structures. The release through an abnormally permeable sarcolemma would depend on the extent of the interaction with intracellular structures. If the release of muscle enzymes is through lesions in the sarcolemma or

from necrotic muscle fibers, an enzyme that is extensively associated with intracellular structures might be degraded within the muscle cell and not released into the circulation in an active form. There is evidence for the association of creatine kinase, aldolase, phosphofructokinase, pyruvate kinase, glucose-phosphate isomerase, lactate dehydrogenase, aspartate aminotransferase, and AMP aminohydrolase with myofibrillar proteins in mammalian muscle tissue (see Table 1). The extent of intracellular association of these proteins with myofibrillar components under the metabolic conditions present in DMD muscle may regulate the extent of release of these enzymes into the circulation.

If the sarcolemma is selectively permeable to some muscle proteins and not others, it might be expected that the sarcolemma would be more permeable to small proteins than to larger ones. The data in Table 1 show that no apparent correlation exists between molecular size and the extent of elevation of muscle enzymes in DMD serum. The absence of the high molecular weight enzymes phosphofructokinase, AMP aminohydrolase, phosphorylase, and hexokinase in DMD serum is consistent with the retention of these enzymes in muscle because of their size. However, creatine kinase, pyruvate kinase, aldolase, lactate dehydrogenase, aspartate aminotransferase, alanine aminontransferase, glyceraldehyde-3--phosphate dehydrogenase, glucose-phosphate isomerase and malate dehydrogenase are elevated in DMD serum despite their large molecular size, and the small protein adenylate kinase is only slightly elevated in DMD serum.

In this thesis I examine aspects of the elevation of muscle enzymes in the serum of dystrophic chickens as a potential model for human DMD,

the role of the extent of association of enzymes with intracellular structures, and the loss of enzyme activity from the circulation on the circulatory levels that are observed. The next section of this literature review discusses aspects of hereditary muscular dystrophy of the chicken.

B. MUSCULAR DYSTROPHY IN THE CHICKEN

Hereditary muscular dystrophies exist in the mouse (125), chicken (126), hamster (127), mink (128), sheep (129), and turkey (130). Most research has involved study of the mouse and chicken models. The primary genetic lesion(s) which causes the muscular dystrophies in any of these animal models is not known. Though both similarities and differences are observed in comparing the animal models to the human muscular dystrophies, it is hoped that the understanding of the animal muscular diseases might shed light on the inherent problem in the human diseases. In this section I review studies on muscular dystrophy of the chicken.

Inherited muscular dystrophy of the chicken was first described by Asmundson and Julian (126) in a commercial flock of New Hampshire Red chickens. A homozygous line of dystrophic chickens (line 301) was selected from matings of affected chickens. Muscular dystrophy of the chicken is an autosomal recessive trait and affects primarily the white fiber glycolytic muscle (131). Selection of affected chickens was based on the inability of affected chickens to right themselves from the supine position. The pedigree and characteristics of dystrophic chickens developed since that time are reviewed by Wilson et al. (132). Several lines of chickens were developed from the line 301 chickens to produce lines that differ in some characteristics. Lines 304 and 307 have high

muscle fat content and early onset of symptoms compared to the line 301 chickens. However, these lines differ in that line 304 is characterized by early pectoralis (breast) muscle hypertrophy and line 307 by breast muscle atrophy. Line 304 chickens were outcrossed to normal New Hampshire Red chickens to produce the dystrophic lines 413 and 455, and the normal control lines 412 and 454. These dystrophic lines are characterized by breast muscle hypertrophy, and low fat content compared to lines 301, 304, and 307. The development of the lines in the 400 series was a significant advancement because for the first time closely related control birds were available for study. The original line 301 chickens were outcrossed to normal White Leghorn chickens to obtain the Connecticut line of dystrophic chickens studied by some investigators.

There are many advantages to using the chicken model for examining aspects of muscular dystrophy. Obviously, chickens can be used for many experiments which for ethical reasons can not be performed on human patients. New methods of chemotherapy can be tested by monitoring the effect of agents on the physical and biochemical symptoms of the disease. Eggs of predicted phenotype are obtained which allow convenient study of the embryonic development of the disease. Post-natal development of the disease occurs rapidly and predictably. The affected pectoralis muscle is large in size and consists almost entirely of white, glycolytic muscle fibers, thus allowing convenient biochemical studies of a near homogenous tissue. Many biochemical similarities are observed between human and chicken muscular dystrophies and results with the chicken model may be applicable to human muscular dystrophy.

Though there has been some controversy as to whether human DMD is myogenic or neurogenic in origin (36), the results of limb bud

transplantation experiments between normal and dystrophic chickens indicate muscular dystrophy in the chicken is myogenic in origin. When limb buds are transplanted between chick embryos 3 1/2 days after incubation of the eggs, the transplanted limb buds attach and develop on the body of the host producing functional wing muscles that are innervated by the host. Transplantation of limb buds between normal and dystrophic chicken embryos show the phenotype of the developed limb is determined by the limb bud and not by the host (132,133).

Dystrophic chicken muscle has some morphological and ultrastructural features that are common to those observed in human DMD (33). There is heterogeneity of muscle fiber diameter (132), large numbers of nuclei situated internally within the muscle fiber rather than in a subsarcolemmal position (132,134), splitting of muscle fibers (134), accumulation of lipid between muscle fibers (132), evidence of regenerating muscle fibers (135), and necrosis and phagocytosis of muscle fibers at later stages of the disease (134). However, characteristics unique to muscular dystrophy in the chicken are the enlargement of the sarcotubular system (132), and vacuolization within muscle fibers at later stages of the disease (134). Histochemical staining of dystrophic chicken muscle with stains indicative of different enzyme activities within muscle fibers (132) reflect the alteration of enzyme activities in muscle homogenates that are discussed below.

No protein or enzyme has been found to be absent, or present in drastically reduced levels, in the muscle of dystrophic chickens. There are decreased levels of creatine kinase (126,136), AMP aminohydrolase (137-140) and all of the glycolytic enzymes examined (132,134,136, 140-147), with the exception of hexokinase which is elevated (134,148).

The levels of glycogen phosphorylase were shown to be decreased by some investigators (134,149), but increased by others (150). Increased muscle levels of citric acid cycle enzymes (134,151), lysosomal enzymes (152-157), enzymes of the hexose monophosphate shunt (147,148), enzymes involved in cyclic AMP metabolism (158,159), enzymes of purine metabolism (137), acetycholinesterase (132,145,160), prostaglandin E9-ketoreductase (161), transferase RNA methylase (162), acetylphosphatase (145), glutathione peroxidase (147), superoxide dismutase (147), glutathione reductase (147), and myoglobin (147) are observed in dystrophic chickens. Few studies report the levels of metabolites in dystrophic chicken muscle, though increased levels of muscle lipids (147,163-165), serine ethanolamine phosphate (166,167), and cystathionone (168) are observed. Increased levels of intracellular sodium and chlorine are observed in muscle from dystrophic chickens (169). However, as is the case in human DMD, the differences in the levels of these muscle components are not dramatic and are likely secondary effects of the disease.

The enzyme level patterns in dystrophic chicken muscle indicate an increase in oxidative metabolism and a decrease in glycolytic metabolism and is reflective of metabolism in embryonic muscle tissue. This trend is also observed in human DMD. There are several studies which examine the isoenzyme patterns of dystrophic chicken muscle to determine whether embryonic forms of muscle enzymes are present. Adult dystrophic chicken breast muscle retains some embryonic-like properties of acetylcholinesterase; the enzyme exists primarily in a low molecular weight form and high levels are observed in the cytoplasm outside of the motor endplate region (31,132,170). Elevated levels of embryonic forms of creatine kinase in adult dystrophic chicken breast muscle were observed

by Weinstock et al. (171), but not by others (172,173). Embryonic-like forms of myosin (174-176) and troponin-t (1976) are observed in adult dystrophic chicken breast muscle. Embryonic isoenzymes may be in part due to a large number of regenerating muscle fibers in dystrophic chicken breast muscle (135). However, embryonic forms of pyruvate kinase are not observed (146,147) in dystrophic breast muscle.

Several studies show altered levels of membrane bound enzymes and abnormalities in the properties of membranes from dystrophic chicken muscle. Increased levels of adenylate cyclase (159,178,179) and guanylate cyclase (179) are observed. There is no apparent difference in the stimulation of adenylate cyclase from normal and dystrophic muscle by catecholamines or NaF (178), in contrast to what is observed in DMD sarcolemma (38,48). Reduced levels of the ouabain-sensitive Na/K,Mg-ATPase are observed in dystrophic chicken muscle (156). One study found no differences in the levels of microsomal Ca-ATPase in dystropic and normal muscle (179,180) though others found decreased levels of Ca/Mg-ATPase (181) and a reduction of calcium uptake by isolated sarcoplasmic reticulum vesicles (182). Increased sarcolemmal viscosity (183) and an altered lipid composition of the sarcoplasmic reticulum of dystrophic chickens are observed (184).

As is the case in human DMD, in dystrophic chickens there are increased serum levels of some muscle proteins. Increased serum levels of aldolase (185), glutamate-oxaloacetate transaminase (185), creatine kinase (136,186-192), pyruvate kinase (136,177,191,192) and acetycholinesterase (31,191,193) are observed in dystrophic chickens. Elevated serum levels of pyruvate kinase (177) and creatine kinase (186) are also observed in heterozygous carriers of the dystrophic trait. The increased

serum levels of pyruvate kinase in dystrophic chickens are due to the muscle isoenzyme (177,194). Increases in the serum levels of creatine kinase and pyruvate kinase are proportional to the increase in body weight throughout the development of dystrophic chickens after hatching (191). The mechanism for the release of enzymes from the muscle of dystrophic chickens is not known. Presumably the loss of muscle enzymes is due to a structural defect in the sarcolemma. One report shows that there are few muscle fibers in dystrophic chickens which are permeable to peroxidase (195), suggesting that there are few lesions that would allow free passage of sarcoplasmic components to the circulation, and that enzymes may be leaked through a sarcolemma with an abnormal permeability to muscle proteins.

Several classes of drugs with vastly different modes of pharmacological action improve or delay the onset of symptoms of muscular dystrophy in the chickens. The effect of drugs on chicken muscular dystrophy is usually monitored by measuring the ability of dystrophic chickens to right themselves from the supine position or by monitoring the serum levels of muscle enzymes. Prednisolone (196) and diphenylhydantoin (196,197) delay the onset of symptoms in dystrophic chickens presumably by stabilizing muscle membranes. Treatment with serotonin agonists are also somewhat successful (190,191,198). Serotonin induces muscle lesions in normal rodents (199,200), and serotonin agonists are thought to be effective chemotherapeutic agents in avian muscular dystrophy by reducing the abnormal accumulation of biogenic amines that are reported in dystrophic tissues (201-203). Because of the high activity of proteinases in dystrophic chicken muscle (152,154-157), peptide proteinase inhibitors were tested as therapeutic agents

and are successful in delaying the onset and severity of symptoms in dystrophic chickens (204,205).

Chemotherapy trials with the sulfhydryl compound penicillamine are also successful in delaying the onset of symptoms in dystrophic chickens (147,189,206,207). Based on these results it was suggested that the defect in avian muscular dystrophy is related to the oxidation-reduction state in muscle (147). Crude pectoralis muscle homogenates from dystrophic chickens have decreased levels of both exposed and total sulfhydryl groups (147,208). The reduced specific activity of glyceraldehyde-3-phosphate dehydrogenase in dystrophic chicken muscle may be primarily due to the oxidation of essential sulfhydryl groups (208). Penicillamine treatment protects glyceraldehyde-3-phosphate dehydrogenase from inactivation (208) and increases the total free sulfhydryl content in dystrophic muscle (147). Penicillamine might stabilize muscle membranes by maintaining the proper oxidation-reduction state of membrane proteins and by reducing the peroxidation of membrane lipids (147).

Despite encouraging results of chemotherapeutic trials with dystrophic chickens, trials with some of these same agents in human patients with DMD have been less encouraging. Prednisolone treatment of DMD patients decreases serum creatine kinase levels, however, no other improvement in the clinical characteristics of the disease are observed (7,94). Serotonin agonists have virtually no effect on the creatine kinase levels or on any other clinical characteristics of DMD patients (203).

CHAPTER II. MUSCLE ENZYMES IN THE SERUM OF DYSTROPHIC CHICKENS

INTRODUCTION

In human DMD the most widely used test for the early diagnosis of the disorder and for the detection of the X-linked trait in female carriers is the increased levels of muscle enzymes in the circulation. The success of chemotherapeutic trials in animal models of muscular dystrophy is often evaluated by monitoring the serum levels of muscle enzymes. For these reasons it is of interest to understand and evaluate those factors which control the levels of muscle enzymes in the circulation of normal and dystrophic chickens.

These studies show that the pattern of elevation of several enzymes in the circulation of dystrophic chickens is the same as is observed for human DMD. The results suggest that the study of those factors which determine the levels of muscle enzymes in the serum in avian muscular dystrophy is relevant to the human condition. Based on studies of the rates of efflux of enzymes from dystrophic muscle, the rates of inactivation of enzymes in the circulation, the rates of circulatory clearance, and the extent of intracellular association of muscle enzymes that are reported here, it appears as if the rates of circulatory clearance are important in determining the levels of these enzymes in the serum of dystrophic animals.

MATERIALS AND METHODS

Materials

Frozen chicken breast muscle was obtained from Pel-Freeze Biologicals, Rogers, Arkansas. Line 412 (normal) and 413 (dystrophic) chickens were raised from fertile eggs obtained from the Department of Avian Sciences, University of California at Davis, Davis, California. White Leghorn chickens were raised from fertile eggs obtained from a local hatchery. All chickens used in these experiments were between 25 and 45 days of age. All salt solutions were prepared with reagent grade biochemicals. All solutions were prepared in distilled, deionized water. Phosphocellulose was from Whatman Inc., Clifton, New Jersey. Ultra-pure $(NH_4)_2SO_4$ from Schwarz-Mann Inc., Spring Valley, New York, was recrystallized from H_2O before use. Aquacide III was from Calbiochem-Behring Corporation, La Jolla, California. Sephadex G-100 was from Pharmacia Inc., Piscataway, New Jersey. 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril was a gift from Professor J.C. Speck. $Na^{125}I$ was from Amersham Corporation, Arlington Heights, Illinois or ICN Chemical and Radioisotope Division, Irvine, California. N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), all substrates, and coupling enzymes were from Sigma Chemical Company, St. Louis, Missouri except glucose which was from Mallinckrodt Chemical Works, St. Louis, Missouri.

Preparation of Muscle Crude Extracts

Muscle crude extracts were prepared by homogenizing tissue in a Waring blender using 3.3 ml of 0.18 M KCl, 0.054 M KH_2PO_4, 0.035 M K_2HPO_4, pH 6.5, per g tissue. The homogenate was centrifuged at 6,000 x g for 20 min and the supernatant assayed for enzyme activity.

Collection of Blood Samples

Blood samples (about 0.5 ml) were collected from the brachial vein into heparinized glass tubes. The plasma was assayed for enzyme activity after removal of red blood cells by brief centrifugation. Samples were chilled on ice and assayed for enzyme activities within 2 hr of collection.

Enzyme Assays

Creatine kinase activity was measured by the procedure of Rosalki (209) using the assay system obtained from Sigma Chemical Company. The assay couples ATP production from ADP and creatine phosphate to NADP reduction with hexokinase and glucose-6-phosphate dehydrogenase, and the increase in absorbance at 340 nm is monitored. Pyruvate kinase was assayed by coupling pyruvate production from phosphoenolpyruvate to NADH oxidation with lactate dehydrogenase as described by Bucher and Pfleiderer (210). The rate of decrease in the absorbance at 340 nm was monitored. Adenylate kinase activity was determined by coupling ATP production from ADP to NADP reduction with hexokinase and glucose-6-phosphate dehydrogenase essentially as described by Oliver (211). The assay mixture contained 3 mM ADP, 5 mM glucose, 5 mM $MgCl_2$, 0.7 mM NADP, 50 mM TRIS, pH 7.6, 1.5 unit ml^{-1} yeast hexokinase, 1.5 unit ml^{-1} yeast glucose-6-phosphate dehydrogenase and 0.1 mg ml^{-1} bovine serum albumin. The rate of increase in absorbance at 340 nm was monitored.

AMP aminohydrolase was assayed by following the increase in absorbance at 290 nm or the decrease in absorbance at 265 nm as AMP is deaminated to form IMP (212). Phosphofructokinase was assayed by coupling ADP production from fructose-6-phosphate and ATP to NADH oxidation with pyruvate kinase and lactate dehydrogenase as described by Emerk and Freiden (213). The decrease in absorbance at 340 nm was monitored. All assays were performed at 30°C and were initiated by the addition of an aliquot of the enzyme solution to 1.0 ml of the assay mixture.

Enzyme Purification

Creatine kinase was purified to homogeneity by the procedure of Eppenberger et al. (214) and pyruvate kinase by the procedure of Cardenas et al. (215) from frozen chicken breast muscle and were stored at -20°C as a suspension in 50% saturated $(NH_4)_2SO_4$ and 50% glycerol until use.

AMP aminohydrolase from frozen chicken breast muscle was purified to homogeneity as described by Suelter et al. (212) and was stored at 4°C in the final column buffer until use. Samples were routinely concentrated with Aquacide III before use.

Adenylate kinase was purified from frozen chicken breast muscle by a modification of the procedure of Schirmer et al. (216). Four hundred and eighty grams of thawed muscle were homogenized in 1 l 10 mM KCl in a blender. Centrifugation, pH fractionation, zinc acetate precipitation, and $(NH_4)_2SO_4$ fractionation were as described previously (216). The precipitate obtained from the $(NH_4)_2SO_4$ fractionation was extensively dialyzed against 10mM imidazole, 10 mM 2-mercaptoethanol, pH 7.5, and applied to a 8 x 2 cm column of phosphocellulose, and washed with dialysis buffer until the absorbance at 280 nm of the eluent was

0.06. Adenylate kinase was eluted with a linear gradient of 0-15 mM potassium pyrophosphate in 10 mM imidazole, 10 mM 2-mercaptoethanol, pH 7.5. Each side of the gradient had a volume of 200 ml. Fractions with a specific activity greater than 400 μmole min^{-1} mg $protein^{-1}$ were pooled and concentrated by precipitation of the enzyme by adding 60.3 g solid $(NH_4)_2SO_4$ per 100 ml solution to bring the final $(NH_4)_2SO_4$ concentration to 90% saturation (calculated at 0°C). The enzyme was resuspended and dialyzed against 0.1 M imidazole, 10 mM 2-mercaptoethanol, pH 7.5, concentrated with Aquacide III, applied to a 80 x 2 cm Sephadex G-100 column and eluted with 0.1 M imidazole, 10 mM 2-mercaptoethanol, pH 7.5. Peak fractions were concentrated by precipitating the enzyme with solid $(NH_4)_2SO_4$ to 90% saturation. Adenylate kinase was stored as a suspension at -20°C in 50% saturated $(NH_4)_2SO_4$ and 50% glycerol containing 10 mM imidazole, 10 mM 2-mercaptoethanol, pH 7.5. The enzyme was homogenous as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis and had a specific activity of 1630 $\mu moles^{-1}$ min^{-1} mg $protein^{-1}$.

Radioiodination of Enzymes

Purified AMP aminohydrolase and pyruvate kinase were iodinated with $Na^{125}I$ using the iodinating agent 1,3,4,6-tetrachloro-3a,6a--diphenylglycoluril by the method of Fraker and Speck (217). Purified adenylate kinase and creatine kinase were iodinated by the lactoperoxidase method as described by Martin et al. (218). Free iodine after the iodination of proteins was removed by extensive dialysis against phosphate-buffered saline (PBS) which contained 0.2 g l^{-1} KCl, 8 g l^{-1} NaCl, 0.2 g l^{-1} KH_2PO_4, and 0.78 g l^{-1} K_2HPO_4. The specific radioactivities of the enzymes used in these

experiments were: AMP aminohydrolase (11 μCi mg^{-1}), pyruvate kinase (51 μCi mg^{-1}), creatine kinase (52 μCi mg^{-1}), and adenylate kinase (14 μCi mg^{-1}). All radioactive samples were counted in a Beckman Biogamma Counter.

Rates of Loss of Enzymes from the Circulation

Prior to injection enzymes were dialyzed twice against 200 volumes of PBS. Any insoluble material was removed by centrifugation. The rates of loss of enzyme activities or ^{125}I were measured after the injection of 0.5 ml of an enzyme solution per 200 g body weight. The concentrations of enzymes used were: 280 units ml^{-1} pyruvate kinase and 8.9 x 10^5 cpm ml^{-1} ^{125}I-pyruvate kinase; 2000 units ml^{-1} AMP aminohydrolase and 8.0 x 10^5 cpm ml^{-1} ^{125}I-AMP aminohydrolase; 245 units ml^{-1} creatine kinase and 9.2 x 10^5 cpm ml^{-1} ^{125}I-creatine kinase; 71 units ml^{-1} adenylate kinase and 8.6 x 10^5 cpm ml^{-1} ^{125}I-adenylate kinase.

Enzyme solutions were injected into the brachial vein. Blood samples were collected into heparinized tubes from the brachial vein of the wing opposite to that injected. Blood samples were collected before injection to determine endogenous enzymatic activities, and at the time intervals after injection given in the results. After brief low speed centrifugation to remove red blood cells, the blood plasma was assayed for enzyme activities and 50 or 100 μl aliquots were counted to determine ^{125}I content.

The loss of enzyme activity and radioactivity of ^{125}I-labeled enzymes from the circulation followed a biphasic exponential loss and was fit to equation [1] with a non-linear curve fitting computer program

(219) after subtraction of the enzyme activity in the circulation prior to injection and background radioactivity.

$$A_t = A_1e^{-k_1t} + A_2e^{-k_2t} \qquad [1]$$

A_t is the total amplitude ($A_1 + A_2$) of enzyme activity of radioactivity disappearing at rates k_1 and k_2. The clearance of adenylate kinase activity was a monophasic exponential decay and was fit to equation [2].

$$A_t = A_1e^{-k_1t} \qquad [2]$$

The parameters were determined by the computer program for each chicken separately and averaged to give the rate constants and amplitudes given in the results.

Measurement of the Inactivation of Enzyme Activity in Serum In Vitro

Blood was collected from chickens and allowed to clot 1 hr at room temperature. The clot was separated from the serum by centrifugation and the serum placed on ice. To 200 µl serum in 1.5 ml polypropylene tubes was added 5 µl 1 M HEPES pH 7.5, and enzyme solution in PBS, to give final concentrations of the enzyme similar to that expected in the plasma after intravenous injection experiments in vivo. Samples (5 µl) were assayed before warming the tubes (t=0), then the tubes were capped and incubated at 41°C (chicken body temperature) in a water bath and 5 µl aliquots were assayed for enzymatic activity at the time intervals given in the Results.

Determination of the Tissue Distribution of ^{125}I-Adenylate Kinase

Thirty minutes after the injection of ^{125}I-adenylate kinase as described above, the chicken was decapitated. Tissues were weighed and aliquots were counted in a Beckman Biogamma Counter.

Preparation of Muscle Press Juices

Press juices of normal and dystrophic muscle tissue were obtained by placing the entire pectoralis muscle in a centrifuge tube, and centrifuging at 40,000 x g for 4 hr at 4°C. The supernatant press juice was removed with a Pasteur pipet and assayed for enzyme activity immediately. The pectoralis muscle opposite to that used to make the press juice was used to make a high ionic strength crude extract as described above. Enzyme activities in the crude extract and press juices were determined as described earlier and protein was determined by the method of Lowry et al. (220).

RESULTS

Levels of Enzymes in the Muscle and Blood Plasma of Normal and Dystrophic Chickens

The data in Table 2 show the levels of several enzymes in the muscle and blood plasma of normal and dystrophic chickens and the ratio of the total enzyme activity in the blood to that in the breast muscle. These results show that the activities of creatine kinase and pyruvate kinase are markedly elevated in the plasma of dystrophic chickens and that the activities of adenylate kinase and AMP aminohydrolase are not elevated. Pyruvate kinase levels are more markedly increased than creatine kinase when expressed as the ratio of the activity in the blood plasma to that in the breast muscle. Increased blood plasma activities of creatine kinase and pyruvate kinase in dystrophic chickens compared to normal chickens are consistent with previous reports (132,191).

Pyruvate Kinase Isoenzymes in the Plasma of Dystrophic Chickens

Because pyruvate kinase is found in abundance in many tissues other than muscle, it is of interest to verify that the pyruvate kinase released into the plasma of dystrophic chickens is from muscle and not some other tissue or organ. This can be determined because the major pyruvate kinase isoenzyme found in adult chicken muscle is electrophoretically and kinetically distinct from the enzyme found in spleen, lungs, liver, kidney, brain and erythrocytes (221). The adult muscle isoenzyme is not activated by fructose-1,6-bisphosphate

TABLE 2. The Activities of Several Enzymes in the Blood Plasma and Breast Muscle of Normal and Dystrophic Chickens[a]

Enzyme	Chicken[b]	Units/ml Plasma	Units/gm Breast Muscle	$\frac{\text{Total Units Plasma} \times 10^3}{\text{Total Units Breast Muscle}}$[c]
Creatine Kinase	N	0.4 ± 0.2 (8)	1610 ± 240 (4)	0.17
	D	9.3 ± 4.6 (10)	1460 ± 430 (4)	2.6
Pyruvate Kinase	N	0.8 ± 0.2 (8)	605 ± 177 (4)	0.90
	D	7.2 ± 2.6 (8)	308 ± 135 (4)	9.5
Adenylate Kinase	N	0.13 ± 0.07 (8)	83 ± 24 (4)	1.1
	D	0.14 ± 0.07 (7)	86 ± 19 (4)	0.60
AMP Aminohydrolase[d]	N	0.005 ± 0.002 (3)	36 ± 3 (4)	0.09
	D	0.005 ± 0.002 (4)	13 ± 1 (4)	0.16

[a]The results are expressed as the average ± standard deviation for the number of chickens tested in parentheses.

[b]N designates line 412 (normal) and D designates line 413 (dystrophic).

[c]Calculated by estimating the plasma volume as 6.0% of the body weight of 250 g (15 ml), using measured values of breast muscle weight of 22 g and 37 g in normal and dystrophic chickens, respectively.

[d]Activity determined by following the change in absorbance at 265 nm with an assay mixture of 100 μM AMP, 0.15 M KCl, 50 mM MES-TRIS, pH 6.5.

(Fru-1,6-P_2) at limiting concentrations of phosphoenolpyruvate as are the pyruvate kinase isoenzymes found in other tissues (221). The results in Table 3 verify this observation. Erythrocyte and liver pyruvate kinases are activated 7.0 and 7.6 fold, respectively, by the addition of 1.0 mM Fru-1,6-P_2 at 0.1 mM phosphoenolpyruvate, while the muscle pyruvate kinases from both normal and dystrophic muscle are not activated. In normal chicken plasma the low pyruvate kinase activity that is present is activated three-fold by Fru-1,6-P_2 and implies that this activity is in part due to isoenzymes from tissues other than muscle. The high pyruvate kinase activity in the plasma of dystrophic chickens is only slightly increased by the addition of Fru-1,6-P_2 to the assay. The slight activation that is observed is attributable to levels of isoenzymes other than the muscle type and is equal to that in normal plasma because the total amount of Fru-1,6-P_2 activated pyruvate kinase activity is the same in both normal and dystrophic chickens. These results indicate that the high levels of pyruvate kinase in the blood plasma of dystrophic chickens are due to release from muscle tissue.

Pyruvate Kinase Clearance and Inactivation

The loss of pyruvate kinase activity from the circulation after the intravenous injection of the purified enzyme is shown in Figure 1. The clearance of pyruvate kinase activity in normal chickens fits a biphasic exponential decay with half-lives of 2.2 and 14 hr (Table 4). The rate constants for the loss of pyruvate kinase activity from the plasma of dystrophic chickens could not be determined because fluctuations in the high background pyruvate kinase activity in the plasma resulted in data too scattered to be fit by the non-linear curve fitting computer program used to calculate the rate constants. However, the data in Figure 1

TABLE 3. Characterization of Pyruvate Kinase Isoenzymes in the Serum and Tissues of Normal and Dystrophic Chickens by Activation with Fructose-1,6-Bisphosphate (Fru-1,6-P_2)[a]

	Activity (-)	Activity (+)	Activity (+)- Activity (-)	$\frac{\text{Activity (+)}}{\text{Activity (-)}}$
Normal[c] Serum (4)	0.73 ± 0.41	2.0 ± 0.6	1.3 ± 0.3	3.02 ± 0.77
Dystrophic[c] Serum (4)	13.8 ± 5.4	15.6 ± 6.2	1.8 ± 0.9	1.13 ± 0.03
Normal[c] Breast Muscle Homogenate (3)	21.4 ± 4.6	22.0 ± 3.4	--	1.04 ± 0.07
Dystrophic[c] Breast Muscle Homogenate (4)	7.3 ± 1.2	7.2 ± 1.9	--	1.00 ± 0.05
Normal[d] Erythrocyte Lysate (1)	3.0	21.0	--	7.0
Normal[d] Liver Homogenate (1)	8.4	64.0	--	7.6

[a]Assays were as described in the Methods except phosphoenolpyruvate was 0.1 mM with (+) or without (-) 1.0 mM Fru-1,6-P_2. Results are expressed as the change in absorbance at 340 nm per minute per ml sample.

[b]These ratios were calculated for each sample separately and averaged to give the values shown, which may differ slightly from the ratios calculated from the average values given in this table.

[c]Normal is line 412, dystrophic is line 413 chickens.

[d]Determined with normal White Leghorn chickens.

Figure 1. The loss of intravenously injected pyruvate kinase activity from the circulation of normal and dystrophic chickens. The loss of activity from normal (Δ) and dystrophic (0) chickens was determined as described in the Methods. Pyruvate kinase activity in the plasma prior to injection was subtracted from all values before plotting. The curve was calculated from the parameters given in Table 4 for the clearance of pyruvate kinase activity form normal chickens using equation [1].

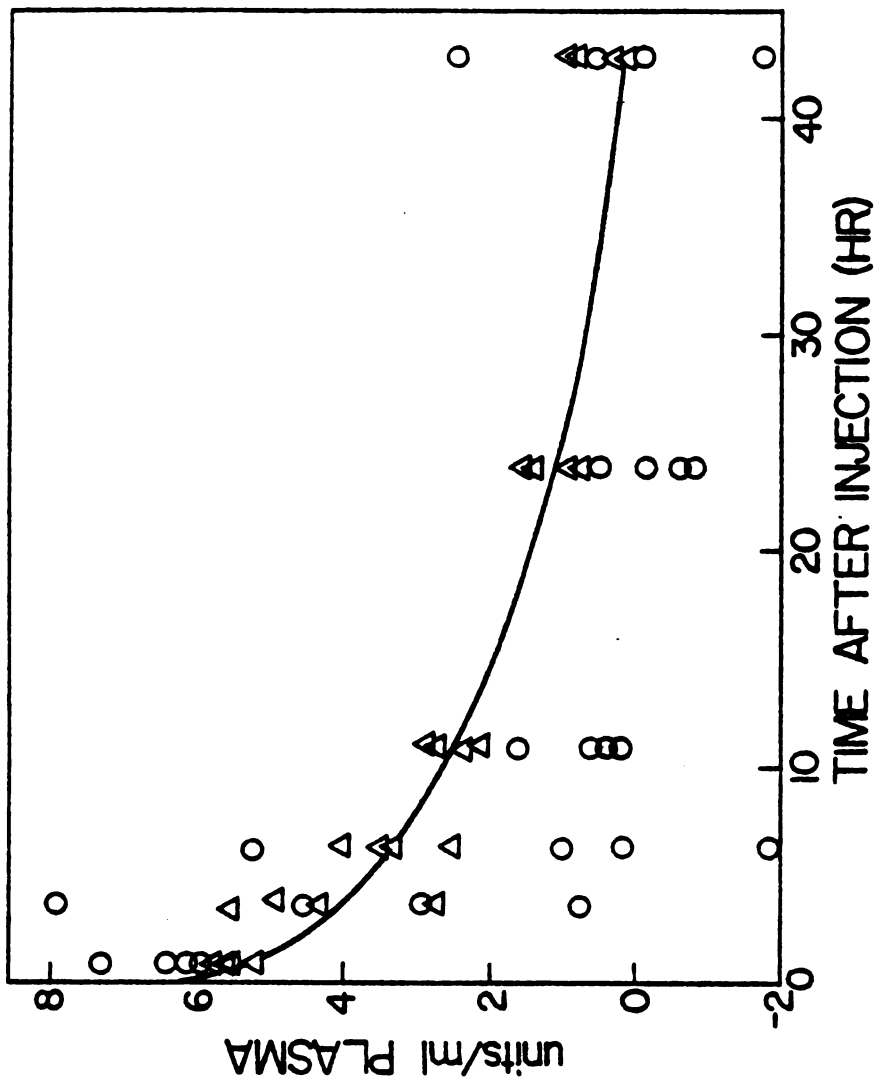

Figure 1.

TABLE 4. Rates of Loss of Pyruvate Kinase Activity and ^{125}I-Pyruvate Kinase from the Circulation of Normal and Dystrophic Chickens[a]

Enzyme	Chicken Type	k_1 (hr^{-1}) [$t_{1/2}$ (hr)]	k_2 (hr^{-1}) [$t_{1/2}$ (hr)]	% Lost In Rapid Phase
Pyruvate Kinase	Normal (3)	0.31 ± 0.14 [2.2]	0.051 ± 0.005 [14]	44 ± 15
Pyruvate Kinase	Dystrophic (0)[b]	--	--	--
^{125}I-Pyruvate Kinase	Normal (4)	0.35 ± 0.14 [2.0]	0.058 ± 0.013 [12]	66 ± 9
^{125}I-Pyruvate Kinase	Dystrophic (2)	0.44 ± 0.20 [1.6]	0.066 ± 0.012 [11]	71 ± 11

[a]Results are expressed as the average ± standard deviation for the number of chickens in parentheses. The clearance of pyruvate kinase and ^{125}I-pyruvate kinase was determined in 4 normal and 4 dystrophic chickens, however, the data from several experiments were too scattered to be fit by the non-linear curve-fitting computer program and were not included in the calculation of the rate constants shown here.

[b]None of the experiments measuring the clearance of pyruvate kinase activity in dystrophic chickens were successful presumably due to fluctuations in the high background pyruvate kinase activity in these chickens.

suggest that the clearance of injected pyruvate kinase activity in dystrophic chickens is similar to, or perhaps slightly more rapid than, the clearance of pyruvate kinase activity in normal chickens. To avoid the problem of high background pyruvate kinase activity in dystrophic chickens, the clearance of ^{125}I-pyruvate kinase was also measured in both normal and dystrophic chickens (Figure 2). The half-lives for the biphasic clearance of radioactivity after the intravenous injection of ^{125}I-pyruvate kinase are 2.0 and 12 hr in normal chickens and 1.6 and 11 hr in dystrophic chickens (Table 4).

When pyruvate kinase is incubated in normal serum *in vitro* at 41°C at concentrations similar to those expected in the blood after intravenous injection experiments *in vivo*, 41% of the pyruvate kinase activity is lost after 19 hr and 72% of the activity is lost after 42 hr. This rate of inactivation is considerably slower than the observed loss of about 90% of the enzyme activity 24 hours after intravenous injection (Figure 1). Furthermore, ^{125}I-pyruvate kinase is cleared at a similar rate as the clearance of pyruvate kinase activity in normal chickens (Table 4). These results indicate that the loss of pyruvate kinase activity from the circulation is primarily due to clearance and not inactivation.

Creatine Kinase Clearance and Inactivation

The loss of creatine kinase activity from the circulation after intravenous injection of the purified enzymes is shown in Figure 3. The clearance of creatine kinase activity fits a biphasic exponential decay with half-lives of 1.4 and 12 hr for the two phases in normal chickens, and 0.69 and 9.9 hr in dystrophic chickens (Table 5). The loss of ^{125}I-creatine kinase from the circulation of normal and dystrophic

Figure 2. The loss of radioactivity from the circulation after the intravenous injection of ^{125}I-pyruvate kinase in normal and dystrophic chickens. The loss of radioactivity from normal (Δ) and dystrophic (0) chickens was determined as described in the Methods. The curve was calculated from the average of the parameters given in Table 4 for the clearance of ^{125}I-pyruvate kinase in normal and dystrophic chickens using equation [1].

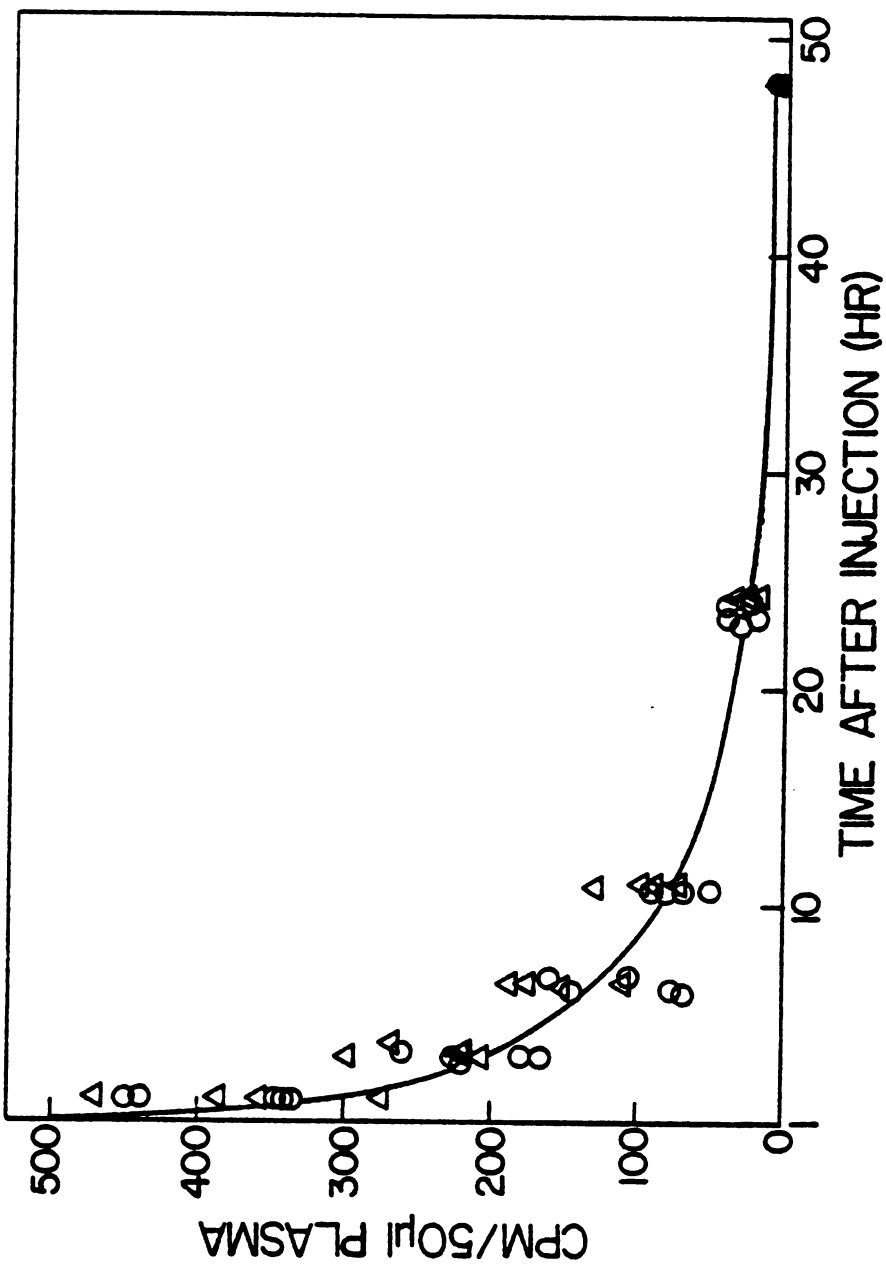
CPM/50µl PLASMA
500
400
300
200
100
0
0
10
20
30
40
50
TIME AFTER INJECTION (HR)

Figure 2.

Figure 3. The loss of intravenously injected creatine kinase activity from the circulation of normal and dystrophic chickens. The loss of activity from normal (Δ) and dystrophic (0) chickens was determined as described in the Methods. Creatine kinase activity in the plasma prior to injection was subtracted from all values before plotting. The curve was calculated from the average of the parameters given in Table 5 using the equation [1].

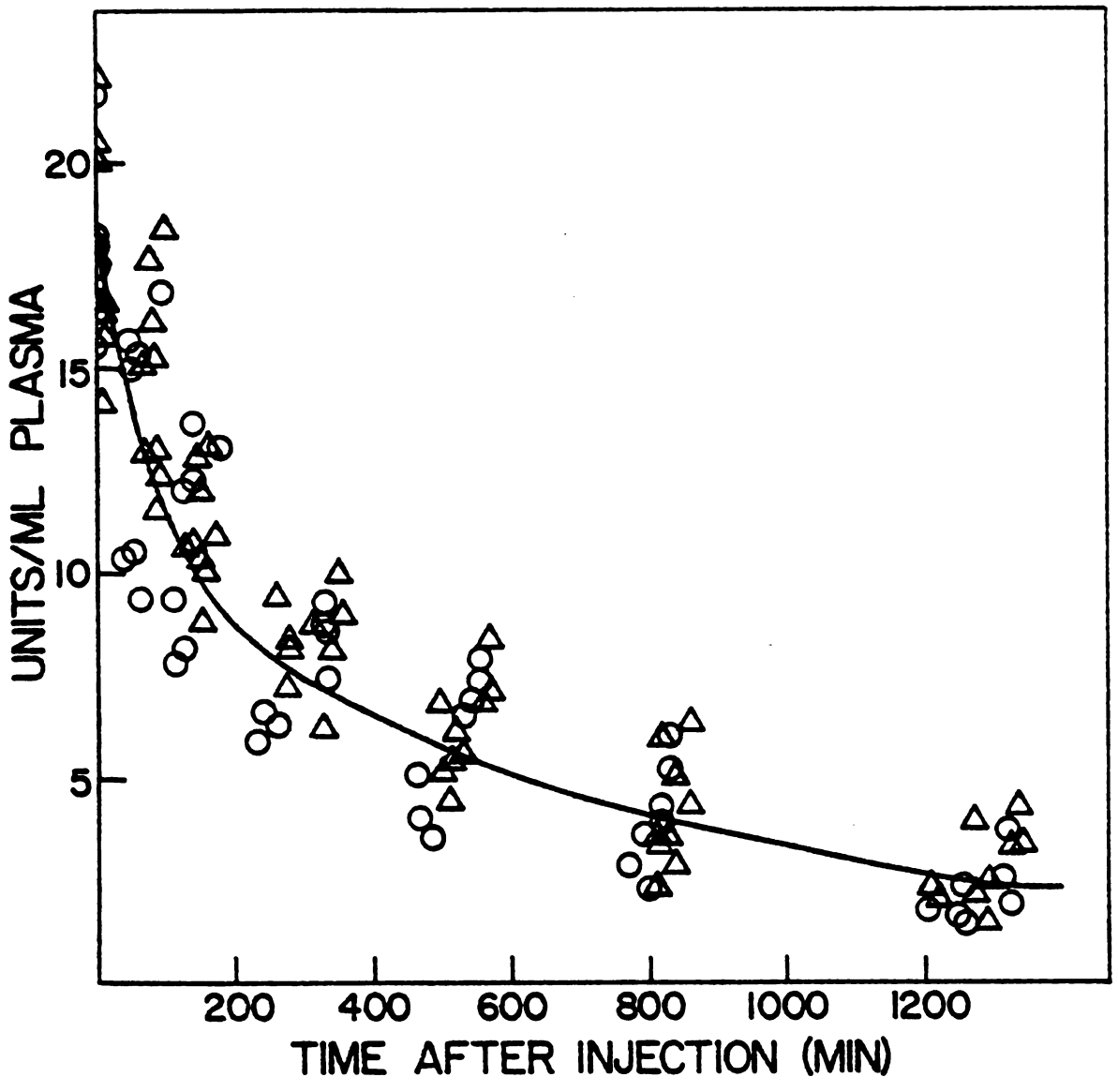
UNITS/ML PLASMA
20
15
10
5
200
400
600
800
1000
1200
TIME AFTER INJECTION (MIN)

Figure 3.

TABLE 5. Rates of Loss of Creatine Kinase Activity and ^{125}I-Creatine Kinase from the Circulation of Normal and Dystrophic Chickens[a]

Enzyme	Chicken Type	k_1 (hr^{-1}) [$t_{1/2}$ (hr)]	k_2 (hr^{-1}) [$t_{1/2}$ (hr)]	% Lost In Rapid Phase
Creatine Kinase	Normal (9)	0.51 ± 0.22 [1.4]	0.057 ± 0.025 [12]	51 ± 18
Creatine Kinase	Dystrophic (7)	1.0 ± 0.9 [0.69]	0.070 ± 0.031 [9.9]	44 ± 21
^{125}I-Creatine Kinase	Normal (4)	1.58 ± 0.15 [0.44]	0.14 ± 0.01 [5.0]	74 ± 2
^{125}I-Creatine Kinase	Dystrophic (3)	1.67 ± 0.04 [0.42]	0.12 ± 0.01 [5.8]	77 ± 4

[a]Results are expressed as the average ± standard deviation for the number of chickens in parentheses.

chickens after intravenous injection is shown in Figure 4. These data also fit a biphasic exponential loss with half-lives of 0.44 and 5.0 hr in normal chickens and 0.42 and 5.8 hr in dystrophic chickens (Table 5).

When creatine kinase is incubated at 41°C in vitro in serum from normal or dystrophic chickens at concentrations similar to those obtained after intravenous injection, 17% of the activity is lost from normal serum and 27% of the activity is lost from dystrophic serum after 22 hr. Twenty-one percent of the endogenous creatine kinase activity is lost from dystrophic serum after 22 hr at 41°C. These in vitro rates of inactivation are relatively slow compared to the loss of 88% of the injected creatine kinase activity 22 hr after intravenous injection (Figure 3).

Adenylate Kinase Clearance and Inactivation

To determine whether a rapid loss of adenylate kinase activity from the circulation explains the data in Table 2 that show adenylate kinase activity is not elevated in the plasma of dystrophic chickens, the rate of loss of adenylate kinase activity from the circulation of normal and dystrophic chickens was determined after the intravenous injection of the enzyme purified from chicken breast muscle. Figure 5 shows that the rapid loss of adenylate kinase activity from the blood plasma fits a monophasic exponential decay with half-lives of 4.6 and 5.3 min in normal and dystrophic chickens, respectively (Table 6).

To determine whether inactivation and/or clearance are responsible for the rapid loss of adenylate kinase, we measured the rates of inactivation of adenylate kinase in normal serum in vitro and the loss of radioactivity after the intravenous injection of ^{125}I-adenylate kinase in normal and dystrophic chickens. Figure 6 (solid circles) shows

Figure 4. The loss of radioactivity from the circulation after the intravenous injection of ^{125}I-creatine kinase in normal and dystrophic chickens. The loss of radioactivity from normal (Δ) and dystrophic (0) chickens was determined as described in the Methods. The curve is calculated from the average of the parameters given in Table 5 using equation [1].

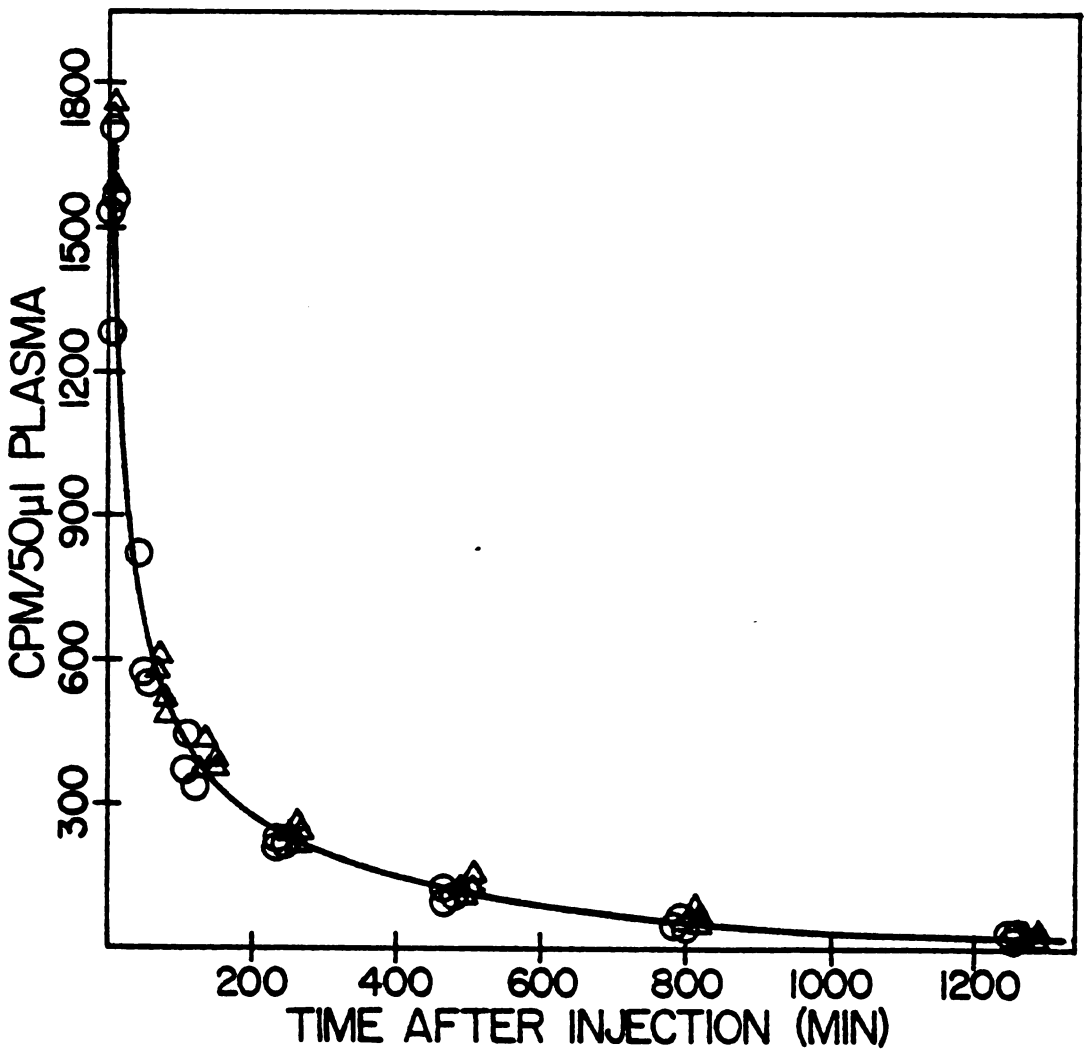
CPM/50µl PLASMA
1800
1500
1200
900
600
300
200
400
600
800
1000
1200
TIME AFTER INJECTION (MIN)

Figure 4.

Figure 5. The loss of intravenously injected adenylate kinase activity from the circulation of normal and dystrophic chickens. The loss of activity from normal (Δ) and dystrophic (O) chickens was determined as described in the Methods. Adenylate kinase in the plasma prior to injection was subtracted from all values before plotting. The curve was calculated from the average of the parameters given in Table 6 using equation [2].

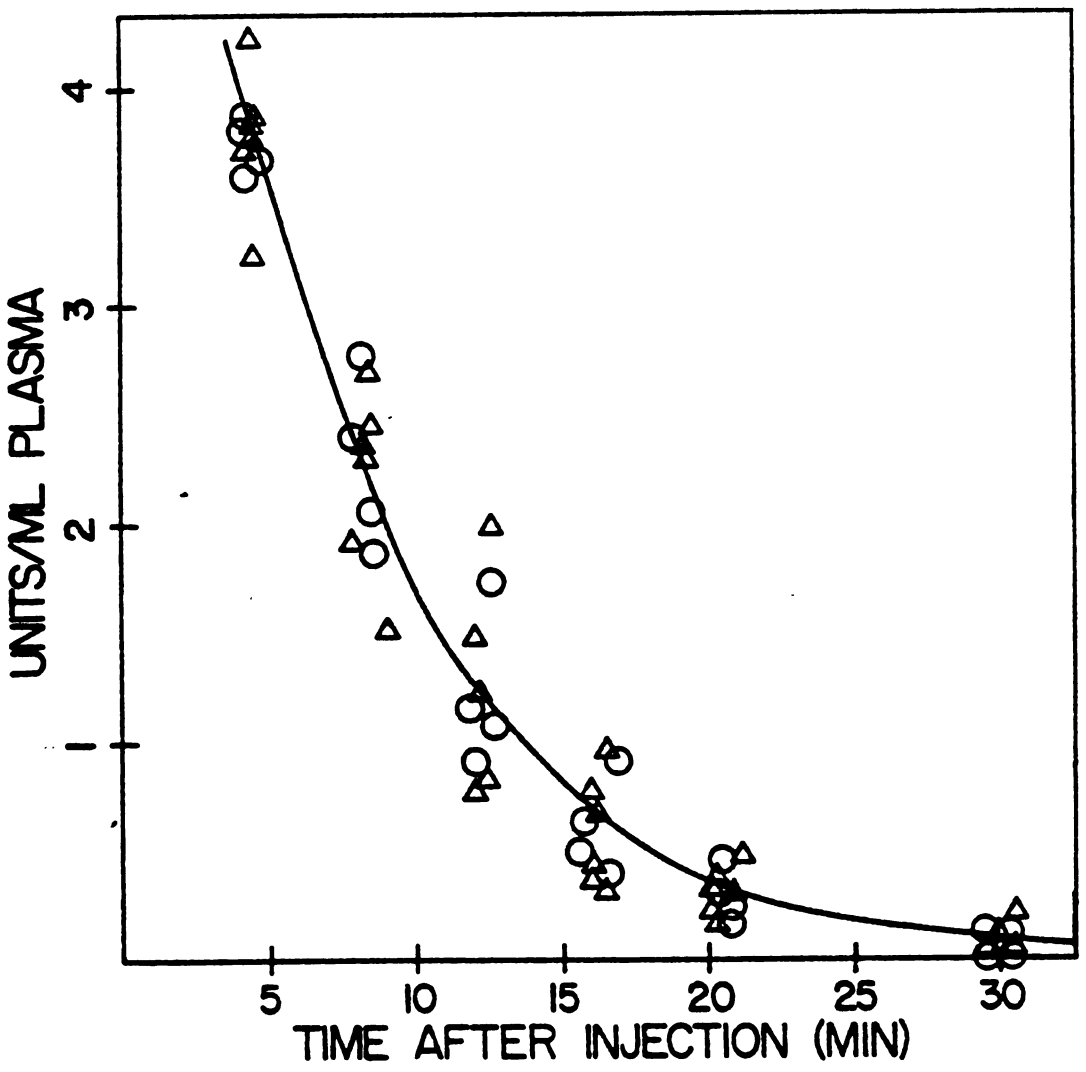

Figure 5.

TABLE 6. Rates of Loss of Adenylate Kinase Activity and ^{125}I-Adenylate Kinase from the Circulation of Normal and Dystrophic Chickens[a]

Enzyme	Chicken Type	k_1 (min^{-1}) [$t_{1/2}$ (min)]	k_2	% Lost In Rapid Phase
Adenylate Kinase	Normal (6)	0.15 ± 0.03 [4.6]	--	100
Adenylate Kinase	Dystrophic (5)	0.13 ± 0.02 [5.3]	--	100
^{125}I-Adenylate Kinase	Normal (4)	0.33 ± 0.17 [2.1]	$t_{1/2}$ > 1 hr	87 ± 1
^{125}I-Adenylate Kinase	Dystrophic (3)	0.24 ± 0.06 [2.9]	$t_{1/2}$ > 1 hr	90 ± 4

[a]Results are expressed as the average ± standard deviation for the number of chickens in parentheses.

Figure 6. The loss of adenylate kinase activity in chicken serum *in vitro* at 41°C. One unit of adenylate kinase was incubated in 200 µl normal chicken serum containing 10 mM HEPES, pH 7.5, and 10 mM dithiothreitol (o); or containing 10 mM HEPES, pH 7.5 (●).

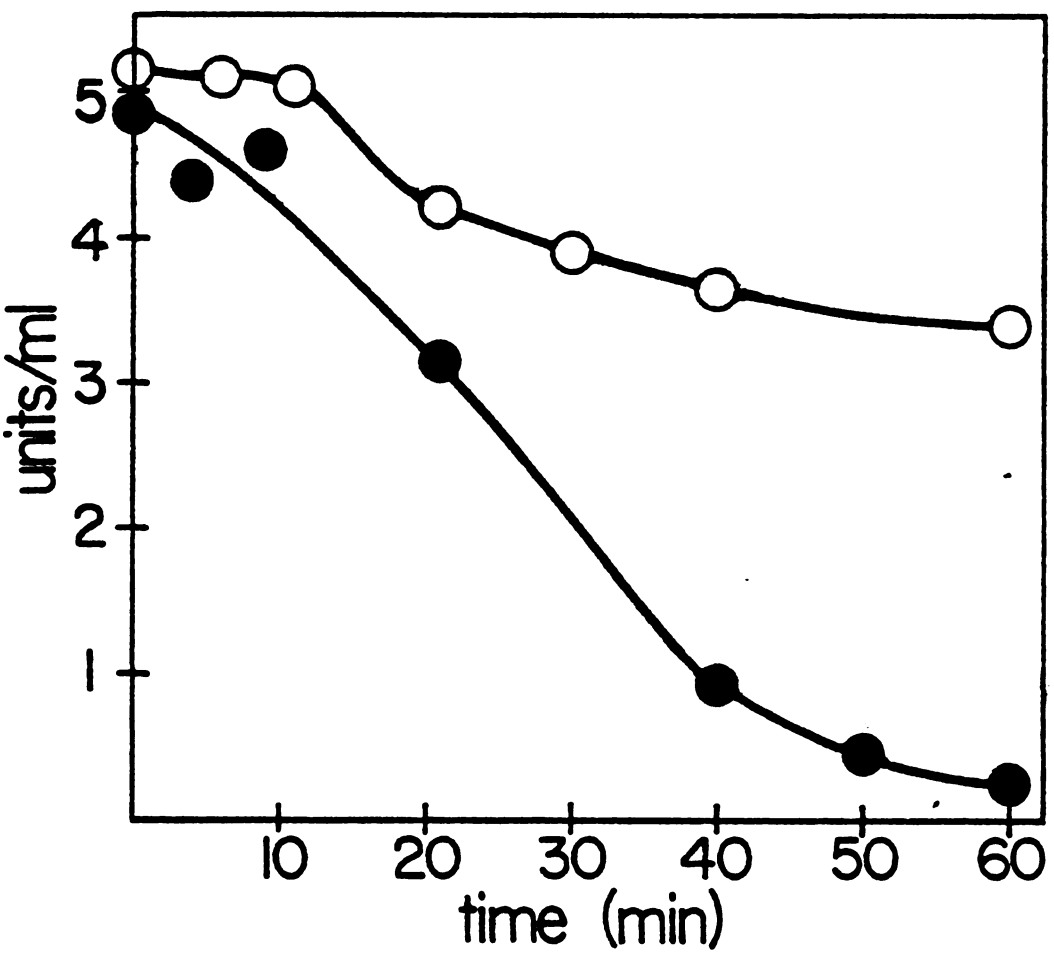

Figure 6.

that adenylate kinase activity is lost rapidly when incubated in serum *in vitro* at 41°C. This rate of loss is decreased significantly by including 10 mM dithiothreitol in the serum (Figure 6, open circles). Thus the loss of adenylate kinase in the circulation after injection is probably due at least in part to the oxidation of sulfhydryl groups essential for catalytic activity, and is consistent with reports that show human (222,223), porcine (220), and rabbit (225) muscle adenylate kinases are inactivated by agents which modify sulfhydryl groups.

The loss of intravenously injected ^{125}I-adenylate kinase is shown in Figure 7. ^{125}I-Adenylate kinase is lost more rapidly than adenylate kinase enzymatic activity (Table 6). The half-lives for the initial loss of ^{125}I-adenylate kinadd are 2.1 and 2.9 min in normal and dystrophic chickens, respectively. About 10% of the ^{125}I is cleared very slowly with a half-life greater than one hour. The tissue distribution of ^{125}I 30 min after injection of ^{125}I-adenylate kinase is shown in Table 7. The largest portion of ^{125}I is recovered in muscle tissue, however, the liver and spleen have the highest concentrations of ^{125}I. Despite the high concentrations of ^{125}I in the spleen and liver, no significant increase in adenylate kinase activity is observed in homogenates of these tissues 40 min after the injection of adenylate kinase (Table 8). These results indicate that adenylate kinase is both rapidly inactivated and cleared from the circulation.

AMP Aminohydrolase Clearance

The loss of AMP aminohydrolase activity after intravenous injection of the purified enzyme is shown in Figure 8. AMP aminohyrolase activity is lost rapidly from the circulation with half-lives of 2.9 and 4.1 min

Figure 7. The loss of radioactivity from the circulation after the intravenous injection of ^{125}I-adenylate kinase in normal and dystrophic chickens. The loss of radioactivity from normal (Δ) and dystrophic (0) chickens was determined as described in the Methods. The curve drawn is calculated from the average of the parameters given in Table 6 using equation [1].

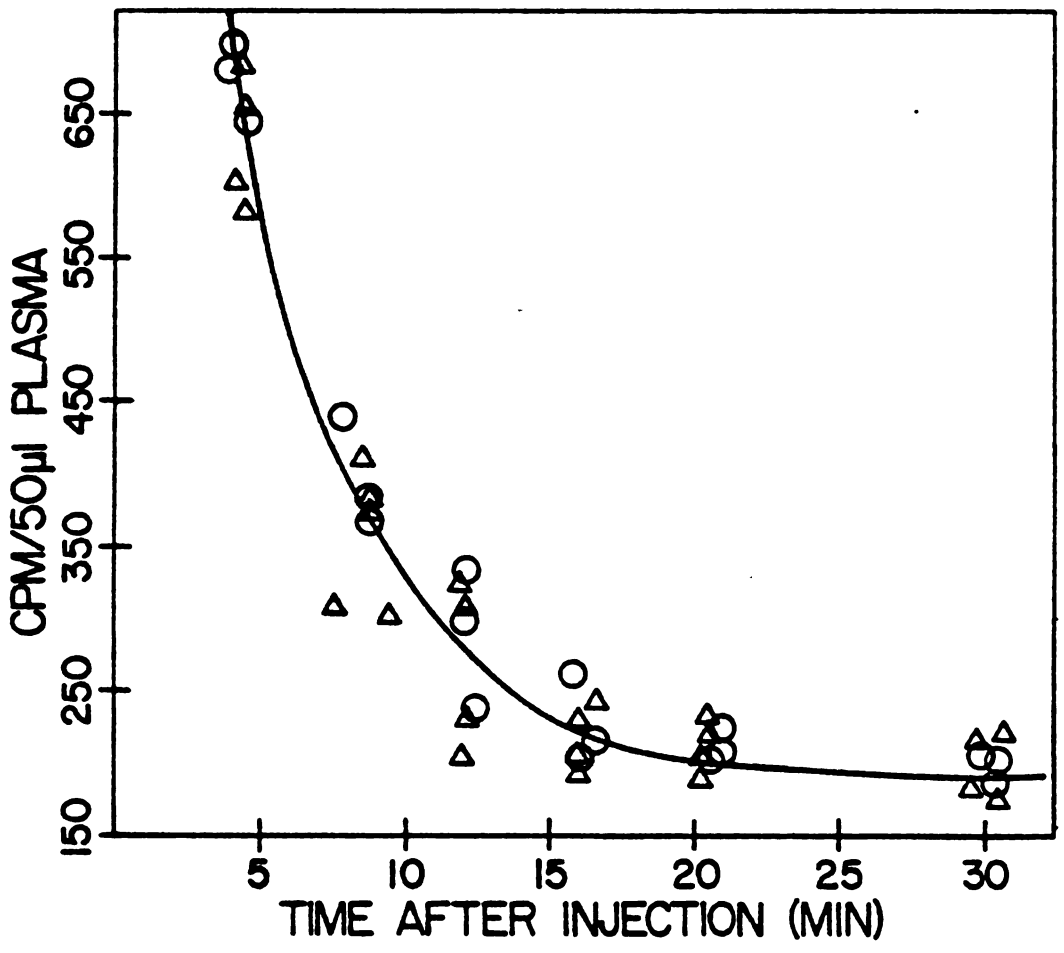
CPM/50µl PLASMA
650
550
450
350
250
150
5
10
15
20
25
30
TIME AFTER INJECTION (MIN)

Figure 7.

TABLE 7. Tissue Distribution of ^{125}I-Adenylate Kinase 30 Minutes After Intravenous Injection

Tissue	Percent of Injected Radioactivity Recovered	Percent of Injected Radioactivity Recovered Per Gram Tissue
Spleen	1.3	3.4
Liver	15.9	2.3
Gizzard	2.2	1.5
Kidneys	2.9	1.4
Lungs	2.3	1.1
Leg, Back and Breast Muscle	28.1	0.6
Leg, Back and Breast Bones	16.6	0.6
Excrement	8.5	--
All Other Tissues	20.5	<0.6[a]
	98.3	

[a]No other single tissue contained more than 0.6% of the injected radioactivity per gram.

TABLE 8. Recovery of Adenylate Kinase (AK) Activity in the Liver and Spleen 40 Minutes After the Intravenous Injection of Adenylate Kinase

Tissue	Total Units AK in Tissue When no AK Injected[a]	Total Units AK in Tissue 30 min After 531 Units AK Injected[a]	Total Units AK in Tissue Expected if Percent Clearance by Tissue as in Table 6
Liver	47	58	131
Spleen	2.2	2.5	9.1

[a]Each determination is the average value for two chickens.

Figure 8. The loss of intravenously injected AMP aminohydrolase activity from the circulation of normal and dystrophic chickens. The loss of activity from normal (Δ) and dystrophic (O) chickens was determined as described in the Methods. The curve was calculated from the average of the parameters for the clearance of AMP aminohydrolase in normal and dystrophic chickens using equation [1].

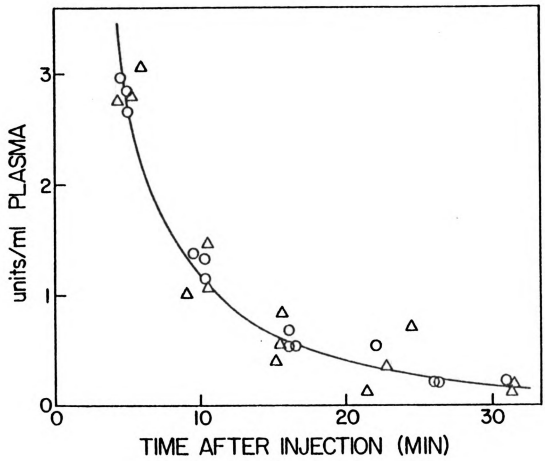

Figure 8.

in normal and dystrophic chickens, respectively (Table 9). A small percentage of the activity is lost in a slower phase with a half-life greater than one hour. The clearance of ^{125}I-AMP aminohydrolase (Figure 9) has a half-life of 3.0 and 3.5 min in normal and dystrophic chickens, respectively (Table 9). Not apparent in Figures 8 and 9 is that about 95% of the injected enzyme activity or radioactivity is lost from the circulation prior to the collection of the first blood sample 4 to 5 min after injection. This is estimated based on the amount of enzyme injected assuming a plasma volume of 6.0% of the body weight (226). Extrapolation of the data shown of Figures 8 and 9 to the time of injection using the rate constants in Table 9 indicates less than 10% of the injected enzyme is lost at the measured rates. Thus, the clearance of AMP aminohydrolase is accomplished by essentially a single pass through the site(s) of clearance. Incubation of AMP aminohydrolase in chicken serum or heparinized whole blood at 41°C at concentrations similar to those expected after intravenous injection results in no measurable loss of enzyme activity for up to 2 hr. The details of the rapid clearance of AMP aminohydrolase are discussed in Chapter III.

Enzyme Activities in Muscle Press Juices

To estimate the extent of association of several muscle enzymes with intracellular structures, the activities of these enzymes were measured in breast muscle press juices and compared to the total enzyme activities in high ionic strength crude breast muscle extracts (Table 10). The centrifugation method for the preparation of muscle press juices (122) results in the disruption of the sarcolemma, and the resulting press juice presumably contains those proteins free in the sarcoplasm. Myofibrillar proteins and those proteins associated with the myofibrils

TABLE 9. Rates of Loss of AMP Aminohydrolase Activity and ^{125}I-AMP Aminohydrolase from the Circulation of Normal and Dystrophic Chickens[a]

Enzyme	Chicken Type	k_1 (min^{-1}) [$t_{1/2}$ (min)]	% Lost In Rapid Phase[b]
AMP Aminohydrolase	Normal (3)	0.24 ± 0.05 [2.9]	96
AMP Aminohydrolase	Dystrophic (3)	0.17 ± 0.01 [4.1]	97
^{125}I-AMP Aminohydrolase	Normal (3)	0.23 ± 0.13 [3.0]	--
^{125}I-AMP Aminohydrolase	Dystrophic (3)	0.20 ± 0.06 [3.5]	--

[a]The results are expressed as the average ± standard deviation for the number of chickens tested in parentheses.

[b]The rate of the slow phase of clearance of AMP aminohydrolase activity has a half-life greater than one hour. The percent of the ^{125}I-AMP aminohydrolase cleared in the rapid phase was not determined because some contaminants in the ^{125}I-AMP aminohydrolase preparation were slowly removed from the circulation and contributed significantly to the observed percent lost in the slow phase. The radioactivity cleared at a slower rate was less than 10% of the injected ^{125}I-AMP aminohydrolase.

Figure 9. The loss of radioactivity from the circulation after the intravenous injection of ^{125}I-AMP aminohydrolase in normal and dystrophic chickens. The loss of radioactivity from normal (Δ) and dystrophic (0) chickens was determined as described in the Methods. The contribution of radioactivity by contaminants of the ^{125}I-AMP aminohydrolase preparation in the circulation have been subtracted to give the values shown.

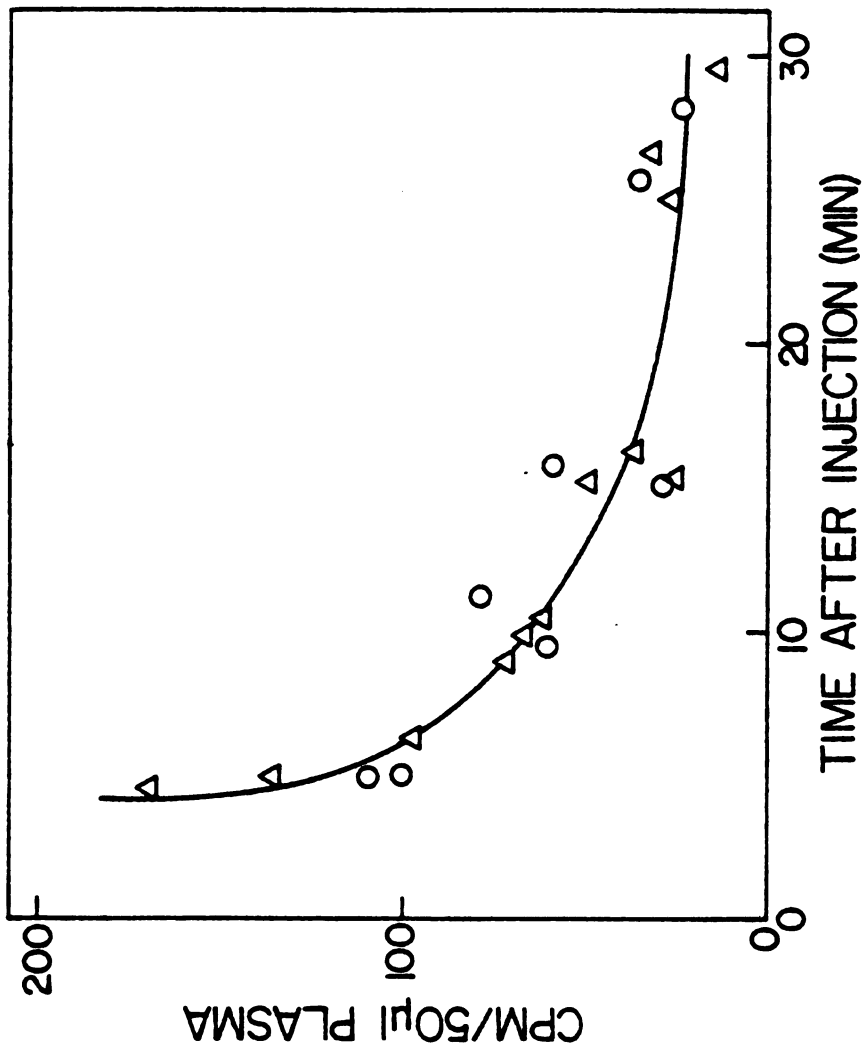

Figure 9.

TABLE 10. Enzyme Activities of Press Juices and Crude Extracts from Normal and Dystrophic Chicken Breast Muscle[a]

Enzyme	Chicken Line[b]	units/ml (press juice)	units/g (muscle)	units/ml (press juice) / units/g (muscle)	units/mg protein (press juice)	units/mg protein (muscle)	units/mg protein (press juice) / units/mg protein (muscle)
Adenylate Kinase	N (5)	154 ± 26	83 ± 24	2.1 ± 0.5	1.50 ± 0.17	1.51 ± 0.53	1.1 ± 0.3
	D (4)	161 ± 41	86 ± 19	1.9 ± 0.4	2.32 ± 0.15	2.04 ± 0.75	1.3 ± 0.6
Creatine Kinase	N (5)	1930 ± 190	1610 ± 240	1.3 ± 0.2	20.4 ± 4.7	27.0 ± 4.2	0.60 ± 0.10
	D (4)	1730 ± 100	1460 ± 430	1.3 ± 0.4	25.8 ± 5.1	32.1 ± 4.8	0.81 ± 0.13
Pyruvate Kinase	N (5)	1420 ± 430	605 ± 177	2.6 ± 0.4	14.1 ± 5.0	10.6 ± 2.3	1.3 ± 0.2
	D (4)	448 ± 128	308 ± 135	1.7 ± 0.9	6.5 ± 1.1	6.6 ± 1.3	1.0 ± 0.2
AMP Amino-hydrolase	N (5)	104 ± 36	203 ± 71	0.56 ± 0.15	1.03 ± 0.41	3.59 ± 1.16	0.29 ± 0.11
	D (4)	24.9 ± 3.4	77 ± 21	0.25 ± 0.12	0.31 ± 0.10	2.12 ± 0.29	0.15 ± 0.07

[a]The results are expressed as the average ± standard deviation for the number of chickens in parentheses. The ratios of enzyme activity in the press juice to that in muscle were calculated separately for each chicken and averaged to give the values shown, which occasionally differ slightly from the ratios obtained if calculated from the average activities in the press juice and muscle given in this table.

[b]N designates normal line 412 chickens, D designates dystrophic line 413 chickens.

are retained in the muscle tissue. On the other hand, muscle crude extracts prepared by the homogenization of tissue in a high ionic strength buffer solubilized myofibrillar and associated proteins. Therefore, we assume that an enzyme with a low ratio of enzyme activity in the press juice to that in the high ionic strength crude extract is associated with intracellular structures to a greater extent than an enzyme with a higher ratio. These ratios are calculated in two ways; the first, as the ratio of the units ml^{-1} in the press juice to the units g^{-1} muscle, and the second as the units $(mg\ protein)^{-1}$ in the press juice to the units $(mg\ protein)^{-1}$ in the crude extract. In both cases the ratio of adenylate kinase activity in the press juice to the activity in the muscle tissue is as high or higher than the ratios for pyruvate kinase and creatine kinase, enzymes which are markedly elevated in the circulation of dystrophic chickens (Table 2). For creatine kinase the ratio of the activity in the press juice to that in the muscle is lower than for adenylate kinase or pyruvate kinase, suggesting that a greater percentage of the creatine kinase is associated with the intracellular structure. Of all the enzymes examined, AMP aminohydrolase is associated with intracellular structures to the greatest extent. The extent of asociation of all enzymes was similar in normal muscle compared to dystrophic muscle, with the exception of AMP aminohydrolase which was associated with intracellular structures to a greater extent in dystrophic muscle.

DISCUSSION

The activities of pyruvate kinase and creatine kinase are markedly elevated in the serum of dystrophic chickens compared to normal chickens, however, the levels of the other abundant muscle enzymes adenylate kinase and AMP aminohydrolase are not increased. This pattern of elevation of enzymes is identical to that observed in human DMD (see literature review in Chapter I) and suggests that the study of the factors which control the circulating levels of muscle enzymes in the dystrophic chicken may be relevant to DMD.

The kinetic analysis of the activation of plasma pyruvate kinase in dystrophic chickens indicates that the enzyme is the muscle isoenzyme, and is consistent with a report by Liu et al. (177) which showed that plasma pyruvate kinase in dystrophic chickens has the same isoelectric point as muscle pyruvate kinase.

Differences in the rates of clearance of different muscle enzymes from the blood plasma of normal and dystrophic chickens offer an explanation for the pattern of elevation of muscle enzymes noted above. Adenylate kinase and AMP aminohydrolase activity are cleared much more rapidly than creatine kinase and pyruvate kinase activity from the circulation of both normal and dystrophic chickens following intravenous injection. These results are consistent with the observation that pyruvate kinase and creatine kinase are elevated in the circulation of dystrophic chickens compared to normal chickens, and that adenylate

kinase and AMP aminohydrolase are not. Pyruvate kinase and creatine kinase are cleared at similar or slightly more rapid, rates in dystrophic chickens compared to normal chickens, indicating that the elevation of these enzymes in the serum of dystrophic chickens is not due to a decreased ability of dystrophic chickens to remove them from the circulation. These results indicate that the relative levels of different muscle enzymes in the circulation of dystrophic chickens may be determined primarily by the rates of clearance of these enzymes from the circulation.

The mechanism by which muscle proteins are released into the circulation of dystrophic animals is not known. If the loss of enzymes into the blood is the result of loss from necrotic muscle fibers, then all soluble muscle constituents would be released into the circulation at similar rates and circulatory clearance and/or inactivation would be of prime importance in determining the relative levels of different muscle enzymes in the circulation of dystrophic animals. On the other hand, the efflux of proteins through abnormally permeable muscle membranes into the circulation could be regulated by the size and charge of the protein as well as by the extent of association of the protein with intracellular components. However, as reviewed by Rowland (38), there is no apparent correlation between molecular size and plasma levels of muscle enzymes in human dystrophies. Our results with normal and dystrophic chickens are consistent with this and offer an explanation for this lack of a correlation. The rapid rate of clearance of both adenylate kinase with a molecular weight of 21,000 (227), and AMP aminohydrolase with a molecular weight of 276,000 (228) would reduce the steady-state levels of both of these enzymes in the plasma of dystrophic chickens to low levels upon

release from dystrophic muscle tissue. Creatine kinase with a molecular weight of 80,000 (229) and pyruvate kinase with a molecular weight of 212,000 (215) are cleared relatively slowly from the circulation, and the activities of these enzymes are elevated markedly in the plasma of dystrophic chickens compared to normal chickens. These observations indicate there is no correlation between molecular size and plasma levels or clearance rates of these enzymes in dystrophic chickens.

That dystrophic muscle has an increased permeability to all soluble muscle enzymes is supported by the data of Dawson (220) which show the same rates of release of several enzymes (including creatine kinase and adenylate kinase) from dissected chicken breast muscle into physiological saline; the rate of efflux of all enzymes from dystrophic muscle was larger than the rate from normal muscle. These results are consistent with the data in Table 10, which show the lack of a significant association of adenylate kinase with intracellular structures compared to creatine kinase or pyruvate kinase. Since the activities of creatine kinase and pyruvate kinase are elevated in dystrophic chicken plasma compared to normal chicken plasma, the low activity of adenylate kinase in the plasma of dystrophic chickens compared to the activities of creatine kinase and pyruvate kinase can not be explained by extensive association of the enzyme with intracellular components. It is possible, however, that metabolic changes in the muscle during the centrifugation necessary for the preparation of the muscle press juices alters the extent of association of these enzymes in the muscle tissue.

The observation that creatine kinase is associated to a greater extent than adenylate kinase or pyruvate kinase (Table 10) is consistent with the association of creatine kinase with myofibrillar proteins

reported by Walliman et al. (115). Pyruvate kinase is also associated with myofibrillar proteins in low ionic strength extracts of muscle tissue (120), though our results suggest the extent of this interaction in vivo is small. On the other hand, the results of the press juice experiment indicate that AMP aminohydrolase is associated with intracellular components to a significant extent in vivo. This is consistent with the binding of AMP aminohydrolase to subfragment-2 of myosin (123), and throughout the A bands in muscle (124). This association, as well as the rapid circulatory clearance rate of AMP aminohydrolase, may contribute to low activities of this enzyme in the plasma of dystrophic chickens.

The rapid clearance of adenylate kinase, the dithiothreitol inhibited inactivation of adenylate kinase in serum in vitro, and the inability to observe increased adenylate kinase enzymatic activity in the liver and spleen shortly after injection, suggest that the enzyme is either inactivated by oxidation of sulfhydryls essential for catalytic activity and is then cleared, or that the enzyme is rapidly inactivated after clearance from the circulation. Because ^{125}I-adenylate kinase is cleared at a more rapid rate than adenylate kinase activity (Table 6), it is possible that the iodinated enzyme is cleared by a process different from that involved in the loss of adenylate kinase activity from the circulation, and that the tissue distribution results (Table 7) are misleading.

Despite the rapid loss of muscle adenylate kinase following intravenous injection, there is a significant level of adenylate kinase activity in the circulation of both normal and dystrophic chickens (Table 2). This activity may be due to adenylate kinase isoenzymes from tissues

other than muscle which are more slowly inactivated and/or cleared. Adenylate kinase isoenzymes from non-muscle tissues in humans are not inactivated by sulfhydryl modifying agents (222,223). Hamada et al. (68) have reported that the increased adenylate kinase activity in the serum of some patients with Duchenne dystrophy is due to an "aberrant" form of the muscle isoenzyme which is not inactivated by sulfhydryl modifying agents but is precipitated by antibodies to muscle adenylate kinase.

The rate of loss of chicken muscle creatine kinase from the circulation in chickens reported here is more rapid than the loss of dog muscle creatine kinase from the circulation after intravenous injection into dogs reported by Kotuku et al. (231). They reported a half-life of 1.5 hours. The loss of creatine kinase activity from the circulation in humans following myocardial infarction has a half-life of about 14 hours (118); however, the primary creatine kinase isozyme in human heart is different from the skeletal muscle isozyme (91) and may be cleared at a different rate. Furthermore, rates of loss following myocardial infarction are difficult to interpret because the precise time at which enzyme ceases to be released from the tissue can not be determined.

The direct measurement of plasma levels and circulatory clearance rates of muscle creatine kinase and pyruvate kinase reported here provide the parameters necessary to estimate the rate of efflux of these enzymes from dystrophic chicken breast muscle. The reasoning and assumptions necessary to calculate the rate of efflux are discussed below.

The biphasic behavior of the loss of creatine kinase and pyruvate kinase from the circulation following intravenous injection can be discussed in terms of the scheme shown in Figure 10 which was previously

Figure 10. A hypothetical model for the distribution of enzymes between body fluids (232).

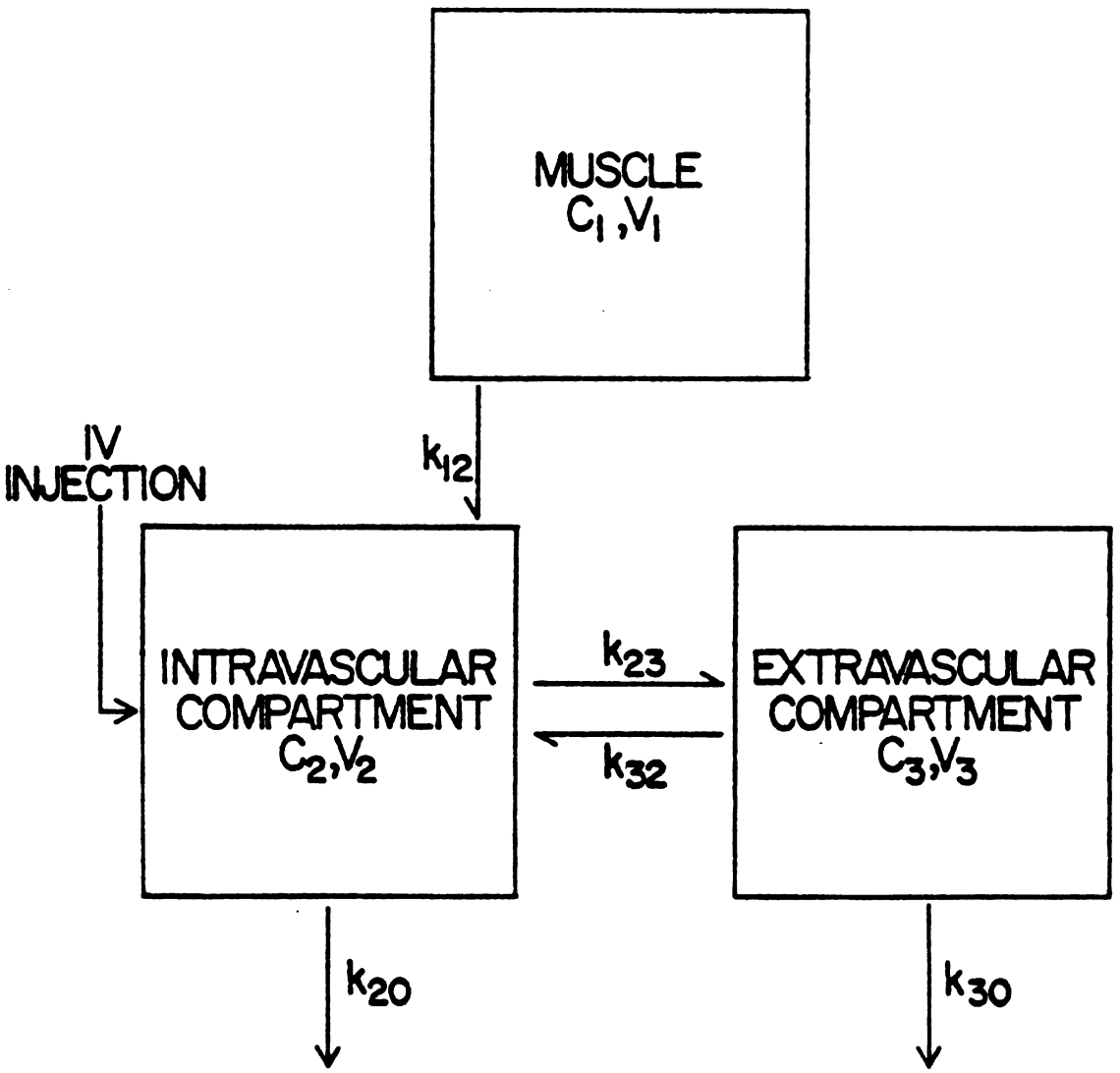
MUSCLE
C_1, V_1
IV
INJECTION
k_{12}
INTRAVASCULAR
COMPARTMENT
C_2, V_2
k_{23}
k_{32}
EXTRAVASCULAR
COMPARTMENT
C_3, V_3
k_{20}
k_{30}

Figure 10.

suggested by Boyd (232). For most enzymes the rate of loss of enzyme activity following intravenous injection follows a biphasic exponential model (233). Presumably, the rapid phase is due to the distribution of the injected enzyme between the intravascular and extravascular spaces (k_{23} and k_{32}). For the purpose of this discussion, intravascular space refers to the blood plasma. It is the loss of enzyme from this space that is measured in the experiments reported here. The extravascular space is the sum of all of the spaces where an enzyme can reversibly equilibrate with the intravascular space, and includes equilibration with the interstitial fluids of all tissues and organs, and the reversible binding to these tissues. The second, slower phase is attributed to the rate of irreversible elimination or inactivation of the enzyme (k_{20} and k_{30}).

The steady-state concentration of an enzyme in the intravascular compartment depends on the difference between the rate of efflux of the enzyme from muscle (k_{12}) and the rate of irreversible loss of enzyme from the intravascular (k_{20}) and extravascular compartments (k_{30}). If the sum of k_{20} and k_{30} is large compared to k_{12}, the steady-state concentration of the enzyme in the intravascular and extravascular compartments will be small or negligible depending on the magnitude of the differences. This may be true in the cases of adenylate kinase and AMP aminohydrolase since these proteins are rapidly cleared and plasma elevations of these enzymes are not observed. The increased steady-state concentrations of creatine kinase and pyruvate kinase in the plasma of dystrophic chickens is consistent with a rate of inactivation or clearance (k_{20} and k_{30}) that is not large compared to the rate of efflux from muscle tissue (k_{12}).

The values of several of the rate constants shown in Figure 10 were calculated as suggested by Boyd (232) from the data for the clearance of intravenously injected creatine kinase and pyruvate kinase (Table 11). For this calculation, it is assumed that the efflux of these enzymes from muscle tissue (k_{12}) is constant and irreversible. Also, since the inactivation of creatine kinase and pyruvate kinase incubated in serum _in vitro_ is slower than the loss of enzymatic activity after intravenous injection, it is assumed that K_{20} is zero.

Using the assumptions made by Boyd (232), the rate of efflux of creatine kinase and pyruvate kinase from dystrophic muscle (k_{12}) is estimated from the relation in equation [3]:

$$k_{12} = \frac{C_2 V_2 k_1 k_2}{k_{32}} \qquad [3]$$

where C_2 is the steady-state concentration of the enzyme in a blood volume V_2, and k_1, k_2 and k_{32} are the rate constants given in Tables 4, 5, and 11. It is assumed that all of the plasma activity of creatine kinase and pyruvate kinase is from breast muscle since primarily white-fiber muscle is affected by muscular dystrophy in the chicken (131) and the breast muscle comprises most of the white-fiber muscle.

Using measured values of 4 week-old line 413 chicken breast muscle weight of 37 g, muscle creatine kinase levels of 1460 units g^{-1} (Table 2), plasma creatine kinase levels of 9.3 units ml^{-1} (Table 2), a plasma volume of 6.0% of the body weight of 250 g (226), and the rate constants for the loss of enzyme activity from the circulation given in

TABLE 11. Rate Constants for the Distribution of Creatine Kinase and Pyruvate Kinase after Intravenous Injection[a]

Enzyme	Chicken Line	k_{30} (hr^{-1})[b]	k_{23} (hr^{-1})[c]	k_{32} (hr^{-1})[d]
Creatine Kinase	Normal (9)	0.11 ± 0.05	0.28 ± 0.12	0.18 ± 0.12
Creatine Kinase	Dystrophic (7)	0.16 ± 0.07	0.52 ± 0.48	0.41 ± 0.40
Pyruvate Kinase	Normal (3)	0.059 ± 0.063	0.20 ± 0.25	0.073 ± 0.083
Pyruvate Kinase	Dystrophic (3)[e]	0.10 ± 0.04	0.35 ± 0.15	0.081 ± 0.044

[a]The results are expressed as the average ± standard deviation for the number of chickens in parentheses.

[b]$k_{30} = k_1k_2/k_{23}$.

[c]$k_{23} = A(k_1-k_2) + k_2$ where A is the fraction of the enzyme lost in the rapid phase.

[d]$k_{32} = k_1+k_2-k_{23}-k_{30}$.

[e]Rate constants for the distribution of pyruvate kinase in dystrophic chickens was determined for the loss of ^{125}I-labeled pyruvate kinase since rate constants could not be determined for the loss of pyruvate kinase activity.

Tables 5 and 11, the rate of efflux of creatine kinase from dystrophic chicken breast muscle is:

$$k_{12} = \frac{(9.3 \text{ units ml}^{-1})(250 \text{ g x } .06 \text{ ml g}^{-1})(1.0 \text{ hr}^{-1})(.07 \text{ hr}^{-1})}{(.41 \text{ hr}^{-1})}$$

$$k_{12} = 23.8 \text{ units hr}^{-1}$$

Therefore, the percent of the total breast muscle creatine kinase released per day is:

$$\frac{(23.8 \text{ units hr}^{-1})(24 \text{ hr day}^{-1})}{(1460 \text{ units g}^{-1})\ (37 \text{ g})} = 1.1\%$$

A similar calculation for pyruvate kinase using pyruvate kinase levels of 308 units g^{-1} (Table 2) and plasma pyruvate kinase levels of 7.2 units ml^{-1} (Table 2), the rate of efflux of pyruvate kinase from dystrophic chicken breast muscle (k_{12}) calculated with equation [3] is 38.7 units hr^{-1}; 8.2% of the total breast muscle pyruvate kinase would need to be released into the circulation daily to maintain the observed circulatory level of pyruvate kinase in dystrophic chickens. This estimate is calculated using the rates of loss of ^{125}I-pyruvate kinase from the circulation of dystrophic chickens because the rate constants for the loss of pyruvate kinase activity in dystrophic chickens could not be calculated because of the fluctuations in the high background activity of pyruvate kinase. However, the rates for the loss of pyruvate kinase activity and ^{125}I-pyruvate kinase from normal chickens are similar suggesting that the rates of loss of ^{125}I-pyruvate kinase from the circulation of dystrophic chickens are similar to the rates of loss of enzyme activity.

The apparent rate of efflux of pyruvate kinase from dystrophic muscle is higher than the rate of efflux of creatine kinase. There are several possible explanations for this observation. The association of creatine kinase with intracellular structures may retard the release of the enzyme from the muscle to the circulation. If the loss of muscle enzymes into the circulation in dystrophic chickens is through an abnormally permeable sarcolemma, these proteins may pass through the sarcolemma at different rates. Also, it is possible that the rates of clearance of ^{125}I-pyruvate kinase are different from the rates of clearance of pyruvate kinase activity, and that the rate of release of pyruvate kinase from dystrophic muscle is an incorrect estimate.

The rate of efflux of creatine kinase is sufficiently small that it seems unlikely that the loss of the enzyme from muscle into the circulation could result in a significant reduction in muscle creatine kinase activity. In fact, creatine kinase activity is not significantly reduced in the muscle of dystrophic chickens at 4 weeks of age (Table 2). Even the estimated loss of up to 8.2% of the muscle pyruvate kinase per day into the circulation may not be sufficient to cause the decreased muscle levels of this enzyme that are observed in dystrophic chickens (Table 9). This loss is relatively small compared to the observation that in normal rabbit muscle 62% of the muscle pyruvate kinase is turned over daily (234).

Since elevated levels of adenylate kinase and AMP aminohydrolase are not observed in dystrophic chickens, there is no direct evidence that these enzymes are released into the circulation from dystrophic chicken breast muscle at rates comparable to creatine kinase or pyruvate kinase. Since we have determined the rates of clearance of AMP aminohydrolase and

adenylate kinase activity from the circulation, using equation [3] it is possible to estimate the steady-state level of the enzyme that would be expected in the circulation if these enzymes were released from dystrophic chicken muscle at rates comparable to pyruvate kinase.

AMP aminohydrolase is cleared even more rapidly than the measured rates since 95% of the injected enzyme is cleared before the collection of the first blood sample 4 to 5 minutes after injection. I will assume that 95% of the enzyme is cleared with a half-life of 1 min since at least 4 half-lives must pass in 4 minutes to clear 95% of the enzyme from the circulation, and that 5% of the enzyme is cleared with a half-life of 4.1 min (Table 9). If 8.2% of the breast muscle AMP aminohydrolase is released into the circulation daily, then: .082 x 77 units g^{-1} x 37 g = 234 units would be released per day; 9.7 units would be released per hour. Rearranging equation [3] to calculate C, only .002 units ml^{-1} of AMP aminohydrolase would be expected in the circulation.

This increase is less than the circulatory levels of AMP aminohydrolase given in Table 2 and could easily go undetected in the serum of dystrophic chickens. In addition, the extensive intracellular association of AMP aminohydrolase with intracellular components in muscle tissue (Table 11) may retard the release from the muscle into the circulation.

A similar calculation with adenylate kinase results in an expected elevation of 0.09 units ml^{-1} in the plasma of dystrophic chickens assuming a rate of efflux comparable to pyruvate kinase. As is apparent from the circulatory levels of adenylate kinase in the plasma of dystrophic chickens (Table 2), this increase may not be detected.

These results indicate that the rapid rates of clearance of AMP aminohydrolase and adenylate kinase may reduce the plasma activities of these enzymes to levels that are sufficiently low that increased levels of these enzymes are not observed in the plasma of dystrophic chickens, even if the rates of release of these enzymes from the dystrophic muscle tissue are comparable to the rates of release of enzymes that are observed to be markedly elevated in the circulation of dystrophic chickens.

CHAPTER III. CIRCULATORY CLEARANCE, UPTAKE, AND DEGRADATION OF MUSCLE AMP AMINOHYDROLASE

INTRODUCTION

In Chapter II it was demonstrated that chicken muscle AMP aminohydrolase (AMPAH) is rapidly cleared from the circulation after intravenous injection of the purified enzyme. This rapid clearance, perhaps in conjunction with the association of the enzyme with intracellular structures in muscle tissue, may be responsible for the observation that the activity of AMPAH is not elevated in the circulation of dystrophic chickens compared to normal chickens as are the levels of several other abundant muscle enzymes.

Because of the extremely rapid clearance of AMPAH from the circulation, it is of interest to characterize this process. The characteristics of this rapid clearance are reported in this chapter. AMPAH is cleared primarily by the spleen and the parenchymal cells of the liver. After clearance, the enzyme is apparently internalized and degraded in lysosomes. The circulatory clearance of AMPAH is inhibited by heparin and other sulfated polysaccharides. Heparin injection after the clearance of AMPAH releases AMPAH into the circulation presumably by releasing AMPAH that is bound to the liver and the spleen and that has not yet been internalized.

MATERIALS AND METHODS

Materials

White Leghorn chickens between 25 and 45 days of age were used in these experiments. [U-^{14}C]Sucrose was obtained from either ICN Pharmaceuticals, Irvine, California (360 mCi mmole^{-1}) or New England Nuclear, Boston, Massachusetts (673 mCi mmole^{-1}). Bio-Gel P-60 was from Bio-Rad Laboratories, Richmond, California. 2,5-Diphenyloxazole and Triton X-100 were from Research Products International, Elk Grove Village, Illinois. Eagle's minimal essential medium, minimal essential medium amino acids, horse serum, chicken serum, and penicillin-streptomycin were from Grand Island Biologicals Company, Grand Island, New York. 4-Methyl-umbelliferyl-N-acetyl-β-D-glucosaminide was from Koch-Light Laboratories, Inc., Colnbrook, Berks, England. Ultra-pure sucrose was from Schwarz-Mann, Orangeburg, New York. Type I collagenase (125 units mg^{-1}) and deoxyribonuclease I (1400 units mg^{-1}) were from Worthington Biochemical Corporation, Freehold, New Jersey. Insulin, AMP fetuin, N-acetylglucosamine, yeast mannan, mannose-6-phosphate, bovine serum albumin, trypan blue, heparin, chondroitin sulfate C, and dextran sulfate (average molecular weight = 8,000) were from Sigma Chemical Company, St. Louis, Missouri. Agalactofetuin was prepared from fetuin as described by Kim et al. (235). All other chemicals were obtained from the sources listed in Chapter II. Scintillation cocktail was 7 g l^{-1} 2,5-diphenyloxazole, 333 ml l^{-1} Triton X-100 in toluene.

Preparation of Radiolabeled Proteins

AMPAH was purified to homogeneity from frozen chicken breast muscle as previously described (212). AMPAH was radioiodinated by the peroxidase method as described by Martin et al. (218). ^{125}I not bound to protein was removed by exhaustive dialysis against PBS. The specific activities of ^{125}I-labeled AMPAH preparations were 4.7-6.5 mCi mmole^{-1} and contained 0.55-0.76 atoms iodine per 287,000 molecular weight tetramer. Approximately 25% of the AMPAH enzymatic activity was lost during the iodination procedure. [^{14}C]Sucrose-labeled AMPAH was prepared by the procedure of Pittman et al. (236). [^{14}C]Sucrose not bound to protein was removed by exhaustive dialysis against PBS. [^{14}C]Sucrose-labeled AMPAH preparations contained 0.35-0.95 molecules sucrose bound per 287,000 molecular weight tetramer and had specific activities of 0.9-1.2 μCi mg^{-1}. Less than 10% of the AMPAH enzymatic activity was lost during the labeling procedure. No detectable small molecular weight radioactive compounds were observed when ^{125}I-labeled AMPAH or [^{14}C]sucrose-labeled AMPAH were chromatographed on a Bio-Gel P-60 column in the presence of 8 M urea.

Clearance and Tissue Distribution of AMPAH

Between 1 and 5 μg AMPAH per g body weight in a volume of 0.2 to 0.75 ml PBS was injected into the brachial vein. A consistent amount was injected in any single experiment. The plasma level of the enzyme expected at t=0 is estimated assuming a plasma volume of 6.0% of the body weight (226).

To determine the rate of clearance of [^{14}C]sucrose-AMPAH, 0.25 ml blood samples were drawn into heparinized glass tubes, and the red blood cells were removed by brief low-speed centrifugation. Aliquots of plasma

(normally 100 µl) were counted in 10 ml scintillation cocktail with a Beckman model LS7000 scintillation counter and corrected for quenching.

The recovery of ^{14}C in tissue samples was determined after preparing 50 to 100 mg tissue aliquots for scintillation counting as described by Baynes and Thorpe (237). Samples were counted in 10 ml scintillation cocktail and corrected for quenching. Recovery of ^{125}I in tissue samples was determined by counting whole tissues in a Beckman Biogamma counter.

Recovery of AMPAH enzymatic activity in tissue samples was determined in the supernatants obtained after tissues were homogenized in five volumes 0.18 M KCl, 0.054 M KH_2PO_4, 0.035 M K_2HPO_4, pH 6.5, and centrifuged at 12,000 x g for 20 min. AMPAH activity was determined as described (212). The average AMPAH activities in the livers and spleens of three uninjected chickens were subtracted to determine the amount of enzyme activity cleared from the circulation by the liver and the spleen. Endogenous AMPAH activities in the liver and spleen were 25% and 7.5%, respectively, of the average activities obtained after injection of enzyme.

Gel-Filtration Chromatography of Tissue Extracts

After injection of radiolabeled enzymes, tissue samples (about 1 g) were homogenized in 3-5 volumes 8 M urea with 20 strokes in a Ten-Broeck homogenizer. The homogenates were clarified by centrifugation at 12,000 x g for 20 min, and 0.5 ml aliquots were applied to a 1.5 x 40 cm Bio-Gel P-60 column equilibrated and eluted with 8 M urea. Two ml fractions were collected and counted in a Beckman Biogamma counter (^{125}I) or added to 10 ml scintillation cocktail and counted in a scintillation counter (^{14}C). Recovery of radioactivity after gel-filtration was between 84

and 106 percent of that applied to the column. The positions of elution of blue dextran and moniodotyrosine were determined by measuring the absorbance at 540 nm and 280 nm, respectively, when samples were chromatographed on the column. The position of elution of [^{14}C]sucrose was determined by measuring ^{14}C in fractions after a small aliquot of [^{14}C]sucrose was chromatographed on the column. Radiolabeled AMPAH eluted with blue dextran in the void volume of the column.

Sucrose Density Gradient Sedimentation

Tissue homogenates were sedimented in sucrose gradients using a modification of the procedure of de Duve et al. (238). Seven hours after the injection of [^{14}C]sucrose-AMPAH tissue samples (about 1 g) were homogenized in four volumes of 0.25 M sucrose, 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM imidazole-propionate pH 7.0, with four strokes of a Teflon-glass homogenizer, rotating at 1,000 rpm. The homogenate was centrifuged at 2,000 x g for 5 min to pellet nuclei, unbroken cells, and cell debris. One ml of the supernatant was layered on top of a 26 ml gradient of 20 to 55% sucrose in 5 mM EDTA, 5 mM imidazole-propionate, pH 7.0, and the gradients were centrifuged in a Beckman SW-25.1 rotor at 25,000 rpm for 4 hr in a Spinco Model L ultracentrifuge. After centrifugation, the gradients were fractionated into twenty 1.5 ml fractions. Ten µl aliquots of each fraction were assayed for N-acetyl-β-D-glucosaminidase activity using the synthetic substrate 4-methyl-umbelliferyl--N-acetyl-β-D-glucosaminide as described by Barrett (239). The remainder of the fraction was added to 10 ml scintillation cocktail and the radioactivity determined. Recovery of radioactivity was between 83 and 87% of that applied to the gradient.

Liver Perfusion and Separation of Parenchymal and Nonparenchymal Cells

Chicken livers were perfused by a modification of the collagenase methods described by Seglen (240) and Obrink et al. (241). Immediately before perfusion, chickens were injected with 1.0 ml of a solution containing 30 mg ml^{-1} heparin in PBS. All solutions used in the perfusion were oxygenated by bubbling 95% O_2/5% CO_2 through the solutions for at least 5 min before use, and were warmed to 41°C by passing the solution through a coil immersed in a 41°C water bath immediately before passage through the cannula. A mesenteric vein was cannulated in situ, and about 200 ml of a solution containing 8.3 g l^{-1} NaCl, 0.5 g l^{-1} KCl, and 2.4 g l^{-1} HEPES adjusted to pH 7.4 with 1 M NaOH, was perfused through the liver at a rate of about 30 ml min^{-1}. This was followed by perfusion with 40 ml of a solution containing 1 mg ml^{-1} collagenase, 10 mg ml^{-1} bovine serum albumin (BSA), 3.9 g l^{-1} NaCl, 0.5 g l^{-1} KCl, 0.7 g l^{-1} $CaCl_2 \cdot 2H_2O$, and 24.0 g l^{-1} HEPES adjusted to pH 7.6 with 1 M NaOH, at a flow rate of about 15 ml min^{-1}. The liver was then removed and placed into 20 ml of the collagenase perfusion solution and shaken for 10 min on a shaker set at about 120 oscillation min^{-1}. The suspension was filtered through a 143 μm nylon mesh to remove clumps of undissociated cells. The filtrate was placed on ice and all subsequent fractionations were at 4°C. The cells were centrifuged for 5 min at 500 x g and washed twice with 15 ml of a medium containing Eagle's minimal essential medium containing twice the normal complement of amino acids, 6% horse serum, 4% chicken serum, 5 μg ml^{-1} insulin, 20 mM glucose, 100 units ml^{-1} penicillin and 100 units ml^{-1} streptomycin.

The cell pellet contained both parenchymal and nonparenchymal cells and the differential centrifugation method of Van Berkel and Van Tol (242) was used to separate parenchymal from nonparenchymal cells. The cell pellet was resuspended in 15 ml medium and centrifuged at 40 x g for 2 min. The supernatant contained primarily nonparenchymal cells and was removed and saved. The pellet contained primarily parenchymal cells and was resuspended in 15 ml medium, the 40 x g centrifugation step was repeated two more times, and the supernatants were saved and combined. The final pellet is the purified parenchymal cell fraction and contained 72% to 94% parenchymal cells as judged by the size of the cells. Parenchymal cells are larger than the nonparenchymal cells and are readily distinguishable by light microscopy (240). Parenchymal cells were always greater than 80% viable as judged by exclusion of trypan blue dye 5 min after an aliquot of the cell suspension was mixed with an equal volume of 0.16% trypan blue in 0.15 M NaCl. Recovery of parenchymal cells was between 43 and 63% of the cells in the initial cell suspension. The combined supernatants from the 40 x g centrifugations contained primarily nonparenchymal cells and were centrifuged at 40 x g for 2 min; the supernatant was removed and centrifuged again at 40 x g for 2 min to remove any residual parenchymal cells. The final supernatant was centrifuged at 500 x g for 5 min and the purified nonparenchymal cell pellet was resuspended in a small volume of medium. Nonparenchymal cell fractions were 92 to 99% nonparenchymal cells as judged by size, and greater than 95% viable as judged by trypan blue exclusion. Recovery of nonparenchymal cells was between 71 and 100% of that in the initial cell suspension.

Effect of Compounds on AMPAH Clearance

Compounds tested to determine their effect on AMPAH clearance were dissolved in PBS at the concentrations given in the results. Solutions (0.5-1.0 ml) were injected into the brachial vein 5 min before the injection of AMPAH, and the loss of AMPAH activity was determined after the intravenous injection of AMPAH as described above.

Release of Cleared AMPAH by Heparin

The release of AMPAH by heparin was determined after the intravenous injection of 0.5 ml per 200 g body weight of a solution containing 60 mg ml^{-1} heparin in PBS. Blood samples were drawn 5 min after the injection of heparin and assayed for AMPAH activity, or the ^{14}C content was determined as described above.

RESULTS

Clearance and Tissue Distribution of AMPAH

The clearance of enzyme activity and radioactivity after intravenous injection of unlabeled or [^{14}C]sucrose-AMPAH is shown in Figure 11. [^{14}C]Sucrose-AMPAH is cleared at about the same rate as the clearance of AMPAH activity. Note also that over 90% of the enzyme is cleared before the first blood sample is taken 5 min after injection of the enzyme. These results are essentially the same as the clearance of ^{125}I-AMPAH and AMPAH activity shown in Figure 8 in Chapter II.

The recovery of AMPAH activity, ^{125}I, and ^{14}C in the spleen and the liver 30 min after injection of labeled or unlabeled AMPAH is shown in Table 12. About 84% of the injected ^{14}C is recovered in the liver and the spleen after the injection of [^{14}C]sucrose-AMPAH. These data are probably the most indicative of the initial tissue distribution of cleared AMPAH because the degradation products of [^{14}C]sucrose-labeled proteins are not permeable to the lysosomal membrane and are retained at the site of degradation (236,243). However, less than 50% of the injected AMPAH enzyme activity or ^{125}I are recovered in the liver and spleen 30 min after the injection of unlabeled or ^{125}I-AMPAH. The lower recovery of AMPAH activity and ^{125}I compared to ^{14}C recovery can be explained by the inactivation and degradation of the enzyme in these tissues, which results in the loss of enzyme activity and efflux of ^{125}I. The recovery of AMPAH enzymatic

Figure 11. The loss of AMPAH activity and ^{14}C from the circulation after intravenous injection of AMPAH or [^{14}C]sucrose-AMPAH. The loss of AMPAH activity (●) and ^{14}C (○) were determined as described in the Methods. Each point is the average of results from four chickens ± standard deviation.

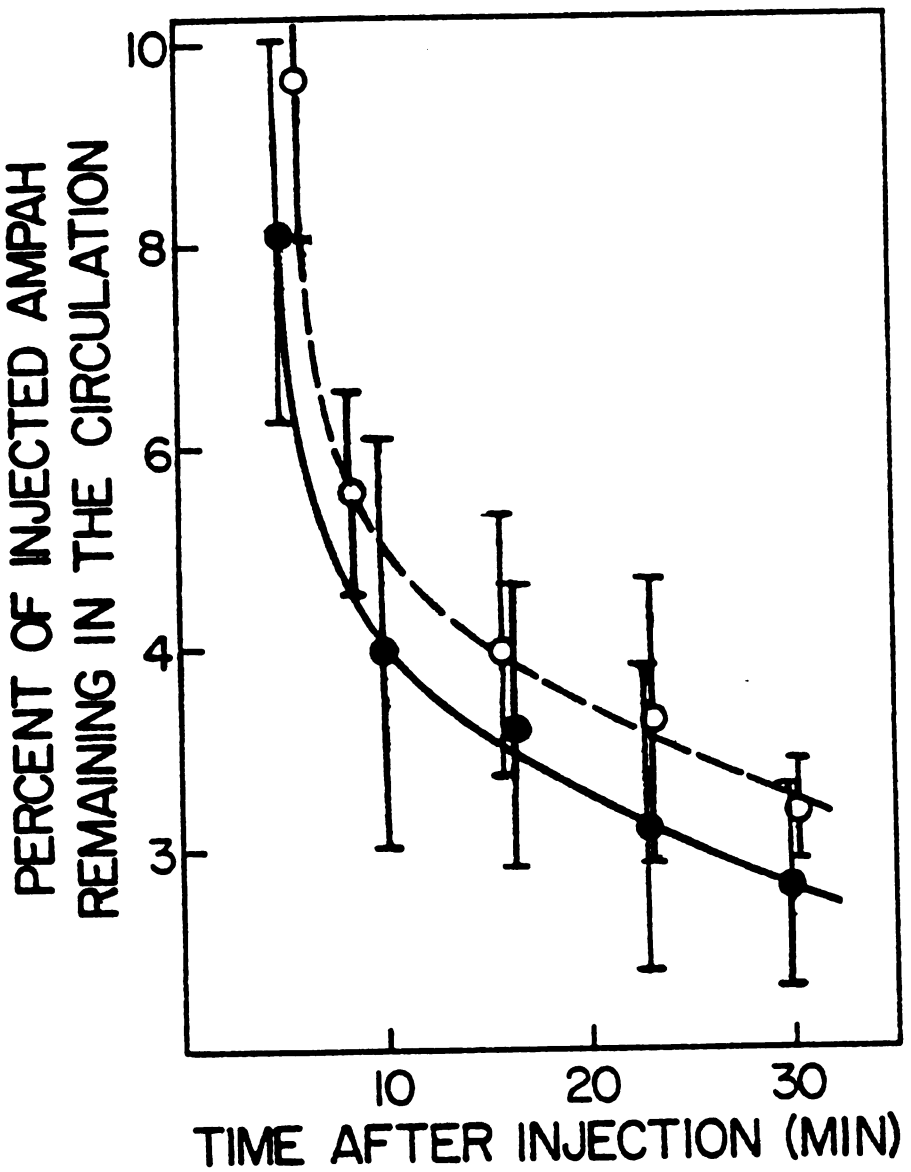
PERCENT OF INJECTED AMPAH
REMAINING IN THE CIRCULATION
10
8
6
4
3
10
20
30
TIME AFTER INJECTION (MIN)

Figure 11.

TABLE 12. Recovery of AMPAH Activity, ^{125}I or ^{14}C in the Spleen and Liver 30 Minutes after the Injection of Unlabeled AMPAH, ^{125}I-AMPAH or [^{14}C]Sucrose-AMPAH[a].

Parameter Measured	Percent of Injected Activity or Radioactivity Recovered in Tissue	
	Liver	Spleen
AMPAH Activity (3)	43 ± 17	4.3 ± 1.4
^{125}I (3)	39 ± 5	8.0 ± 2.0
^{14}C (7)	76 ± 14	8.1 ± 2.6

[a]The results are the average ± standard deviation for the number of experiments in parentheses.

activity in the liver and the spleen indicates that the enzyme is not inactivated before clearance from the circulation, and is consistent with the observation in Chapter II that the incubation of AMPAH in serum at 41°C for 2 hr does not result in any loss of AMPAH activity.

The recovery of ^{14}C and ^{125}I in the liver and the spleen at several times after the injection of [^{14}C]sucrose-AMPAH or ^{125}I-AMPAH is shown in Figure 12. These data verify that these tissues retain ^{14}C after the clearance of [^{14}C]sucrose-AMPAH. The rapid loss of ^{125}I from the liver and the spleen suggests that ^{125}I-AMPAH is degraded in these tissues and that the degradation products of the enzyme are rapidly lost. These data are consistent with the data in Table 12 which show a lower recovery of ^{125}I than ^{14}C in the liver 30 min after the injection of the radiolabeled enzymes.

Degradation of AMPAH in the Liver and the Spleen

To verify that AMPAH is degraded in the liver and spleen, the size distribution of radioactivity in tissue extracts after the injection of radiolabeled AMPAH was determined by gel-filtration chromatography. There are low molecular weight degradation products of ^{125}I-AMPAH in the liver and the spleen as soon as 30 min after the injection of the enzyme (Figure 13). Eventually ^{125}I is excreted as low molecular weight degradation products (Figure 13e). No more than 3.5% of the injected ^{125}I is recovered in the intestines and intestinal contents combined, at any time after the injection of ^{125}I-AMPAH. However, 52% of the injected ^{125}I is recovered in the excrement 7 hours after the injection of ^{125}I-AMPAH indicating that low molecular weight degradation products of ^{125}I-AMPAH are released into the circulation,

Figure 12. Recovery of ^{14}C or ^{125}I in the liver and spleen at various times after the injection of [^{14}C]sucrose-AMPAH or ^{125}I-AMPAH. The recovery of ^{14}C (●) and ^{125}I (■) in the liver (a) and the spleen (b) were determined as described in the Methods. Each point is the average of results from 2 chickens.

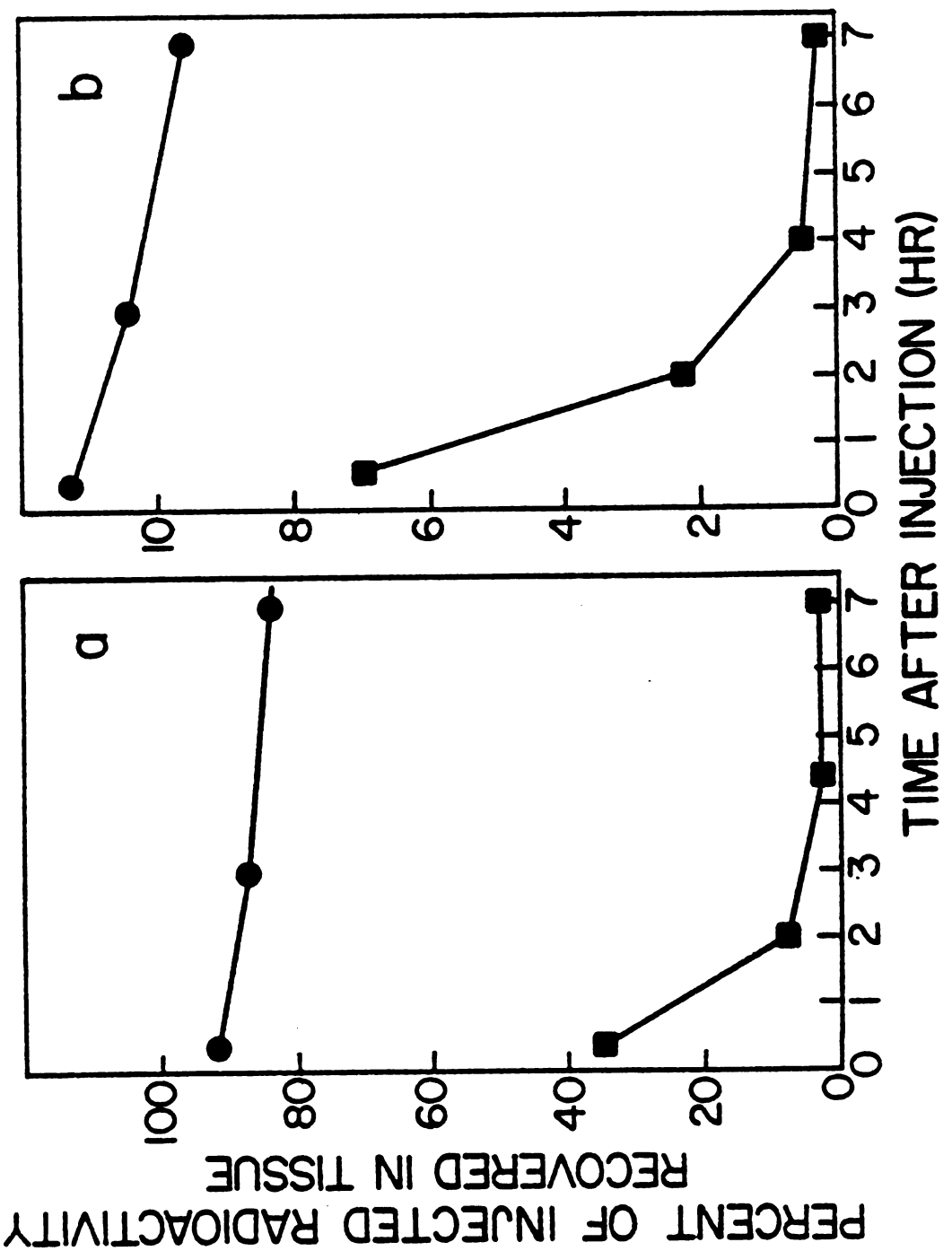
PERCENT OF INJECTED RADIOACTIVITY
RECOVERED IN TISSUE
a
b
TIME AFTER INJECTION (HR)

Figure 12.

Figure 13. Bio-Gel P-60 elution profiles showing the size distribution of ^{125}I in the liver, spleen, and excrement after the injection of ^{125}I-AMPAH. (a) Liver 30 min after injection; (b) liver 2 hr after injection; (c) spleen 30 min after injection; (d) spleen 2 hr after injection; (e) combined excrement collected 7 hrs after injection. MIT = monoiodotyrosine.

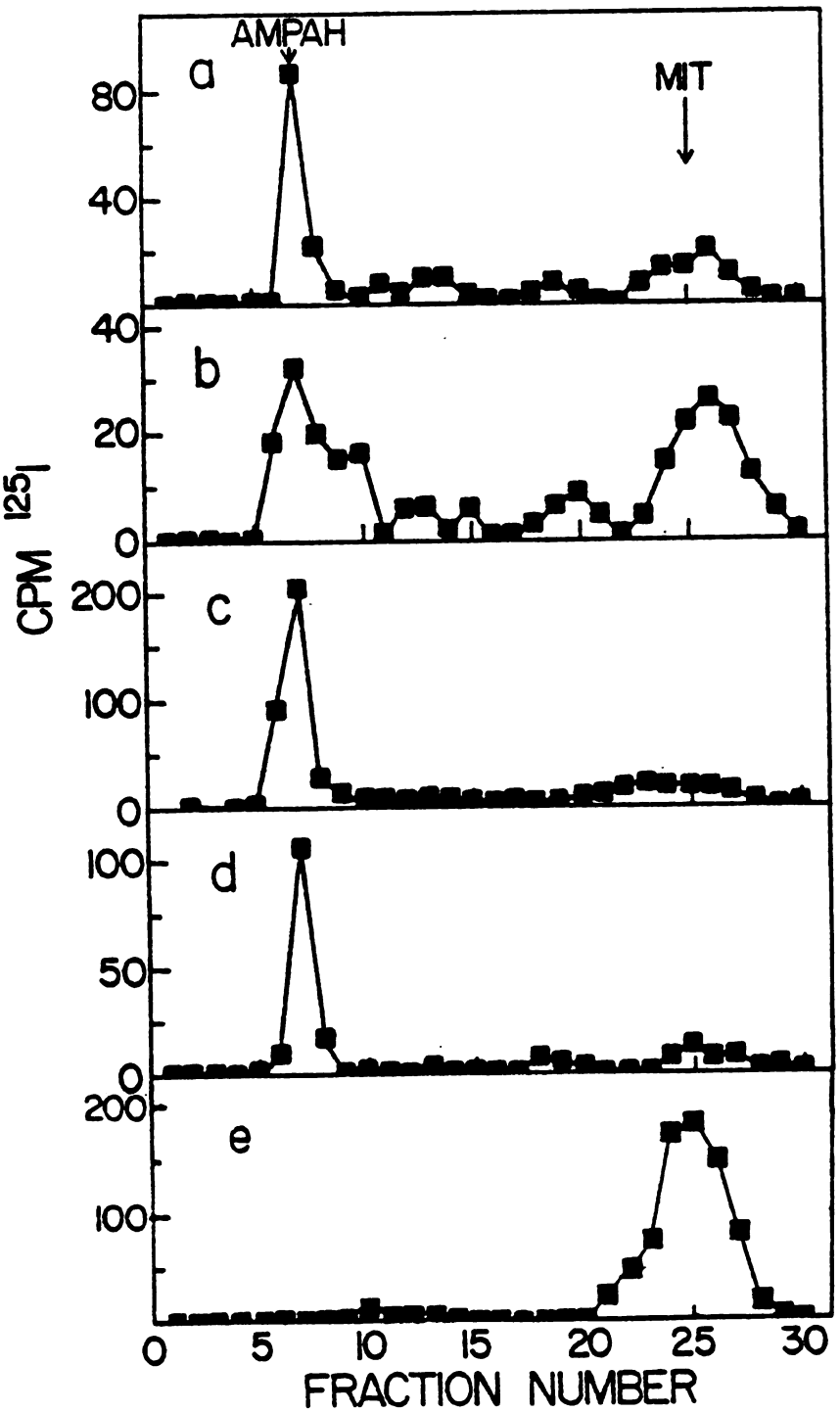
AMPAH
MIT
a
b
c
d
e
80
40
40
20
0
200
100
0
100
50
0
200
100
CPM 125I
0
5
10
15
20
25
30
FRACTION NUMBER

Figure 13.

cleared by the kidneys and excreted in the urine. Seven hours after the injection of [^{14}C]sucrose-AMPAH most of the radioactivity in the liver and the spleen elutes in the low molecular weight region of the gel-filtration profile (Figure 14). The position of elution of ^{14}C on the gel-filtration column indicates the size of this material is larger than [^{14}C]sucrose and is probably [^{14}C]sucrose attached to an amino acid or small peptide. This material would not likely be able to diffuse through the lysosomal membrane. This is consistent with the data in Figure 12 that show ^{14}C is retained in the liver and the spleen after the intravenous injection of [^{14}C]sucrose-AMPAH.

Subcellular Localization of Cleared AMPAH

To determine the subcellular localization of the degradation products of [^{14}C]sucrose-AMPAH, homogenates of liver and spleen were fractionated on sucrose density gradients 7 hr after the injection of the radiolabeled enzyme. The distribution of ^{14}C and the lysosomal marker N-acetyl-β-D-glucosaminidase was determined (Figure 15). The ^{14}C profile coincides with the N-acetyl-β-D-glucosaminidase profile in both the liver and the spleen. The ^{14}C and N-acetyl-β-D-glucosaminidase activity at the top of the gradient is probably from lysosomes broken during the homogenization procedure. A similar experiment with ^{125}I-AMPAH resulted in only a small amount of ^{125}I sedimenting with lysosomes. The remainder of the radioactivity was low molecular weight radioactivity at the top of the gradient and was probably low molecular weight degradation products of the ^{125}I-AMPAH that diffused out of the lysosomes during the preparation procedures.

Figure 14. Bio-Gel P-60 elution profiles showing the size distribution of ^{14}C in the liver and spleen four hours after injection of [^{14}C]sucrose-AMPAH. Tissue extraction and Bio-Gel P-60 chromatography of samples from the spleen (a) and liver (b) were as described in the Methods.

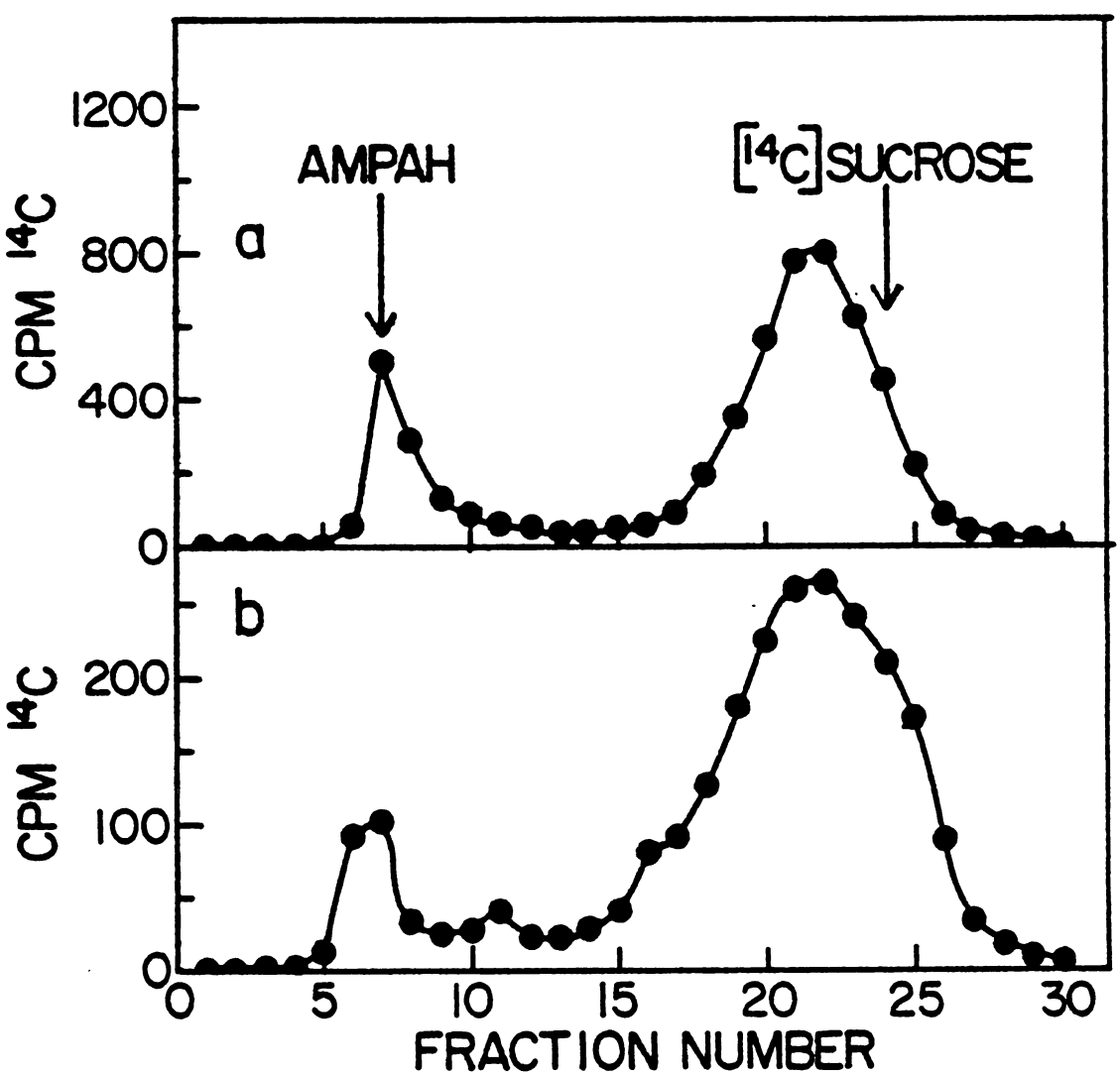
1200
800
400
0
CPM 14C
AMPAH
[14C]SUCROSE
a
b
200
100
0
0
5
10
15
20
25
30
FRACTION NUMBER

Figure 14.

Figure 15. Sucrose density gradient sedimentation profiles of homogenates of the liver and spleen 7 hours after the injection of [^{14}C]sucrose-AMPAH. (●) Relative fluorescence from N-acetyl-β-D-glucosaminidase assay; (X) counts per minute ^{14}C in gradients of homogenates from the liver (a) and the spleen (b). The top of the gradients is to the left.

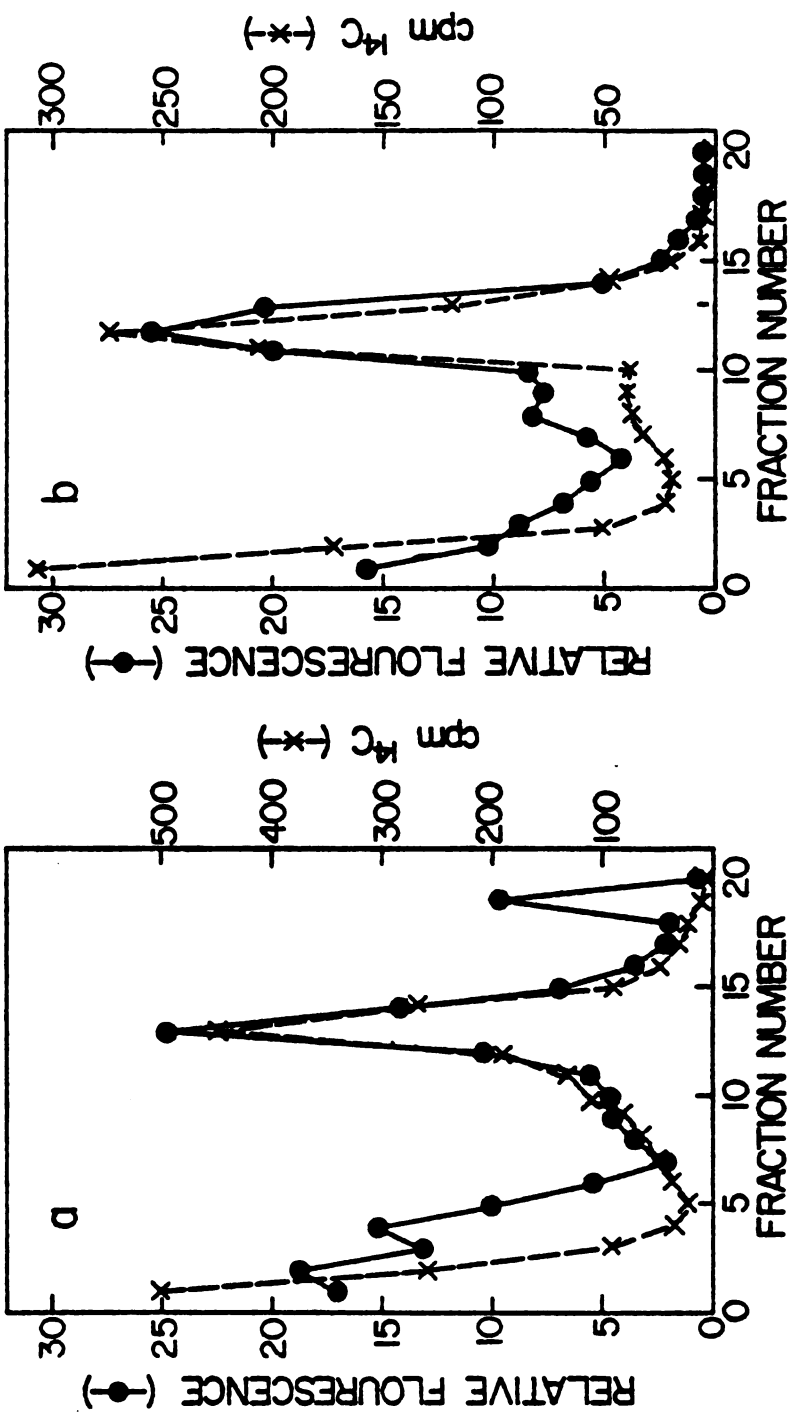
a
b
RELATIVE FLUORESCENCE (—●—)
cpm ^{14}C (—×—)
FRACTION NUMBER
0
5
10
15
20
25
30
100
200
300
400
500
50
150
250

Figure 15.

Parenchymal and Nonparenchymal Cell Distribution of [^{14}C]Sucrose-AMPAH Cleared by the Liver

The results in Table 13 show that most of the ^{14}C is recovered in the parenchymal cell fraction of the liver when liver cells are fractionated after the clearance of [^{14}C]sucrose-AMPAH from the blood. The recovery of ^{14}C per mg cell protein in parenchymal cells is 2.3 times that of nonparenchymal cells. These results demonstrate that the parenchymal cells are primarily responsible for the clearance of AMPAH by the liver.

Inhibition of AMPAH Clearance

To investigate the process involved in the rapid clearance of AMPAH, the effect of various compounds on the rate of clearance was examined. The compounds were injected intravenously 5 min before the injection of AMPAH and the clearance of AMPAH was monitored. Agalactofetuin, N-acetylglucosamine, yeast mannan, mannose-6-phosphate, heparin, chondroitin sulfate, and dextran sulfate were tested for reasons that will be outlined in the Discussion. Of these compounds, only the sulfated polysaccharides heparin, chondroitin sulfate, and dextran sulfate inhibit the clearance of AMPAH activity (Figure 16).

Release of Cleared AMPAH into the Circulation by Heparin

In addition to the inhibition of AMPAH clearance by heparin, injection of heparin releases AMPAH into the circulation after the enzyme is cleared (Figure 17). The activity of AMPAH in the circulation 5 min after heparin injection is higher than that observed only 5 min after AMPAH is injected. Little or no AMPAH activity is released into the circulation by heparin injection into chickens that had not previously received AMPAH injections.

TABLE 13. Distribution of ^{14}C in Parenchymal Cells (PC) and Nonparenchymal Cells (NPC) of the Liver Four Hours After the Injection of [^{14}C]Sucrose-AMPAH. These results are the average ± standard deviation for four determinations.

cpm PC/cpm NPC[a]	$\frac{\text{cpm PC/mg protein PC}}{\text{cpm NPC/mg protein NPC}}$
8.5 ± 2.8	2.3 ± 0.3

[a]The cpm in each cell fraction was corrected for the recovery of cells in the final fraction compared to the initial cell suspension.

Figure 16. The effects of several compounds on the loss of AMPAH activity from the circulation. The compounds were injected 5 minutes before AMPAH injection and the loss of AMPAH activity was monitored. The compounds injected were: (●) none; (○) 20 mg N-acetylglucosamine; (△) 10 mg agalactofetuin; (□) 10 mg mannose-6-phosphate; (*) 15 mg yeast mannan; (■) 30 mg heparin; (X) 30 mg dextran sulfate; (▲) 30 mg chondroitin sulfate C; all in 0.5 to 1.0 ml PBS.

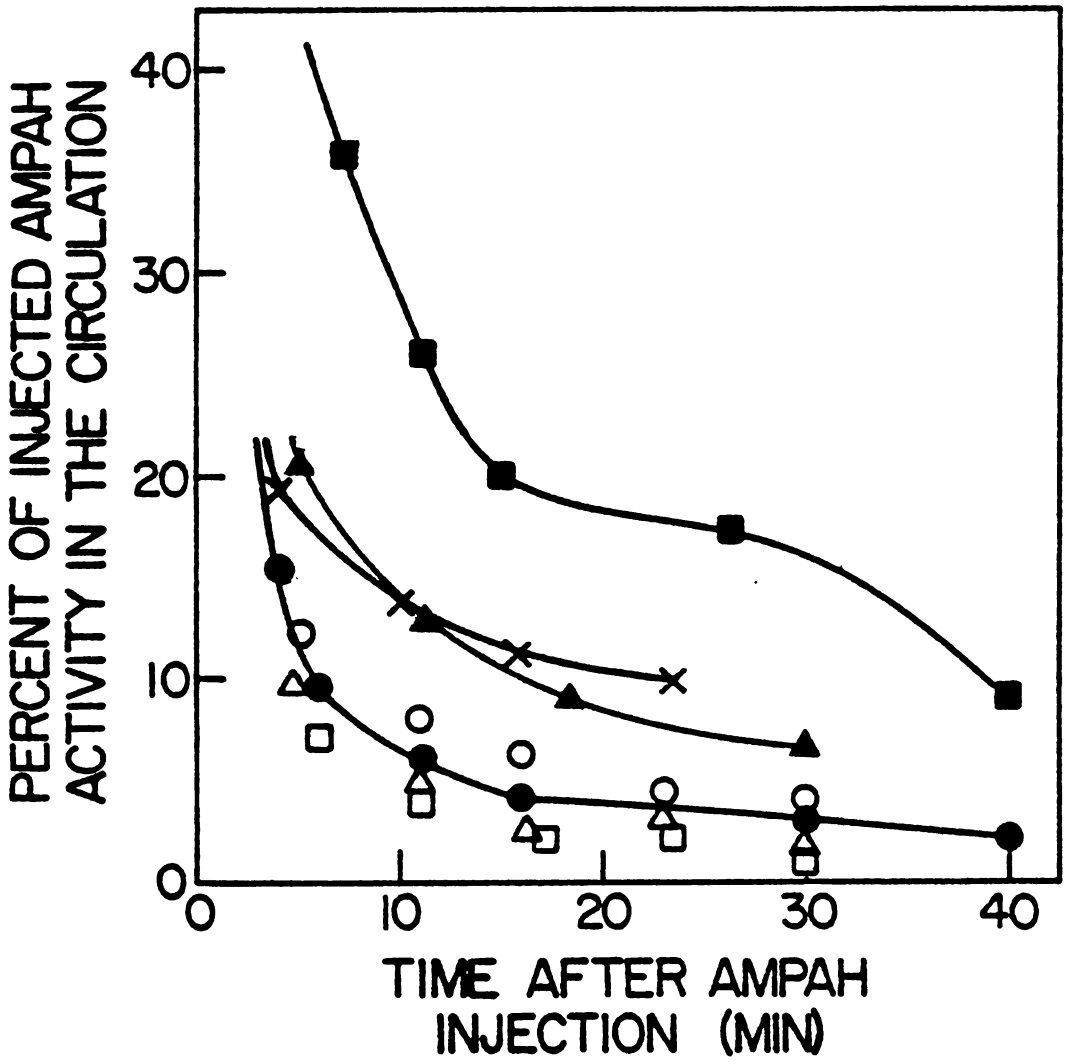
PERCENT OF INJECTED AMPAH
ACTIVITY IN THE CIRCULATION
40
30
20
10
0
0
10
20
30
40
TIME AFTER AMPAH
INJECTION (MIN)

Figure 16.

Figure 17. Release of AMPAH activity into the circulation by heparin injection after the clearance of intravenously injected AMPAH. This experiment is representative of 3 similar experiments.

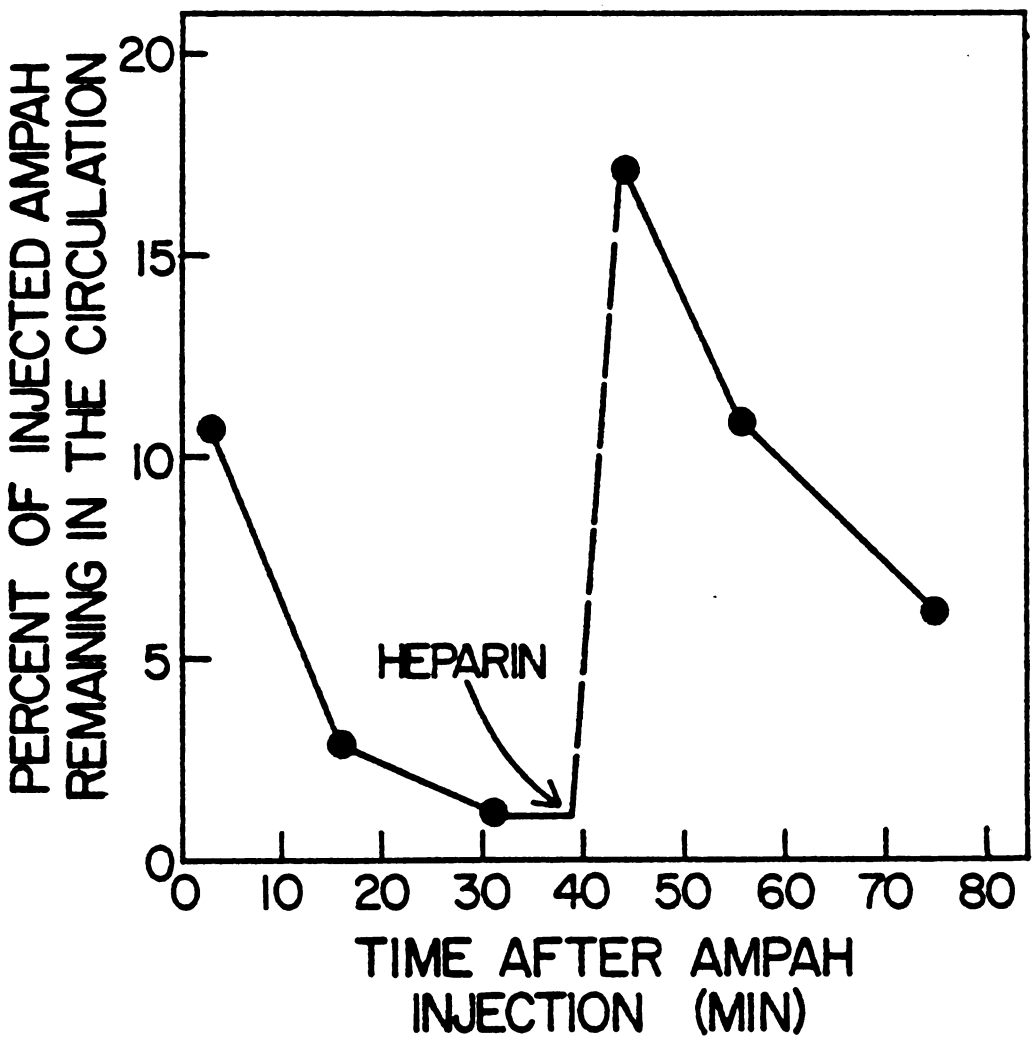
PERCENT OF INJECTED AMPAH REMAINING IN THE CIRCULATION
20
15
10
5
0
HEPARIN
0
10
20
30
40
50
60
70
80
TIME AFTER AMPAH INJECTION (MIN)

Figure 17.

The results in Table 14 show that AMPAH released into the circulation by heparin after clearance of the enzyme is primarily from the spleen and the liver. The increase in circulatory ^{125}I when heparin is injected 30 min after ^{125}I-AMPAH injection is entirely attributable to the loss of ^{125}I from the liver and the spleen. The ^{125}I remaining in these tissues after heparin injection is probably due to internalized ^{125}I-AMPAH and in part due to the ^{125}I present in the blood within these tissues. It is possible that some ^{125}I-AMPAH is bound in a manner that is resistant to release by heparin.

Figure 18 shows that there is AMPAH activity, and radioactivity from [^{14}C]sucrose-AMPAH, released into the circulation by heparin injection for at least 4 hr after injection of the enzyme. The inset in Figure 18 shows that the loss of heparin-releasable AMPAH with time after AMPAH injection is a first-order process with a rate constant of 0.76 hr^{-1} ($t_{1/2}$ = 0.98 hr).

TABLE 14. Release of ^{125}I from the Liver and Spleen into the Circulation by Heparin Injection 30 Minutes After ^{125}I-AMPAH Injection

	Percent of Injected Radioactivity Recovered in Tissue		
	Blood	Liver	Spleen
Chickens Not Injected with Heparin	3.2	45.3	10.1
Chickens Injected With Heparin	28.2	23.9	1.8
Difference (% of Injected Released by Heparin)	25.0	21.4	8.3

Figure 18. Release of AMPAH activity or ^{14}C into the circulation by heparin injection at several times after the injection of unlabeled AMPAH or [^{14}C]sucrose-AMPAH. AMPAH activity (●) and ^{14}C (○) were determined as described in the Methods. Each point is the average from 2 chickens.

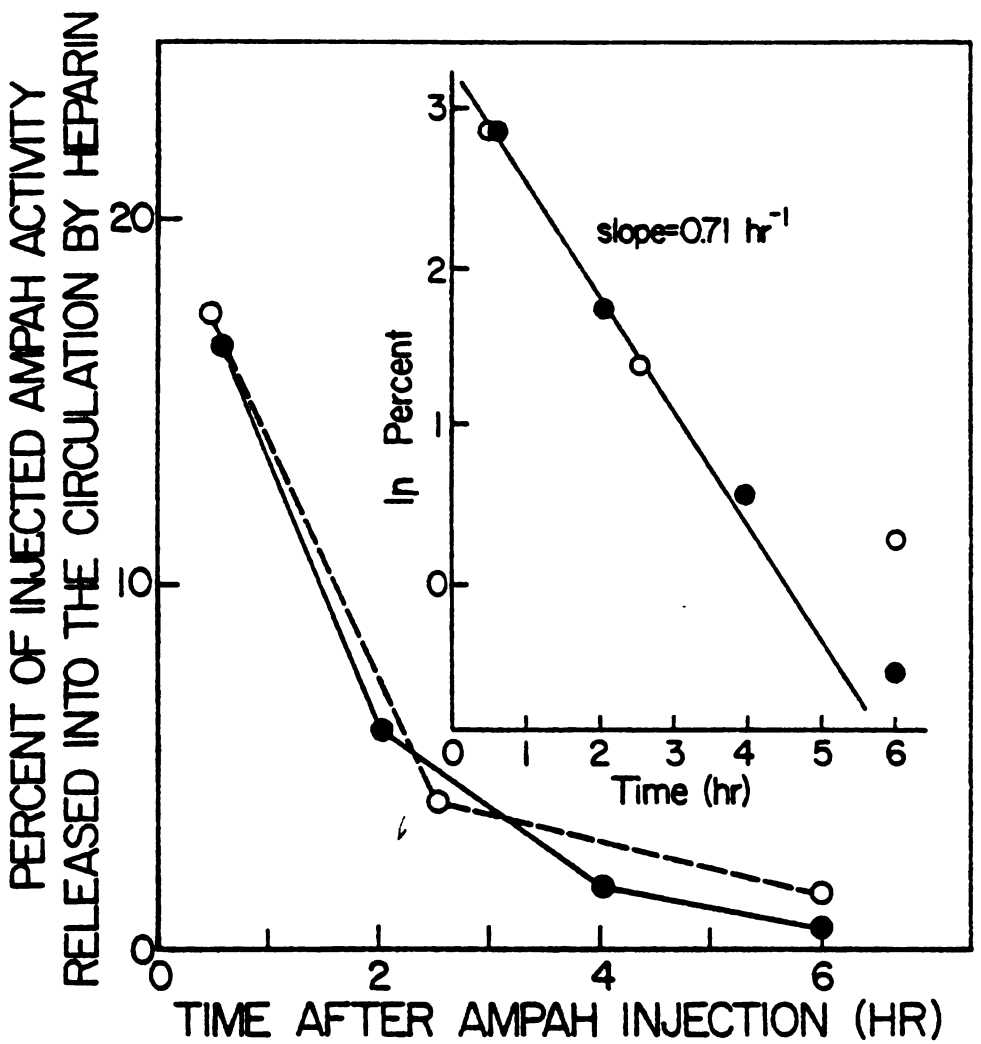
PERCENT OF INJECTED AMPAH ACTIVITY
RELEASED INTO THE CIRCULATION BY HEPARIN
TIME AFTER AMPAH INJECTION (HR)
0
10
20
0
2
4
6
ln Percent
slope=0.71 hr⁻¹
Time (hr)
0
1
2
3
4
5
6

Figure 18.

DISCUSSION

Aspects of the rapid circulatory clearance of AMPAH from the circulation after the injection of ^{125}I-AMPAH or [^{14}C]sucrose-AMPAH were examined to characterize the process of uptake and degradation of AMPAH. Labeling of AMPAH with [^{14}C]sucrose does not alter the rate of clearance (Figure 11) or the primary tissue sites of clearance (Table 12) when compared to the clearance of AMPAH activity or ^{125}I-AMPAH, indicating that the introduction of the [^{14}C]sucrose label does not change the mode of clearance of the enzyme.

Most of the [^{14}C]sucrose-AMPAH is cleared by the liver and the spleen (Table 12) where the enzyme is apparently internalized and degraded in lysosomes (Figures 13 and 15). ^{14}C is retained in the liver and the spleen presumably because the lysosomal membrane is not permeable to the degradation products of [^{14}C]sucrose-labeled proteins (236,243). It is therefore advantageous to use [^{14}C]sucrose-AMPAH to determine the sites of binding, uptake, and degradation of AMPAH rather than ^{125}I-AMPAH because the degradation products of the ^{125}I-labeled enzyme can rapidly diffuse from the site of degradation.

The loss of ^{14}C from the liver and the spleen based on the loss measured over a 7 hr period (Figure 12) has half-lives of 56 hr and 30 hr in the liver and spleen, respectively. Based on the results of Pittman *et al.* (243), the loss of ^{14}C from rat liver after the clearance of

[^{14}C]sucrose-labeled low density lipoprotein had a half-life of about 5 days.

The results in Table 14 verify that heparin releases cleared AMPAH from the liver and spleen into the circulation. Decreasing amounts of AMPAH are released into the circulation with time after the injection of unlabeled or [^{14}C]sucrose-AMPAH (Figure 18), however, the total ^{14}C content in the liver and the spleen decreases only slightly during this 7 hr period and ^{14}C accumulates in a fraction that cosediments with lysosomes in a sucrose density gradient. These results indicate that heparin releases cell surface bound AMPAH and that the ^{14}C not released by heparin is internalized and accumulates in lysosomes. Based on this assumption, the 0.98 hr half-life for the loss of heparin-releasable AMPAH calculated from the results in Figure 18 is the rate of internalization of AMPAH bound to the cell surface.

Clearance of [^{14}C]sucrose-AMPAH in the liver is primarily by the parenchymal liver cells (Table 13). The amount of radiolabel recovered per mg cell protein in the parenchymal cell fraction is 2.3 times that in the nonparenchymal cell fraction. Similarly, Carew et al. (244) have found that [^{14}C]sucrose-labeled low density lipoprotein is cleared most specifically by the spleen and the liver, and that the parenchymal cells of the liver are primarily responsible for the hepatic clearance.

Since AMPAH preparations contain some covalently bound carbohydrate (245), the potential role of several documented carbohydrate mediated uptake mechanisms was determined by examining the effect of inhibitors of these systems on the clearance of AMPAH. Avian liver contains receptors for glycoproteins with terminal N-acetylglucosamine residues (246), but lacks the galactose specific receptor found in mammalian liver (247).

Neither N-acetylglucosamine or agalactofetuin inhibit the clearance of AMPAH (Figure 16) indicating that the N-acetylglucosamine specific recognition system is not responsible for the clearance of AMPAH. The lack of inhibition of clearance by mannose-6-phosphate or yeast mannan (Figure 16) and the predominantly parenchymal cell clearance of AMPAH (Table 13) indicates that neither the non-parenchymal cell receptors that have been identified in mammalian liver for mannose-6-phosphate (248) nor mannose (249) are responsible for the clearance of AMPAH.

The mechanism for the rapid clearance of AMPAH and the basis for the inhibition of clearance by heparin is not known. Fishbein et al. (250) have shown evidence for a factor in human serum which inactivates AMPAH, and that the inactivation is reversed by heparin. Whether this factor is relevant to the heparin-inhibited clearance of AMPAH is not known.

Several aspects of the clearance of AMPAH are similar to the clearance of lipoprotein lipase. The clearance of intravenously injected ^{125}I-labeled lipoprotein lipase has a half-life of about 1 min, clearance is inhibited by heparin, and 70% of the injected enzyme is recovered in the liver 10 min after injection where the enzyme is apparently degraded (251). The release of lipoprotein lipase into the circulation after heparin administration is well documented (252,253). It has been suggested that this occurs because heparin competes with a cell-surface heparin-like molecule for the binding of lipoprotein lipase (252,254). The release of lecithinase (255), diamine oxidase (256), acid ribonuclease (257), and β-glycerophosphatase (257) into the circulation after heparin administration have also been reported.

These studies demonstrate the clearance of AMPAH by the spleen and the parenchymal cells of the liver, and the uptake and degradation of AMPAH by lysosomes. The rapid clearance of the enzyme does not appear to be by a carbohydrate mediated uptake mechanism, but may be similar to other heparin-inhibitable mechanisms for the circulatory clearance of proteins. A method for the study of the interaction of AMPAH with cultured cells would be advantageous for the detailed study of the nature of the interaction of AMPAH with the cells responsible for the rapid circulatory clearance of the enzyme.

CHAPTER IV. INTERNALIZATION AND DEGRADATION OF AMP AMINOHYDROLASE BOUND TO HEPATOCYTE MONOLAYERS

INTRODUCTION

In Chapters II and III it was shown that chicken muscle AMPAH is rapidly cleared from the circulation of chickens after intravenous injection of the purified enzyme. The enzyme is cleared primarily by the spleen and the parenchymal cells of the liver, and is internalized and degraded in lysosomes. Heparin and other sulfated polysaccharides inhibit the rapid clearance of the enzyme.

To study the mechanism for the rapid clearance of AMPAH, an *in vitro* system for the detailed study of the binding of AMPAH to hepatocytes was developed. The results presented in this chapter demonstrate the interaction of AMPAH with monolayer cultures of chicken hepatocytes. The binding of AMPAH to hepatocytes is saturable, has a high affinity, and results in the internalization and degradation of the bound enzyme. Enzyme bound under conditions where internalization is minimal (4°C) is released by heparin.

MATERIALS AND METHODS

Materials

Tissue culture dishes were either 35 mm Corning tissue culture dishes from Corning Glass Works, Corning, New York, or 35 mm 6-well Costar dishes obtained from Rochester Scientific, Rochester, New York. All other materials were from the sources listed in Chapters II and III.

Preparation of Radiolabeled Proteins

^{125}I-AMPAH and [^{14}C]sucrose-AMPAH were prepared as described in the Methods section in Chapter III. [^{14}C]Sucrose-labeled BSA was prepared by the same procedure as was [^{14}C]sucrose-labeled AMPAH and contained 0.3 molecules of sucrose per 68,000 molecular weight monomer and had a specific activity of 1.5 μCi mg^{-1}.

Preparation of Monolayer Cultures of Chicken Parenchymal Liver Cells

The procedure for the preparation of chicken hepatocyte monolayer cultures from minced chicken liver is a modification of the procedure described by Tarlow et al. (258). All procedures were completed under sterile conditions in a laminar flow hood at room temperature unless otherwise noted. All solutions were autoclaved or filter sterilized before use. Two or three 2-4 week old white Leghorn chickens were completed decapitated and the livers immediately removed and rinsed in PBS. The livers were minced with mincing scissors and placed in a 50 ml polypropylene culture tube. To the mince was added 25 ml of a solution containing 150 units ml^{-1} collagenase, 0.38 mg ml^{-1}

deoxyribonuclease I, and 40 mM glucose in PBS, that was previously warmed to 37°C. The mince was shaken for 20 min at 37°C on a shaker set at about 120 oscillations min^{-1}. The undissociated tissue was allowed to settle for about 30 seconds, and the supernatant was removed with a pipet, placed in a polypropylene centrifuge tube and centrifuged at 400 x g for 5 minutes. The supernatant was discarded and the pellet resuspended in 10 ml of an erythrocyte lysis solution containing 0.14 M NH_4Cl, 17 mM Tris(Cl), pH 7.2. After 10 minutes, 10 ml cell culture medium was added. The cell culture medium was Eagle's minimal essential medium supplemented with amino acids to give twice the normal amino acid concentrations, 20 mM glucose, 100 units ml^{-1} penicillin-streptomycin, 5 μg ml^{-1} insulin and 5% chicken serum. The suspension was centrifuged at 400 x g for 5 min and the supernatant discarded. The cell pellet contained both parenchymal and nonparenchymal cells and the differential centrifugation method of Van Berkel and Van Tol (242) was used to purify parenchymal cells as described in Chapter III. Cell suspensions usually contained 1-3 x 10^8 parenchymal cells and were 80-90% viable as judged by exclusion of trypan blue dye. Contamination by nonparenchymal cells was less than 10%. Cells were plated at a density of 5 x 10^6 cells per 35 mm dish in 2 ml medium and were maintained at 37°C in a humidified incubator under 5% CO_2-95% air. The cells attached to the culture dishes within 2 hr after plating and were confluent monolayers by 24-48 hr after plating. The medium was replaced with 2 ml fresh medium daily.

Measurement of the Binding of AMPAH to Hepatocyte Monolayers

Hepatocyte monolayers were used for all experiments 2-4 days after the cells were plated. AMPAH does not bind to freshly prepared

hepatocytes presumably due to the destruction of cell surface binding sites by the enzymatic treatment necessary to dissociate liver cells.

In the standard assay for the binding of AMPAH to hepatocyte monolayers, culture dishes were placed on ice, the medium was removed, and the following components were added: 1) 0.50 ml culture medium; 2) 50 µl 100 mg ml^{-1} BSA in PBS: 3) 25 µl 1 M HEPES pH 7.4; 4) and a total of 100 µl PBS and unlabeled AMPAH, ^{125}I-AMPAH, or [^{14}C]sucrose-AMPAH in PBS. The plates were gently shaken either in a 4°C room or a 37°C room on a shaker set at about 30 oscillations min^{-1}. Except for where indicated otherwise in the Results, the AMPAH was incubated with hepatocytes for 1.5 hr.

After the binding of AMPAH, the cells were washed extensively at 4°C by the following procedure. The medium was removed from the dishes with a pasteur pipet, and the dishes were rapidly rinsed 3 times with 2 ml wash buffer containing 1 mg ml^{-1} BSA in 0.14 M NaCl, 10 mM HEPES, pH 7.4. The dishes were washed 3 more times with 1.5 ml wash buffer with each wash shaken at about 30 oscillations min^{-1} for 10 min. To determine the release of bound AMPAH by heparin, 1.0 ml of 1.0 mg ml^{-1} heparin in wash buffer was added to the dish after the six washes described above were completed. The dishes were shaken at 30 oscillations min^{-1} for 5 min. The wash was removed and the dish was rinsed rapidly with 0.5 ml of the heparin wash solution and the two heparin washes were combined. For the determination of radioactive AMPAH not released by the wash, dishes were rapidly rinsed with 1.5 ml wash buffer without BSA (no significant AMPAH was released by this wash), and the cells were dissolved in 1.0 ml 0.1 N NaOH and removed from the culture dish. The dish was rinsed with 0.5 ml 0.1 N NaOH and the NaOH

fractions were combined. The total amount of radiolabeled AMPAH bound was determined as the sum of that released by heparin and that in the NaOH solubilized cell fraction. In some experiments the release by heparin was not measured, and the total cell associated radioactivity was determined by solubilizing the cell fraction as described above immediately after the 6 initial washes with the wash buffer and one rapid wash with wash buffer without BSA.

AMPAH enzymatic activity released by heparin was assayed as previously described (212). The increase in absorbance at 290 nm was monitored when 50 µl of the heparin wash was added to 0.95 ml of an assay mixture containing 5 mM AMP, 0.5 M KCl, and 50 mM MES-TRIS, pH 6.5. ^{125}I in wash fractions and NaOH solubilized cell fractions was determined by counting the entire fraction in a Beckman Biogamma Counter. ^{14}C in wash fractions, and NaOH solubilized cell fractions neutralized with 1.0 N HCl, were determined by counting aliquots in 10 ml scintillation cocktail. Protein content in the NaOH solubilized cell fractions was determined by the procedure of Lowry et al. (220). All results are the average of duplicate determinations.

Bio-Gel P-60 Chromatography in 8 M Urea

Solid urea was added to wash or media samples to a concentraton of about 8 M before chromatography. Cells were dissolved in 1 ml 8 M urea, homogenized with 20 strokes in a Ten-Broeck homogenizer and clarified by centrifugation at 12,000 x g for 20 min. One ml aliquots of the supernatant were fractionated on a Bio-Gel P-60 column and the radioactivity in each fraction determined as described in Chapter III.

RESULTS

Binding of AMPAH to Hepatocyte Monolayers

The data in Figure 19 show the concentration dependent binding of unlabeled AMPAH to chicken hepatocyte monolayers at 4°C. Table 15 is a summary of the values obtained for the dissociation constants, and maximum amount of AMPAH bound for the binding of unlabeled AMPAH, ^{125}I-AMPAH and [^{14}C]sucrose-AMPAH to hepatocyte monolayers. The binding of unlabeled AMPAH is saturable and has a dissociation constant of 11.3 x 10^{-8} M and a maximum of 3.5 μg AMPAH bound per mg cell protein. The affinities and maximum binding of ^{125}I-AMPAH and [^{14}C]sucrose-AMPAH are similar. The binding of ^{125}I-AMPAH and [^{14}C]sucrose-AMPAH is inhibited by an excess of unlabeled AMPAH at low concentrations of radiolabeled AMPAH (Table 16). These results indicate that the non-specific binding of the radiolabeled enzymes is small and that covalent modification of AMPAH with ^{125}I or [^{14}C]sucrose does not alter the mode of binding of the enzymes to hepatocyte monolayers. Therefore, the radiolabeled enzymes can be used to examine aspects of the binding, uptake, and degradation of AMPAH by hepatocyte monolayers.

Inhibition of AMPAH Binding and Release of Bound AMPAH by Heparin

The effect of heparin on the binding of AMPAH to hepatocytes was examined because heparin inhibits the circulatory clearance of AMPAH *in vivo* (see Chapter III). The data in Table 17 show that the binding of

Figure 19. Concentration dependent binding of AMPAH to chicken hepatocyte monolayers. Cells were incubated at 4°C for 1.5 hr with unlabeled AMPAH (1400 units mg^{-1}) at the concentrations indicated. The procedure for the binding, washing and determination of total bound AMPAH activity is as described in the Methods. Inset is the data plotted as described by Scatchard (260).

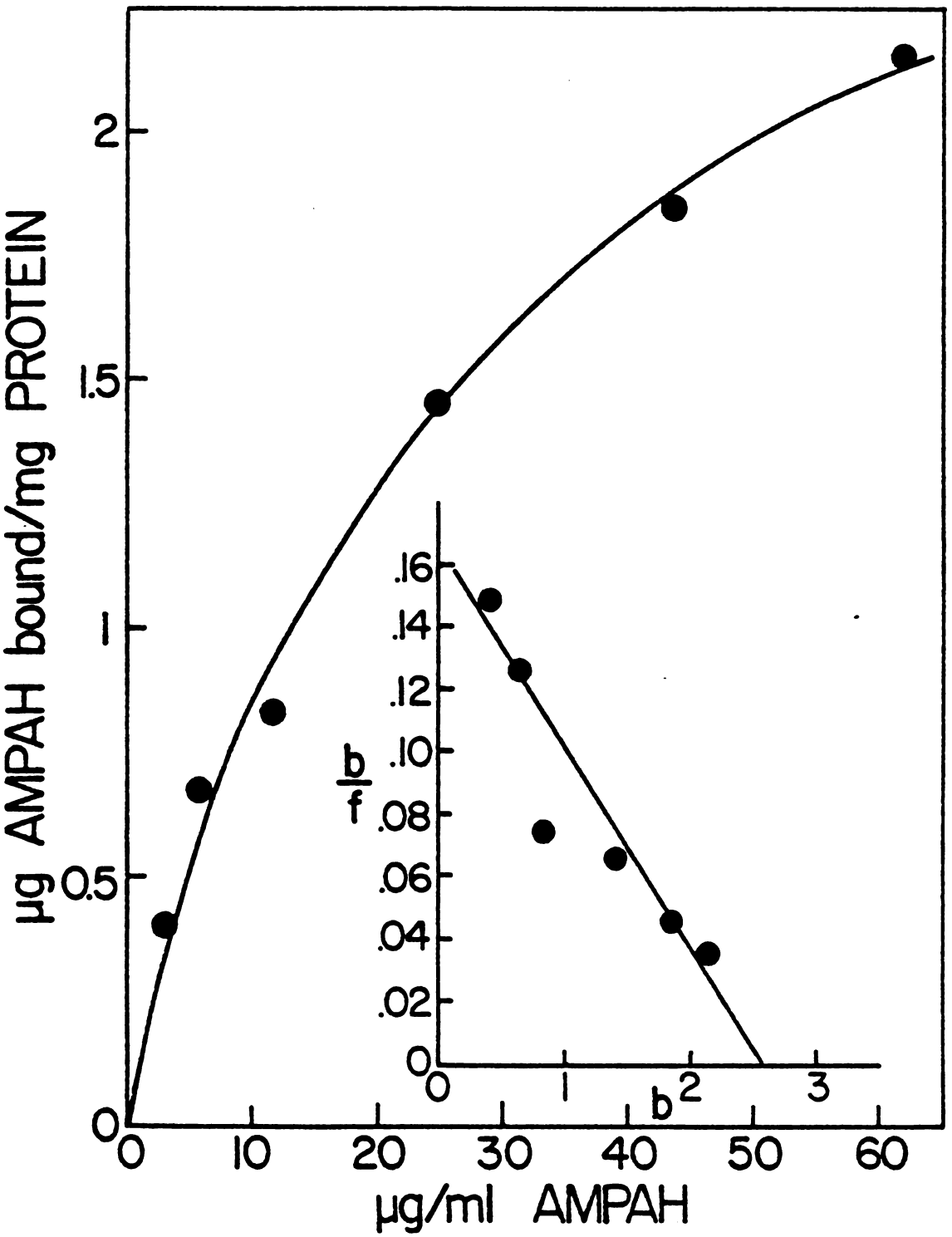
µg AMPAH bound/mg PROTEIN
0
0.5
1
1.5
2
0
10
20
30
40
50
60
µg/ml AMPAH
b/f
.16
.14
.12
.10
.08
.06
.04
.02
0
0
1
2
3
b

Figure 19.

TABLE 15. Characterization of the Binding of AMPAH to Hepatocyte Monolayers. The amount of unlabeled AMPAH, ^{125}I-AMPAH and [^{14}C]sucrose-AMPAH bound to hepatocyes for 1.5 hr at 4°C was determined at several concentrations of enzyme from 0-70 μg ml^{-1} as described in the Methods. The dissociation constants (K_D) and maximum binding (B_{max}) were calculated using a computer program which uses the weighting suggested by Wilkinson (264).

	Number of Determinations	K_D (x 10^8 M)	B_{max} $\frac{\mu g \text{ AMPAH Bound}}{mg \text{ cell protein}}$
Unlabeled AMPAH	3	11.3 ± 4.2	3.5 ± 0.5
^{125}I-AMPAH	3	5.6 ± 2.0	2.7 ± 1.6
[^{14}C]Sucrose-AMPAH	2	11.8 ± 1.3	3.3 ± 0.5

TABLE 16. Inhibition by Unlabeled AMPAH of the Binding of ^{125}I-AMPAH and [^{14}C]Sucrose- AMPAH to Hepatocyte Monolayers. For the ^{125}I-AMPAH inhibition experiment, conditions for binding were as described in the Methods except that to each dish was added either 150 µl PBS or 150 µl 2.2 mg ml^{-1} unlabeled AMPAH in PBS in addition to the other components of the binding assay. ^{125}I-AMPAH was 0.72 mg ml^{-1} with a specific activity of 83 cpm ng^{-1}. Ten µl was added to each dish. The concentration of unlabeled AMPAH was 32 times that of ^{125}I-AMPAH. For the [^{14}C]sucrose-AMPAH inhibition experiment, conditions were as described in the Methods except that to each dish was added either 100 µl PBS or 100 µl 1.3 mg ml^{-1} unlabeled AMPAH in PBS in addition to the other components of the binding assay. [^{14}C]Sucrose-AMPAH was 1.5 mg ml^{-1} with a specific activity of 2405 cpm µg^{-1}. Five µl was added to each dish. The concentration of unlabeled AMPAH was 18 times that of [^{14}C]sucrose-AMPAH. The total cell associated ^{125}I or ^{14}C was determined following washes as described in Methods.

	µg AMPAH Bound/mg Protein		
	No Unlabeled AMPAH	Excess Unlabeled AMPAH	Percent Inhibition
^{125}I-AMPAH	2.30	0.18	92
[^{14}C]Sucrose-AMPAH	1.05	0.17	84

TABLE 17. Effect of Heparin on the Binding of ^{125}I-AMPAH to Hepatocyte Monolayers and on the Release of Bound ^{125}I-AMPAH. The binding of ^{125}I-AMPAH and the washing of the cells was at 4°C as described in the Methods. Each dish was incubated with 10 µl 1.3 mg ml^{-1} ^{125}I-AMPAH in PBS with a specific activity of 115 cpm ng^{-1}. Where indicated, a final heparin concentration of 1 mg ml^{-1} was included in the binding assay or the final wash buffer.

1 mg ml^{-1} Heparin in Binding Mixture	ng ^{125}I-AMPAH Bound / mg Cell Protein	ng of Bound ^{125}I-AMPAH Released per mg Protein by Washing Cells with:[a]	
		Heparin	Wash Buffer
No	1246	886 (71)	75 (6)
Yes	58	23 (40)	4 (7)

[a]The percent of the total bound ^{125}I released by heparin is in parentheses.

^{125}I-AMPAH to hepatocyte monolayers is 95% inhibited by 1 mg ml^{-1} heparin. Of the ^{125}I-AMPAH bound to hepatocytes in the absence of heparin, 71% is released by washing the cells for 5 min with wash buffer containing 1 mg ml^{-1} heparin. Only 6% of the bound ^{125}I-AMPAH is released by washing the cells for 5 min with wash buffer without heparin. Ninety percent of the total bound ^{125}I-AMPAH is released by longer wash periods with wash buffer containing heparin, however, the additional release is not heparin-specific.

Binding of ^{125}I-AMPAH and [^{14}C]Sucrose-AMPAH to Hepatocyte Monolayers at 4°C and 37°C

Figure 20 is the time course for the total and heparin-releasable binding of ^{125}I-AMPAH to hepatocyte monolayers at 4°C and 37°C. After 1 hr at 4°C there is a constant level of total (solid squares) and heparin-releasable (open squares) ^{125}I bound to the hepatocytes. At 37°C there is also a constant level of heparin-releasable ^{125}I bound to hepatocytes that does not increase after 20 minutes of incubation (open circles), however, there is a continual accumulation of total bound ^{125}I over the three hour time period (solid circles). These results suggest that there is a steady-state level of ^{125}I-AMPAH bound to the cell surface that is heparin-releasable, that is continually internalized at 37°C and thus not susceptible to release by heparin. At 4°C the endocytosis of molecules bound to the surface of cells is minimal (260) and this is consistent with the observation that ^{125}I-AMPAH does not accumulate in the cells with time at 4°C.

The concentration dependence of the heparin-releasable binding of ^{125}I-AMPAH at 4°C and 37°C was examined to explain the observation

Figure 20. Time course for the binding of ^{125}I-AMPAH to hepatocyte monolayers at 4°C and 37°C. Hepatocyte monolayers were incubated at 4°C or 37°C as described in the Methods with 10 μg ml^{-1} ^{125}I-AMPAH (10.4 cpm ng^{-1}). Total ^{125}I bound at 4°C (■) and at 37°C (●), and heparin-releasable ^{125}I bound at 4°C (□) and 37°C (○), were determined as described in the Methods.

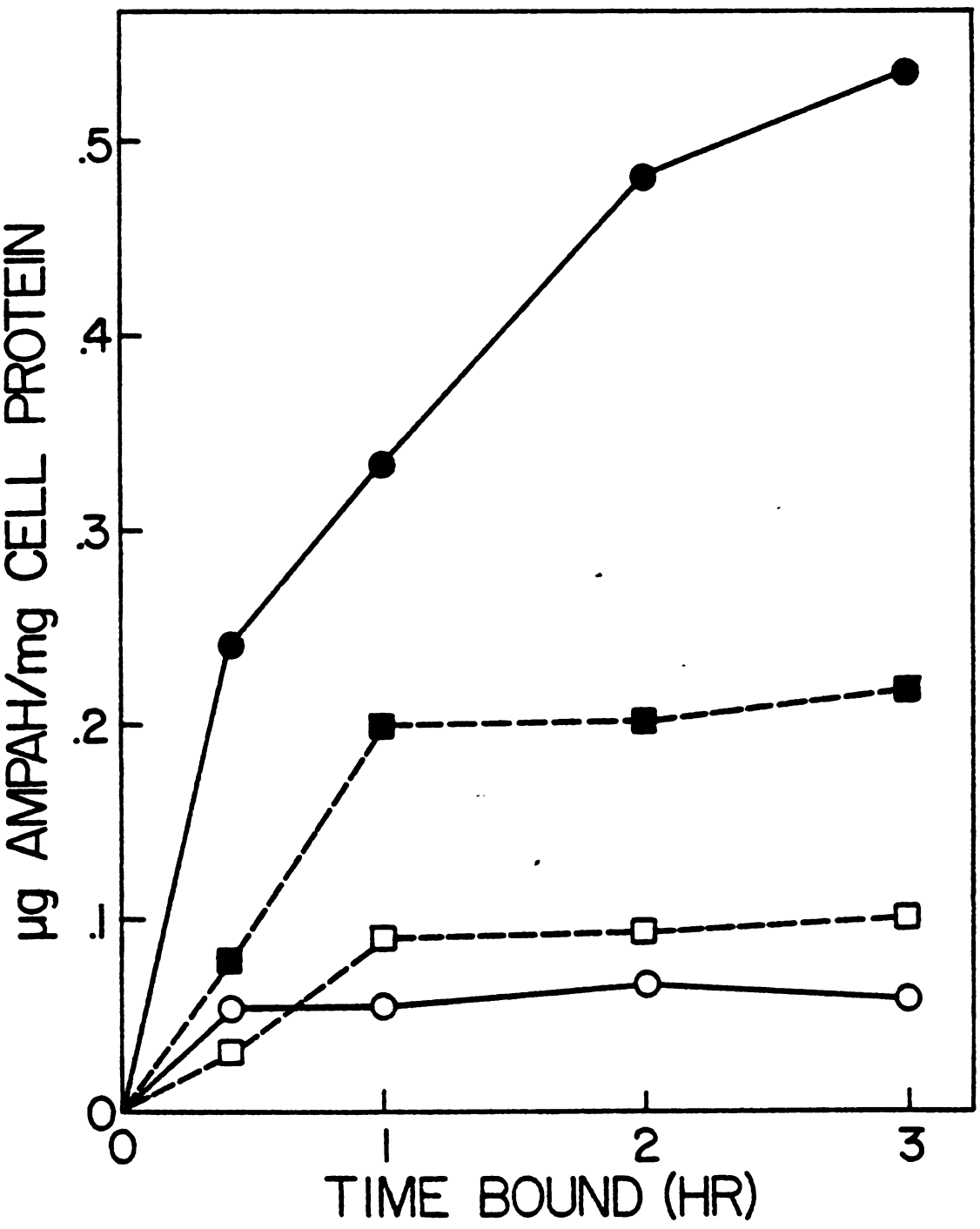

Figure 20.

that the total amount of heparin-releasable ^{125}I AMPAH observed at 37°C is only 60% of that at 4°C (Figure 20). The results in Figure 21 show that when hepatocyte monolayers are incubated with ^{125}I-AMPAH for 1.5 hr at 4°C or 37°C, the apparent affinities of heparin-releasable ^{125}I-AMPAH are similar at both temperatures, and that the diminished binding of ^{125}I-AMPAH at 37°C is due to fewer binding sites for ^{125}I-AMPAH. The reason for this phenomenon is not known.

The time course for the uptake of [^{14}C]sucrose-AMPAH at 37°C is shown in Figure 22. As discussed in Chapter III, the degradation products of [^{14}C]sucrose-labeled proteins are retained at the site of degradation, presumably due to the impermeability of these products to the lysosomal membrane. Therefore, the accumulation of ^{14}C in the hepatocytes is a measure of the rate of uptake of the enzyme bound to the cell surface. The amount of ^{14}C released from the cells by heparin is essentially constant between 1 and 4 hrs, as was observed with ^{125}I-AMPAH (Figure 20). This is consistent with a steady-state level of the enzyme bound to the cell surface that is released by heparin. The amount of total bound ^{14}C increases linearly over the 4 hr time period after a lag of 30 min to 1 hr. The linear accumulation is consistent with the internalization of [^{14}C]sucrose-AMPAH and the retention of the degradation products of the enzyme by the hepatocytes.

In a control experiment, the binding and uptake of [^{14}C]sucrose--BSA was measured to determine the extent of non-specific binding and uptake of proteins from the medium (Figure 22). The amount of total (solid triangle) and heparin-releasable (open triangle) ^{14}C bound is less than 10% of that for [^{14}C]sucrose-AMPAH when equal concentrations of the two [^{14}C]sucrose-labeled proteins are incubated with

Figure 21. Concentration dependent binding of heparin-releasable ^{125}I-AMPAH to hepatocyte monolayers at 4°C and 37°C. Cells were incubated with increasing concentrations of ^{125}I-AMPAH (85 cpm ng^{-1}) for 1.5 hr at 4°C (●) or 37°C (○). The binding, washes, and determination of heparin-releasable ^{125}I was as described in the Methods. Inset: Data plotted as described by Scatchard (260).

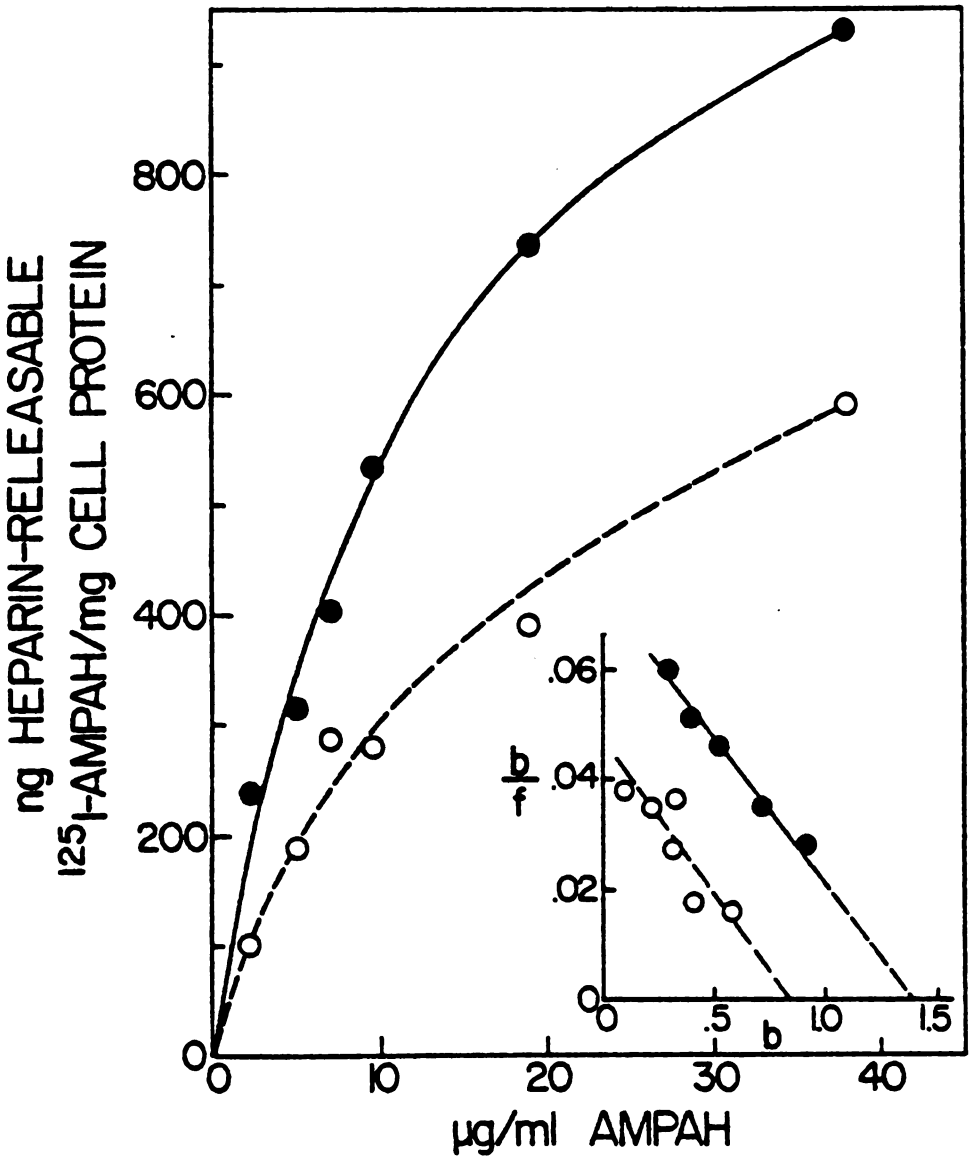
ng HEPARIN-RELEASABLE
125I-AMPAH/mg CELL PROTEIN
800
600
400
200
0
0
10
20
30
40
µg/ml AMPAH
b/f
.06
.04
.02
0
0
.5
1.0
1.5
b

Figure 21.

Figure 22. Time course for the binding of [^{14}C]sucrose-AMPAH and [^{14}C]sucrose-BSA to hepatocyte monolayers at 37°C. Hepatocyte monolayers were incubated at 37°C and the cells were washed as described in the Methods. Cells were incubated with 7 μg ml^{-1} [^{14}C]sucrose-AMPAH (2405 cpm μg^{-1}) and heparin-releasable ^{14}C (O) and total ^{14}C (●) were determined; or cells were incubated with 7 μg ml^{-1} [^{14}C]sucrose-BSA (2416 cpm μg^{-1}) and heparin-releasable ^{14}C (Δ) and total ^{14}C (▲) were determined.

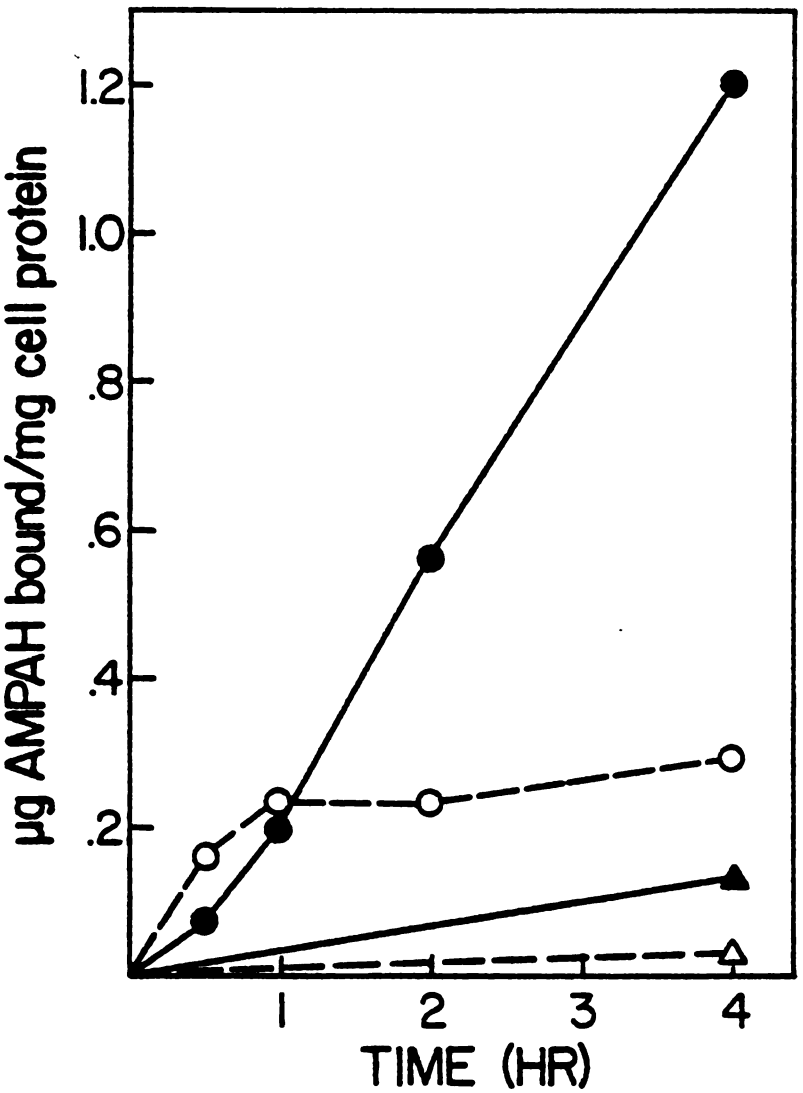

Figure 22.

hepatocyte monolayers at 37°C for 4 hr. This result, as well as the data in Table 16, demonstrate the specificity of binding of [^{14}C]sucrose-AMPAH to hepatocytes.

Size Distribution of ^{125}I-AMPAH Bound to Hepatocytes at 37°C

The size distribution of heparin-releasable and heparin-resistant radioactivity bound to hepatocyte monolayers was determined by gel-filtration chromatography in 8 M urea after the cells were incubated for 4 hr at 37°C with either ^{125}I-AMPAH or [^{14}C]sucrose-AMPAH (Figure 23). All of the heparin-releasable ^{125}I comigrates with ^{125}I-AMPAH in the excluded volume of the column (Figure 23a). However, the ^{125}I that remains associated with the cells after the heparin wash contains a small amount of lower molecular weight degradation products of the enzyme (Figure 23b). Similarly, when the cells are incubated with [^{14}C]sucrose-AMPAH, all of the heparin-releasable ^{14}C comigrates with [^{14}C]sucrose-AMPAH (Figure 23c). However, the heparin-resistant radioactivity is predominantly low molecular weight (Figure 23d). The accumulation of the low molecular weight degradation products of [^{14}C]sucrose-AMPAH is consistent with retention of the degradation products of the [^{14}C]sucrose-labeled enzyme at the site of degradation. The gel-filtration profile in Figure 23d shows that the major degradation products are larger than [^{14}C]sucrose and therefore are likely not permeable to the lysosomal membrane and are retained in the hepatocytes.

Only a small amount of low molecular weight degradation products of ^{125}I-AMPAH which comigrates with monoiodotyrosine (MIT) on the gel-filtration column is observed in hepatocytes. This may be due to the rapid loss of these degradation products from the cells. To verify this

Figure 23. Size distribution of heparin-releasable and heparin-resistant radioactivity after hepatocytes are incubated at 37°C with radiolabeled AMPAH. Hepatocyte monolayers were incubated for 4 hr at 37°C with ^{125}I-AMPAH or [^{14}C]sucrose-AMPAH. Cells were washed as described in the Methods. Heparin wash fractions, and cells after heparin washes, were prepared and chromatographed as described in the Methods on Bio-Gel P-60 in 8 M urea. The positions of elution of radiolabeled AMPAH and monoiodotyrosine (MIT) are indicated. (a) Heparin-releasable ^{125}I; (b) heparin-resistant ^{125}I; (c) heparin-releasable ^{14}C; (d) heparin-resistant ^{14}C.

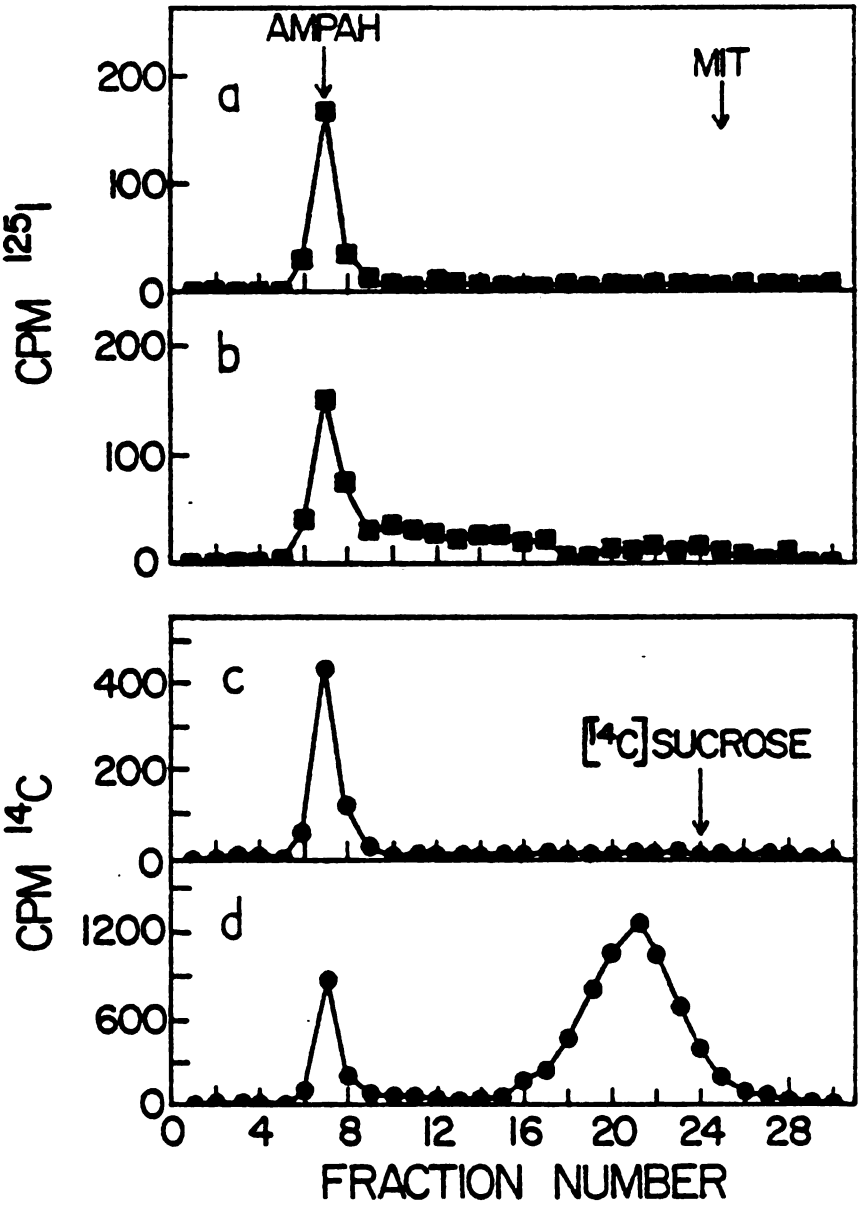
AMPAH
MIT
a
b
c
d
200
100
0
200
100
0
400
200
0
1200
600
0
CPM 125I
CPM 14C
[14C]SUCROSE
0 4 8 12 16 20 24 28
FRACTION NUMBER

Figure 23.

suggestion the release of low molecular weight ^{125}I into the media was examined. ^{125}I-AMPAH was bound to cells at 4°C, the cells were washed by the standard procedure, and then warmed to 37°C in medium. The gel-filtration profile of medium 210 minutes after warming the cells to 37°C is shown in Figure 24. Fifty-four percent of the radioactivity comigrates with ^{125}I-AMPAH. This may be enzyme which dissociated from the cells before internalization and degradation. The remainder of the radioactivity comigrates with monoiodotyrosine and is presumably degradation products of ^{125}I-AMPAH which has been internalized and degraded. No radioactivity of intermediate molecular weight is observed in the medium. This is consistent with the internalization, degradation and release of low molecular weight degradation products into the medium. If the enzyme bound to the cell surface was degraded extracellularly, degradation products of intermediate molecular weight might be expected. The amount of low molecular weight radioactivity in the medium increases with time after warming the cells to 37°C (Figure 24, inset). No significant low molecular weight ^{125}I is observed when ^{125}I-AMPAH is incubated at 37°C for 210 min in the absence of hepatocytes in medium previously incubated over hepatocyte monolayers, or in fresh medium.

Figure 24. Release of low molecular weight ^{125}I into the media after the binding of ^{125}I-AMPAH to hepatocyte monolayers. Hepatocyte monolayers were incubated at 4°C for 1.5 hr with 10.5 μg ml^{-1} ^{125}I-AMPAH (109 cpm ng^{-1}). The procedure for the binding and washing of the cells was as described in the Methods. After the final wash the plates were rinsed twice with 0.5 ml medium, then 0.5 ml medium was added and the dishes were incubated at 37°C for 210 min. The size distribution of ^{125}I in the medium was determined at each time point by gel-filtration chromatography in 8 M urea, as described in the Methods. The elution profile of the medium sample at 210 min is shown. The total small molecular weight radioactivity in the medium is the sum of radioactivity in fractions 21-28 of the Bio-Gel P-60 elution profile. The inset shows the accumulation of small molecular weight ^{125}I in the medium with time after warming the cells to 37°C.

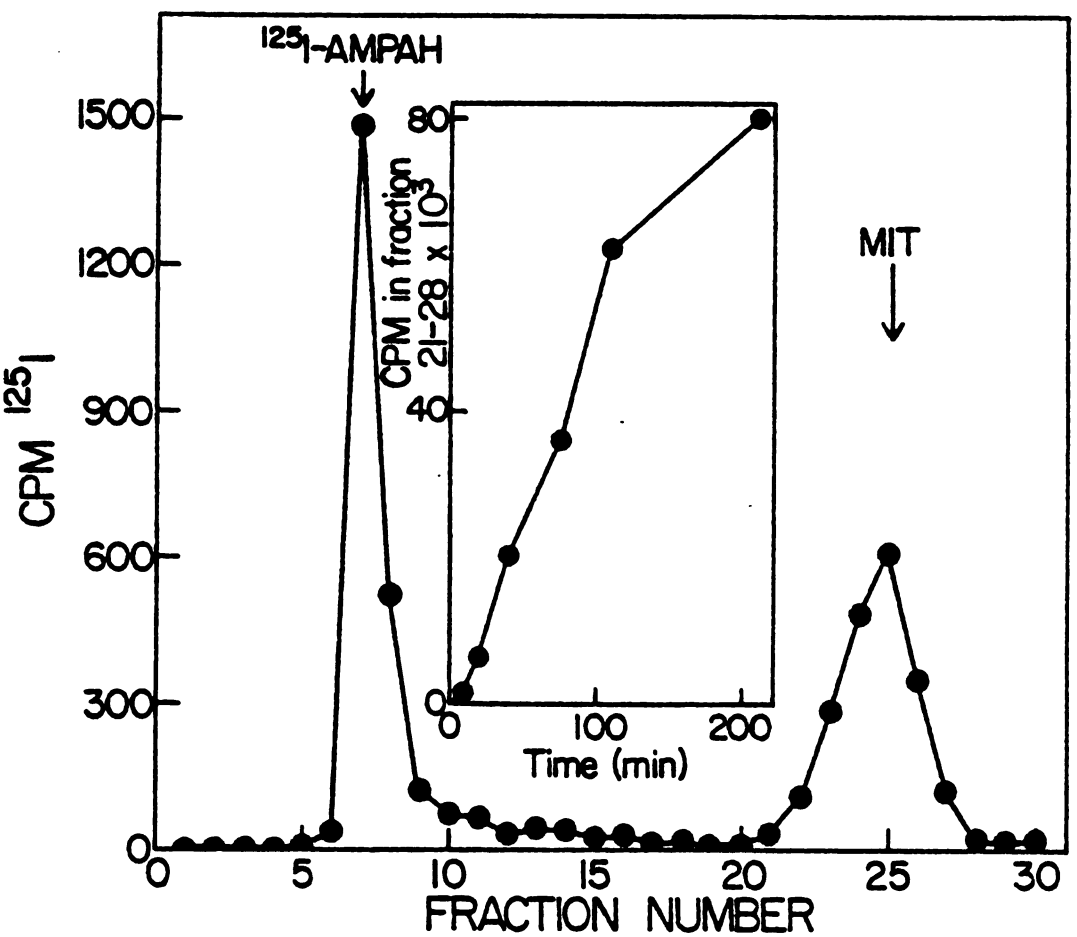
125I-AMPAH
MIT
CPM 125I
1500
1200
900
600
300
0
0
5
10
15
20
25
30
FRACTION NUMBER
CPM in fraction 21-28 x 10³
80
40
0
0
100
200
Time (min)

Figure 24.

DISCUSSION

The binding of unlabeled AMPAH, ^{125}I-AMPAH, and [^{14}C]sucrose-AMPAH to chicken hepatocyte monolayers is specific, has a high affinity, and is saturable. The concentration dependence for the binding of radiolabeled enzymes is similar to that of unlabeled AMPAH, and the binding of radiolabeled enzymes is inhibited by an excess of unlabeled AMPAH. The maximum binding of AMPAH at saturating concentrations of the enzyme is 3.5 μg AMPAH bound per mg total cell protein. Assuming a molecular weight of 287,000 for chicken muscle AMPAH (228) and using a value of 3.5×10^{-7} mg protein per hepatocyte determined by counting nuclei and protein measurements in hepatocyte monolayer cultures, this corresponds to 2.6×10^{6} binding sites for AMPAH per hepatocyte.

Several lines of evidence show that bound AMPAH is internalized and degraded. When hepatocyte monolayers are incubated at 4°C with ^{125}I-AMPAH, 76% of the bound enzyme is released by washing the cells with heparin. However, when the incubation is 37°C, ^{125}I-AMPAH accumulates in the cells with time and is primarily heparin-resistant. The amount of ^{125}I that is released by heparin remains constant with time at both 4°C and 37°C. This behavior is consistent with the suggestion that the AMPAH bound to the cell surface is released by heparin, and that AMPAH is internalized at 37°C and is not susceptible to release by heparin. Furthermore, the size distribution of heparin-releasable and

heparin-resistant ^{125}I-AMPAH or [^{14}C]sucrose-AMPAH bound to hepatocytes indicates that heparin-releasable radioactivity is undegraded enzyme bound to the cell surface, and that the heparin-resistant radioactivity is partially degraded enzyme. When ^{125}I-AMPAH is bound to hepatocytes at 4°C, then warmed to 37°C, increasing amounts of low molecular weight ^{125}I appear in the medium with time, further verifying the internalization and degradation of bound ^{125}I-AMPAH. Furthermore, low molecular weight degradation products of heparin-resistant [^{14}C]sucrose-AMPAH bound to hepatocytes increases linearly with time. This implies the internalization and trapping of these degradation products in lysosomes.

The time course for the uptake of [^{14}C]sucrose-AMPAH by hepatocytes (Figure 24) indicates that the process of internalization is slow. Assuming that heparin-resistant ^{14}C is internalized [^{14}C]sucrose-AMPAH derived from heparin-releasable [^{14}C]sucrose-AMPAH at the cell surface, and that the degradation products of [^{14}C]sucrose-AMPAH are quantitatively retained in the hepatocytes; then in the 3 hr linear portion of the uptake curve (Figure 23) between 1 and 4 hr, 3.9 times as much enzyme is internalized than is bound to the cell surface. Therefore, 3 hr/3.9 or 0.77 hr is the average time that elapses between the time a molecule of AMPAH binds to the cell surface, is internalized, and is replaced at the cell surface by another molecule of AMPAH from the medium. By comparison, the rate of internalization of AMPAH <u>in vivo</u> reported in Chapter III has a half-life of 0.98 hr. This rate of internalization is much slower than the 3 min average time of residency of asialoglycoproteins bound to the cell surface of rat hepatocytes (261,262).

When AMPAH is intravenously injected into chickens the enzyme is rapidly cleared from the circulation by the spleen and the parenchymal cells of the liver where the enzyme is internalized and degraded (see Chapter III). The clearance is inhibited by heparin and other sulfated polysaccharides. The heparin inhibited high affinity binding, internalization, and degradation of AMPAH bound to hepatocyte monolayers, indicates that the binding of AMPAH to hepatocytes studied *in vitro* is by the same mechanism as that responsible for the rapid clearance of the enzyme *in vivo*.

CHAPTER V. INVESTIGATIONS INTO THE NATURE OF THE INTERACTION OF AMPAH WITH HEPATOCYTES

INTRODUCTION

In Chapter IV the specific, high affinity binding of AMPAH to hepatocytes was demonstrated. At 37°C the bound enzyme is apparently internalized and degraded. Heparin inhibits the binding of AMPAH to hepatocytes and releases enzyme bound to the cell surface. The binding of AMPAH to cultured hepatocytes may be by the same mechanism as that responsible for the rapid *in vivo* circulatory clearance of intravenously injected AMPAH.

The binding of AMPAH to hepatocytes is inhibited by sulfated polysaccharides, effectors of AMPAH activity, and salts. Carbohydrates which inhibit the binding of glycoproteins to carbohydrate binding proteins in the liver have little effect on the interaction.

The mechanism for the heparin inhibition of the interaction of AMPAH with hepatocytes was investigated further. Heparin interacts strongly with AMPAH. It is suggested that the binding of heparin to AMPAH may reduce the affinity of AMPAH for the cell surface binding site. Alternatively, AMPAH may interact with a cell surface heparin-like molecule, and heparin may inhibit the interaction by competing for the binding of AMPAH.

MATERIALS AND METHODS

Materials

Carbohydrates, nucleotides, Type III RNA, chondroitin sulfates, dextran sulfate, dextran, and hyaluronic acid were obtained from Sigma Chemical Company, St. Louis, Missouri. Sodium polyphosphate ($n \approx 5$) was from the Monsanto Co., St. Louis, Missouri. CnBr and $(CH_3)_4NCl$ were obtained from Aldrich Chemical Company, Milwaukee, Wisconsin. $(CH_3)_4NCl$ was recrystallized from isopropyl alcohol before use. Sepharose 4B was obtained from Pharmacia Inc., Piscataway, New Jersey. Heparan sulfate (barium salt) was a gift from Dr. Paul O'Connell at The Upjohn Company, Kalamazoo, Michigan, and was purified and the sodium salt prepared as previously described (263). All other materials were from the sources listed in Chapters II-IV.

Measurement of the Effect of Compounds on the Binding of AMPAH to Hepatocyte Monolayers

^{125}I-AMPAH was prepared as described in Chapter III. The preparation of monolayer cultures of chicken parenchymal cells and the measurement of the binding of AMPAH to hepatocyte monolayers was as described in Chapter IV. The effect of compounds on AMPAH binding and the release of bound ^{125}I-AMPAH from hepatocytes was determined as described for the inhibition of binding and release of bound AMPAH by heparin in Chapter IV.

Measurement of Heparin Inhibition of AMPAH Activity

Purified AMPAH was exhaustively dialyzed against 0.15 M KCl, 50 mM MES-TRIS, pH 6.5. Assays were started by adding 5 µl of AMPAH (0.35 µg) to a cuvette containing 1.0 ml 50 mM MES-TRIS, pH 6.5, and varying concentrations of KCl, AMP, and heparin as indicated in the Results. All assays were at 30°C and the increase in absorbance at 290 nm as AMP is deaminated to form IMP was monitored. K_m(apparent) and V_{max} were calculated at each heparin concentration with a computer program which uses the weights suggested by Wilkinson (264).

Binding of AMPAH to Heparin-Sepharose 4B

Heparin was immobilized by reacting heparin with CnBr-activated Sepharose 4B as described by Iverius (265). Control Sepharose 4B was prepared to which no heparin was added. Columns (4 x 30 mm) were washed with 5 ml aliquots of: 1) 5 mg ml^{-1} BSA in 1.0 M KCl, 50 mM MES-TRIS, pH 6.5; 2) 1.0 M KCl, 50 mM MES-TRIS, pH 6.5; and 3) 0.15 M KCl, 50 mM MES-TRIS, pH 6.5. 0.5 ml aliquots of AMPAH (600 units ml^{-1}) in 0.15 M KCl, 50 mM MES-TRIS, pH 6.5 were applied and the column was washed with 5 ml of 0.15 M KCl, 50 mM MES-TRIS, pH 6.5. Under these conditions, AMPAH is quantitatively bound to the heparin-Sepharose 4B column. AMPAH was eluted as described in the Results; 0.5 ml fractions were collected.

RESULTS

Inhibition of the Interaction of AMPAH with Hepatocyte Monolayers by Sulfated Polysaccharides and Other Polyanions

Table 18 shows the effect of sulfated polysaccharides and other polyanions on the binding of ^{125}I-AMPAH to hepatocyte monolayers at 4°C, and the release of bound ^{125}I-AMPAH by these compounds after binding is carried out in the absence of inhibitors. Heparin, heparan sulfate, and dextran sulfate inhibit the binding and release bound ^{125}I-AMPAH. The chondroitin sulfates are less effective. Dextran sulfate inhibits the interaction of AMPAH with hepatocytes, however, dextran, sodium sulfate, and glucose-6-sulfate are relatively ineffective. This implies that the intact sulfated polysaccharide is necessary to inhibit the interaction of AMPAH with hepatocytes. The concentration dependence for the release of bound ^{125}I-AMPAH is shown in Figure 25. Release of bound AMPAH is most sensitive to dextran sulfate followed by heparin, heparan sulfate, and chondroitin sulfate C, in that order. This corresponds to the degree of sulfation of these polysaccharides (266,267); the interaction of AMPAH with hepatocytes is most effectively inhibited by those sulfated polysaccharides with the highest sulfate content. At low concentrations, chondroitin sulfate C is as effective as heparin in releasing ^{125}I-AMPAH bound to hepatocyte monolayers, though at higher concentrations chondroitin sulfate C is less effective. The reason for this phenomenon is not known.

TABLE 18. Inhibition of ^{125}I-AMPAH Binding and Release of ^{125}I-AMPAH Bound to Hepatocytes by Sulfated Polysaccharides and Other Polyanions. The binding of ^{125}I-AMPAH to hepatocytes and the wash procedures were as described in the Methods. Each dish contained 13 µg ^{125}I-AMPAH (115 cpm ng^{-1}). The percent inhibition of binding is the total amount of ^{125}I-AMPAH bound to hepatocyte monolayers when inhibitors were included in the binding mixture, compared to the amount bound when no inhibitor was included. The percent of ^{125}I-AMPAH released by various compounds is the percent of the total bound ^{125}I released by washing the cells with the indicated compound, after ^{125}I-AMPAH was bound to hepatocyte monolayers in the absence of inhibitors. The concentrations of all compounds were 1 mg ml^{-1} except Na_2SO_4 and glucose-6-sulfate which were 10 mM.

Compound	Percent Inhibition of AMPAH Binding	Percent of Total Bound AMPAH Released
None	0	7
Heparin	92	74
Heparan Sulfate	90	74
Chondroitin Sulfate A	22	38
Chondroitin Sulfate B	70	59
Chondroitin Sulfate C	39	44
Dextran Sulfate	77	76
Dextran	26	14
Na_2SO_4	34	14
Glucose-6-Sulfate	ND[a]	13
RNA	16	34
Polyphosphate	33	32
Hyaluronic Acid	26	25

[a]ND, not determined.

Figure 25. Concentration dependence for the release of ^{125}I-AMPAH bound to hepatocytes by sulfated polysaccharides. The experimental procedures were as outlined in Table 18. The percent of the total bound ^{125}I-AMPAH released at different concentrations of dextran sulfate (O), heparin (Δ), heparan sulfate ( ), and chondroitin sulfate C (O) was determined.

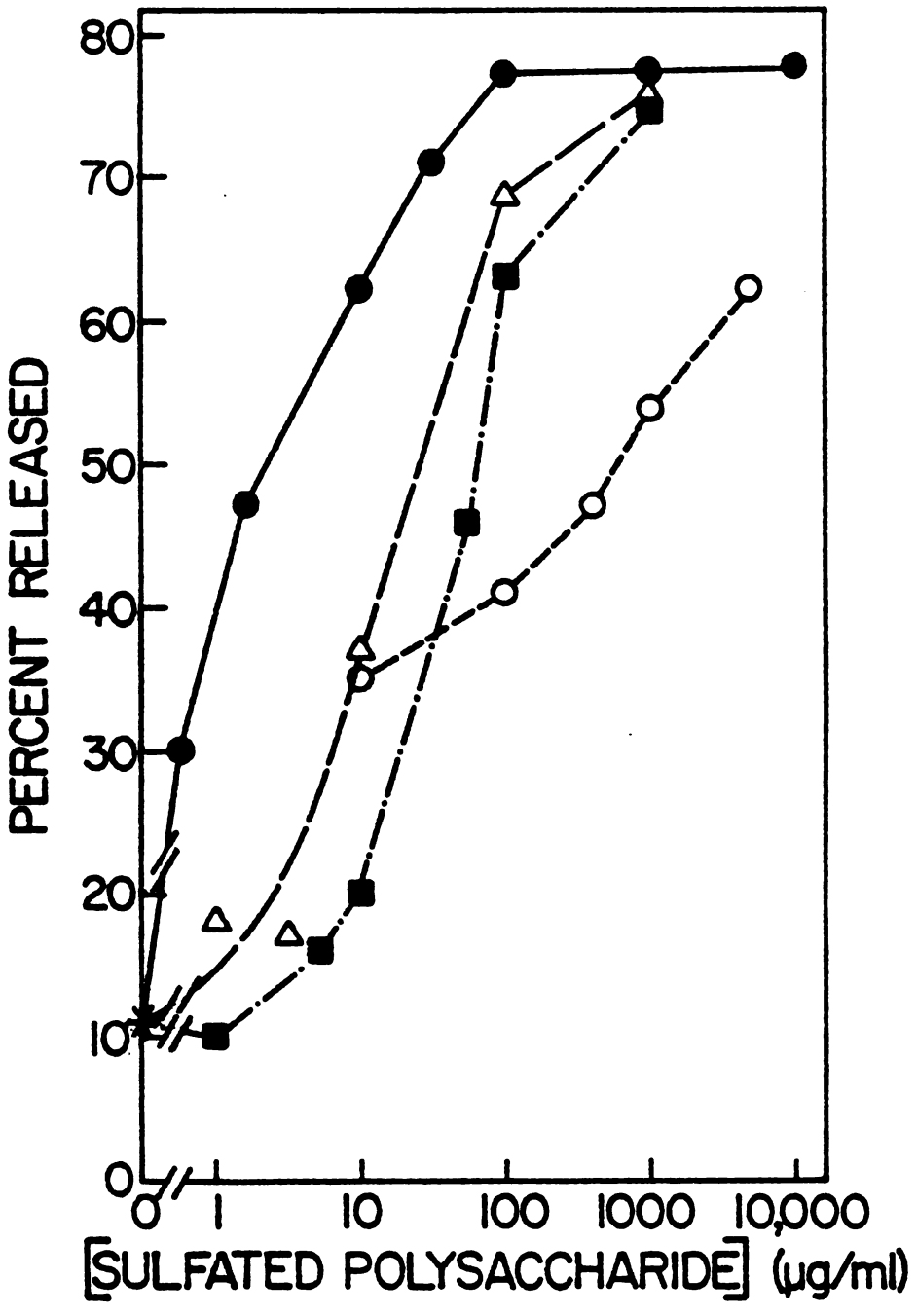
PERCENT RELEASED
80
70
60
50
40
30
20
10
0
0
1
10
100
1000
10,000
[SULFATED POLYSACCHARIDE] (µg/ml)

Figure 25.

The effect of other polyanions on the binding and release of bound ^{125}I-AMPAH was also examined to determine whether the inhibition of the interaction of ^{125}I-AMPAH is specific for sulfated polysaccharides (Table 18). Hyaluronic acid and the phosphate-containing polyanions, RNA and polyphosphate, inhibit the interaction of ^{125}I-AMPAH with hepatocytes but are less effective than the sulfated polysaccharides.

Inhibition of the Interaction of AMPAH with Hepatocytes by Effectors of AMPAH Enzymatic Activity

The effect of allosteric effectors of AMPAH on the interaction of ^{125}I-AMPAH with hepatocyte monolayers was examined to determine whether molecules which bind AMPAH and modulate enzymatic activity (112) affect the interaction. Inhibition of ^{125}I-AMPAH binding and release of bound ^{125}I-AMPAH is observed for pyrophosphate and all of the nucleotides tested (Table 19). Orthophosphate, which is a relatively low affinity inhibitor of AMPAH activity, has little effect. The concentration dependence for release of ^{125}I-AMPAH bound to hepatocytes by pyrophosphate and several nucleotides is shown in Figure 26. Release of bound ^{125}I-AMPAH is most sensitive to GTP and is apparent at 50 μM. ADP is slightly more effective than ATP with release by these nucleotides evident at 1.5-2.0 mM. AMP and pyrophosphate are less effective, with release of bound ^{125}I-AMPAH apparent only at 5-10 mM.

Release of AMPAH Bound to Hepatocyte Monolayers by Salts

The effect of increasing ionic strength on the release of ^{125}I-AMPAH bound to hepatocyte monolayers was examined to determine whether the binding is electrostatic in nature, and also to determine

TABLE 19. Inhibition of ^{125}I-AMPAH Binding and Release of ^{125}I-AMPAH Bound to Hepatocytes by Allosteric Effectors of AMPAH Activity. The experimental procedures were as described in the legend to Table 18. The concentration of all compounds was 10 mM.

Compound	Percent Inhibition of AMPAH Binding	Percent of Total Bound AMPAH Released
None	0	8
P_i	10	12
PP_i	66	63
AMP	ND[a]	48
ADP	89	74
ATP	88	56
GTP	90	70
CTP	ND	59
ITP	ND	46

[a]ND, not determined.

Figure 26. Concentration dependence for the release of ^{125}I-AMPAH bound to hepatocytes by effectors of AMPAH activity. The experimental procedures were as outlined in Table 18. The percent of the total bound ^{125}I-AMPAH released by different concentrations of GTP (O), ADP (▲), ATP (□), AMP (●), and pyrophosphate (Δ) was determined.

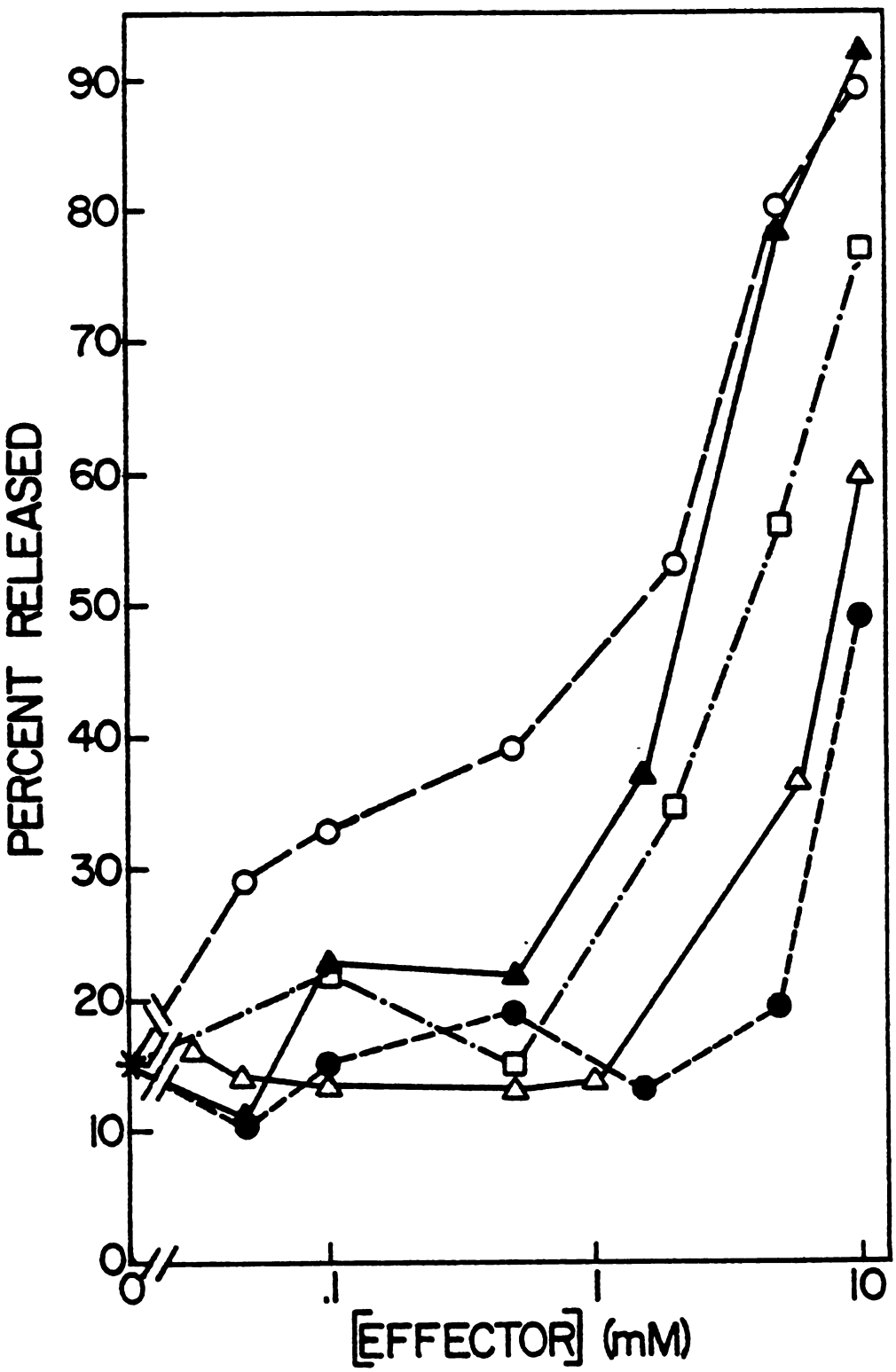

Figure 26.

whether the release of the bound enzyme by the compounds shown in Tables 18 and 19 is in part due to an increase in ionic strength. The release of ^{125}I-AMPAH by increasing concentrations of KCl, NaCl, and $(CH_3)_4NCl$ is shown in Figure 27. The hepatocytes are greater than 80% viable after this brief salt treatment, as judged by trypan blue exclusion at the highest KCl concentration tested. Little or no release of bound ^{125}I-AMPAH is observed at added salt concentrations of 50 mM or below. Therefore, the release of ^{125}I-AMPAH by the compounds in Tables 18 and 19 is not due to an increase in ionic strength. The release of bound ^{125}I-AMPAH by salts indicates that the interaction of AMPAH with hepatocytes is primarily electrostatic. However, ^{125}I-AMPAH is released at slightly lower concentrations of NaCl and KCl than $(CH_3)_4NCl$ possibly due to the interaction of Na^+ and K^+, but not $(CH_3)_4N^+$, with AMPAH (112).

Inhibition of the Interaction of AMPAH with Hepatocytes by Carbohydrates

AMPAH preparations contain covalently bound carbohydrates (245). Small quantities of N-acetylneuraminic acid, N-acetylglucosamine, and glucose are detected. To determine whether any of the carbohydrate recognition systems of the liver (247) are responsible for the binding of AMPAH to hepatocytes, the inhibition of AMPAH binding and the release of bound AMPAH by several carbohydrates was examined. The data in Table 20 show that some of the carbohydrates tested partially inhibit the binding of AMPAH to hepatocytes, but have a lesser effect on the release of bound AMPAH. N-Acetylglucosamine, mannose, and agalactoorosomucoid (a glycoprotein with terminal N-acetylglucosamine residues) should inhibit the interaction of AMPAH with hepatocytes if the N-acetylglucosamine/mannose receptor of avian liver (246,268,269) is responsible for the binding of

Figure 27. Concentration dependence for the release of ^{125}I-AMPAH bound to hepatocytes by salts. The experimental procedures were as outlined in Table 18. The percent of the total bound ^{125}I-AMPAH released by different concentrations of KCl (□), NaCl (Δ), and $(CH_3)_4NCl$ (O) was determined.

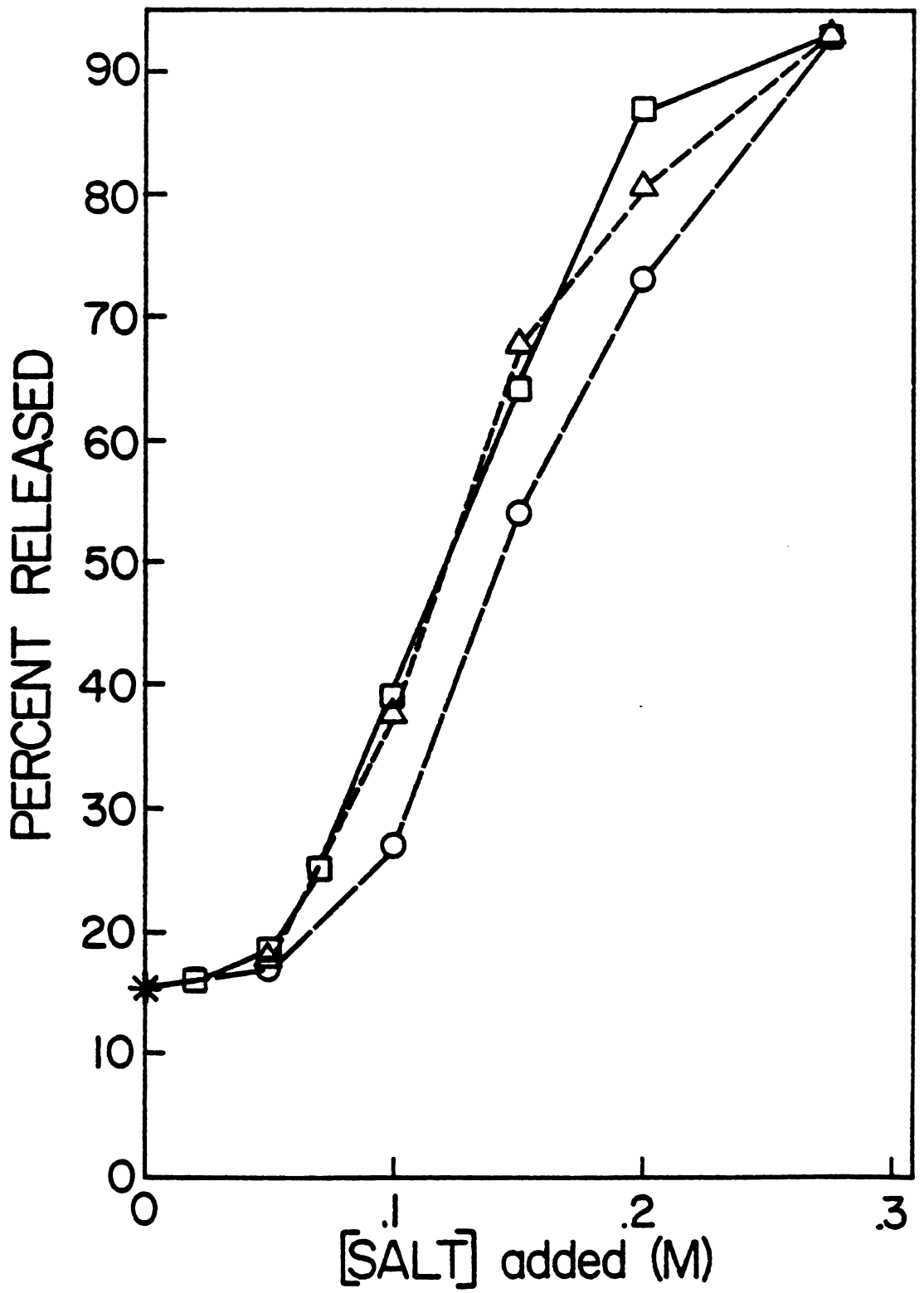

Figure 27.

TABLE 20. Inhibition of ^{125}I-AMPAH Binding and Release of ^{125}I-AMPAH Bound to Hepatocytes by Carbohydrates. The experimental procedures were as described in the legend to Table 18. The concentration of all compounds was 10 mM except agalactoorosomucoid which was 1 mg ml^{-1}.

Compound	Percent Inhibition of AMPAH Binding	Percent of Total Bound AMPAH Released
None	0	7
Galactose	24	11
N-Acetylgalactosamine	21	11
N-Acetylglucosamine	21	12
Mannose	15	ND[a]
Mannose-6-Phosphate	12	11
N-Acetylneuraminic Acid	13	15
Glucose	20	ND
Agalactoorosomucoid	9	ND

[a]ND, not determined.

AMPAH. Galactose and N-acetylgalactosamine inhibit the interaction with the galactose specific receptor of mammalian liver, though this receptor is apparently absent in avian liver (246). A hepatic receptor that recognizes terminal mannose-6-phosphate residues on lysosomal enzymes is observed in bovine liver (270). Mannose-6-phosphate inhibits the binding to this receptor but has no effect on the binding of AMPAH to hepatocytes. Since these carbohydrates have only a small effect on the interaction of AMPAH with hepatocytes at the 10 mM concentrations tested, and because the inhibition of binding of AMPAH by these carbohydrates is relatively non-specific, it is unlikely that the carbohydrate components of AMPAH are of primary importance in the recognition of AMPAH by hepatocytes. These results are essentially in agreement with in vivo experiments in Chapter III which show that the rate of clearance of intravenously injected AMPAH is not affected by compounds which inhibit the binding to these carbohydrate recognition systems.

Inhibition of AMPAH Activity by Heparin and Other Polyanions

Because the interaction of AMPAH with hepatocytes is inhibited by molecules which bind AMPAH and modulate enzymatic activity (Table 19, Figure 26), it is of interest to determine whether heparin, other sulfated polysaccharides, and other polyanions interact with AMPAH. This might explain the release of AMPAH bound to hepatocytes by heparin and sulfated polysaccharides. The data in Table 21 show the effect of several compounds on AMPAH activity. All of the polyanions tested inhibit AMPAH enzymatic activity presumably by the interaction of these molecules with AMPAH. Dextran sulfate inhibits AMPAH activity, though dextran, Na_2SO_4, or glucose-6-sulfate do not. This indicates that the intact sulfated polysaccharide is necessary for inhibition of AMPAH activity.

TABLE 21. Polyanion Inhibition of AMPAH Activity. The inhibition of AMPAH was determined by measuring the activity of AMPAH when 1.4 μg of AMPAH was added to 1.0 ml of an assay solution containing 100 μg ml^{-1} inhibitor, 0.96 mM AMP, 0.15 M KCl, and 50 mM MES-TRIS, pH 6.5. Assays were at 30°C and the increase in absorbance at 290 nm was monitored.

Inhibitor	Percent of Control AMPAH Activity
None (control)	100
Heparin	34
Heparan Sulfate	30
Chondroitin Sulfate A	41
Chondroitin Sulfate B	37
Chondroitin Sulfate C	58
Dextran Sulfate	22
Dextran	101
Na_2SO_4	101
RNA	20

The inhibition of AMPAH by heparin was examined in detail. The data in Figure 28 show that KCl relieves the inhibition of AMPAH activity by heparin. This observation is similar to that of Wheeler et al. (271), who showed that KCl relieves the inhibition of AMPAH by pyrophosphate, triphosphate, ATP, and GTP. The decrease of AMPAH activity at high KCl concentrations both in the presence and absence of heparin is probably due to inhibition of AMPAH by the chloride anion (272). Figure 29a shows the double reciprocal plots for the effect of heparin on AMPAH activity at varying concentrations of AMP. Figures 29b and 29c show heparin decreases the affinity of AMPAH for AMP (increases K_m(apparent)) and has no effect on the maximal velocity (V_{max}). The Dixon plot (273) in Figure 30 shows that the K_i for the inhibition of AMPAH by heparin is about 20 ng ml^{-1}. This inhibition differs from classical competitive inhibition in that high concentrations of heparin do not fully inhibit the enzyme activity.

Interaction of AMPAH with Heparin-Sepharose 4B

AMPAH applied to heparin-Sepharose 4B as described in the Methods is quantitatively bound to the column. No AMPAH binds to Sepharose 4B that is not derivatized with heparin. AMPAH is eluted with either KCl, pyrophosphate, or heparin (Figure 31, solid circles). No activity elutes from the control Sepharose 4B column that is not derivatized with heparin (Figure 31, open circles). The pyrophosphate-induced release of AMPAH bound to heparin-Sepharose 4B indicates that either pyrophosphate competes with heparin for the binding of AMPAH, or that pyrophosphate binds an alternate site on the enzyme and reduces the affinity of AMPAH for heparin.

Figure 28. The effect of KCl on the inhibition of AMPAH activity by heparin. AMPAH activity was measured when 0.45 μg AMPAH was added to 1.0 ml assay mixture containing KCl as indicated, 5 mM AMP, 50 mM MES-TRIS, pH 6.5, with (●) or without (○) 1 mg ml^{-1} heparin.

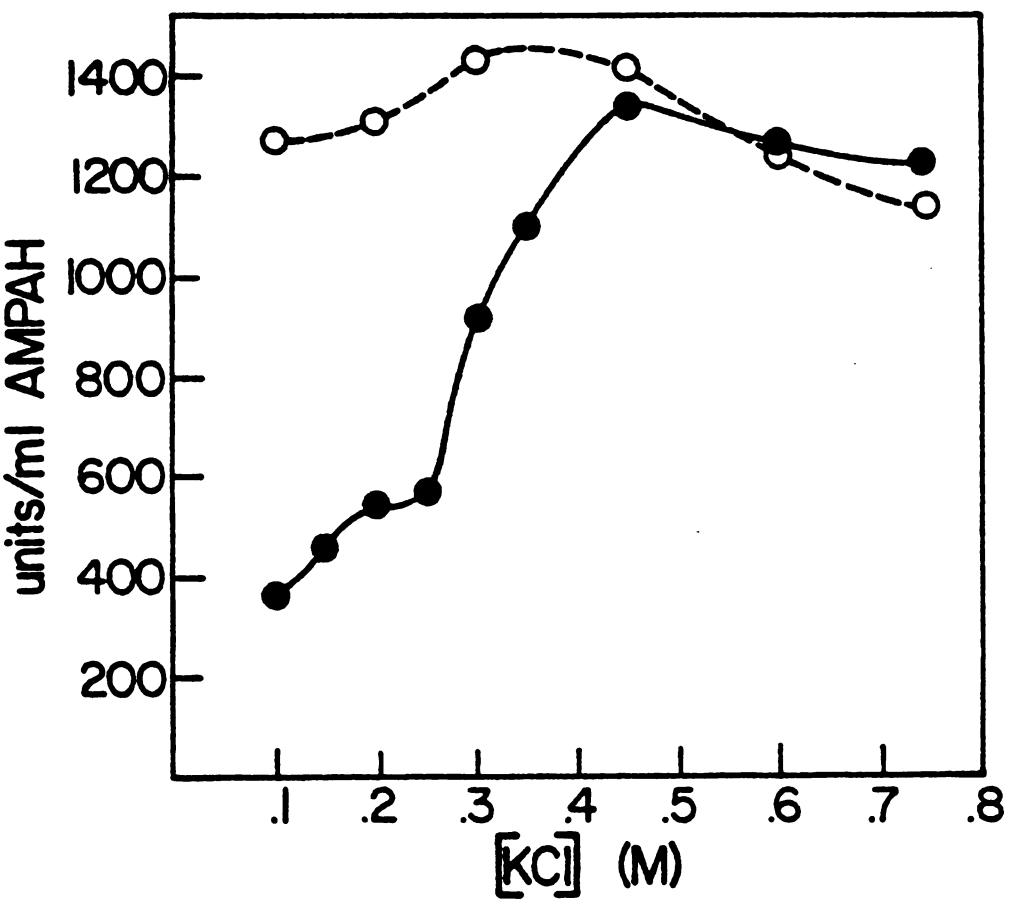

Figure 28.

Figure 29. Effect of heparin on AMPAH kinetic parameters. AMPAH was added to the assay mixture as described in the Methods at varying concentrations of heparin and AMP, and 0.15 mM KCl, 50 mM MES-TRIS, pH 6.5. a) Double reciprocal plot of 1/velocity vs. 1/[AMP], b) effect of heparin concentration on V_{max}, c) effect of heparin concentration on K_m(apparent).

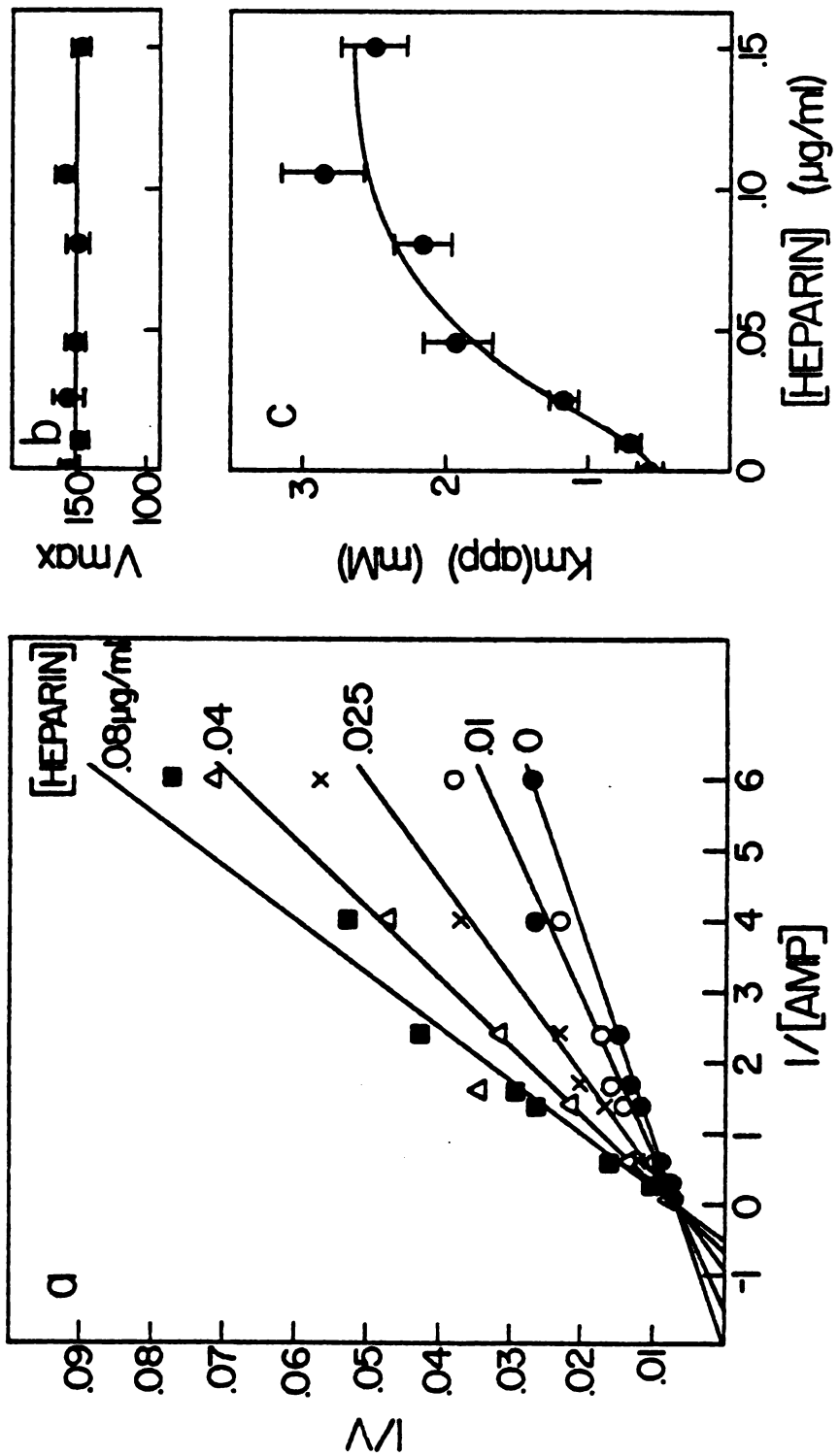
a
[HEPARIN]
.08µg/ml
.04
.025
.01
0
1/V
1/[AMP]
b
Vmax
c
Km(app) (mM)
[HEPARIN] (µg/ml)

Figure 29.

Figure 30. Dixon plot for the inhibition of AMPAH by heparin.

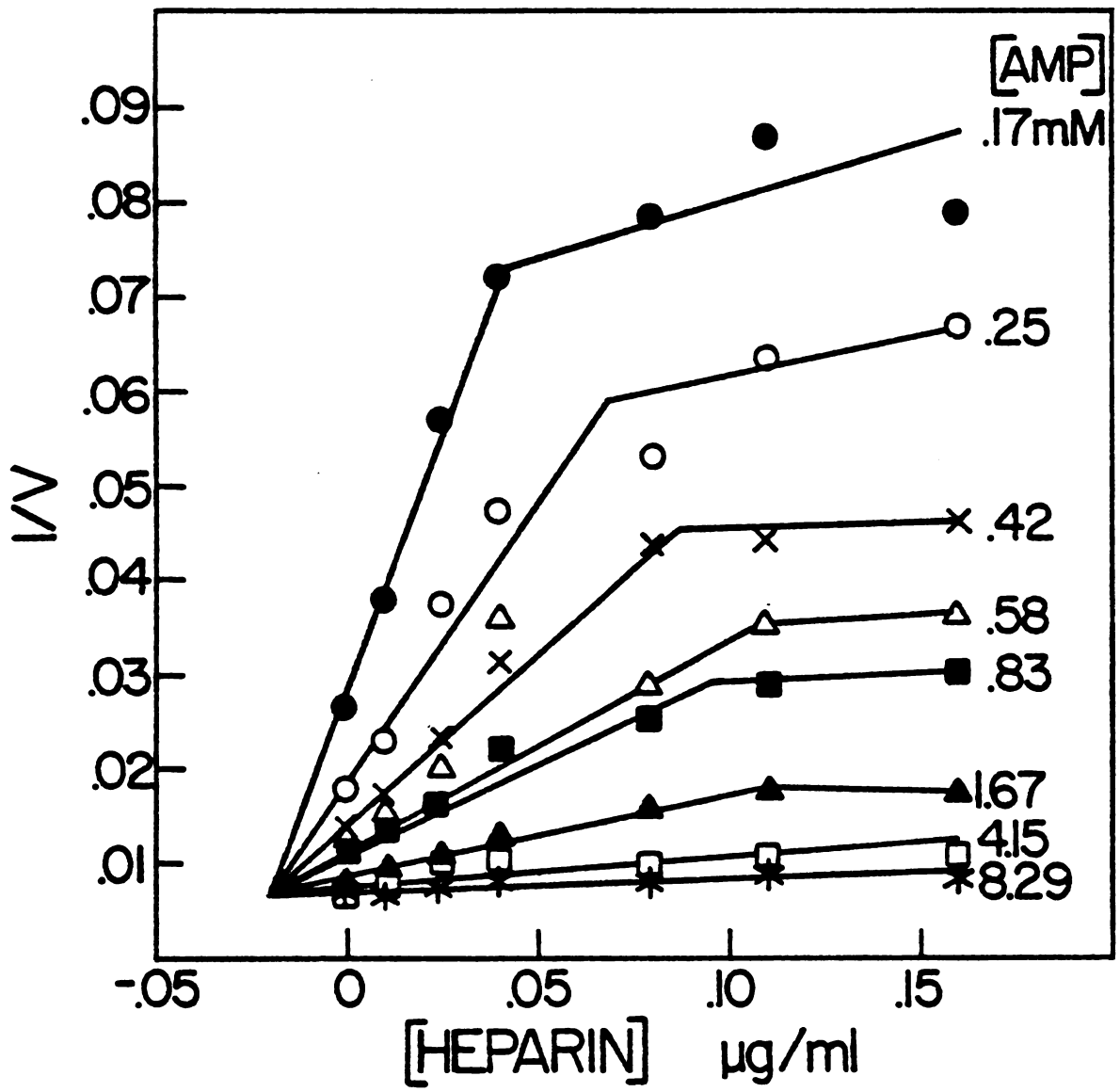
[AMP]
.17mM
.25
.42
.58
.83
1.67
4.15
8.29
1/V
.09
.08
.07
.06
.05
.04
.03
.02
.01
-.05
0
.05
.10
.15
[HEPARIN] µg/ml

Figure 30.

Figure 31. Elution of AMPAH from heparin-Sepharose 4B. AMPAH was applied to heparin-Sepharose 4B (●) or underivatized Sepharose 4B (○) as described in the Methods and eluted as indicated in the figure with either a KCl gradient of 0.15-1.2 M KCl, 1.2 M KCl, 1 mg ml^{-1} heparin, or 20 mM pyrophosphate, all in 50 mM MES-TRIS, pH 6.5. The heparin and pyrophosphate elution buffers also contained 0.15 M KCl.

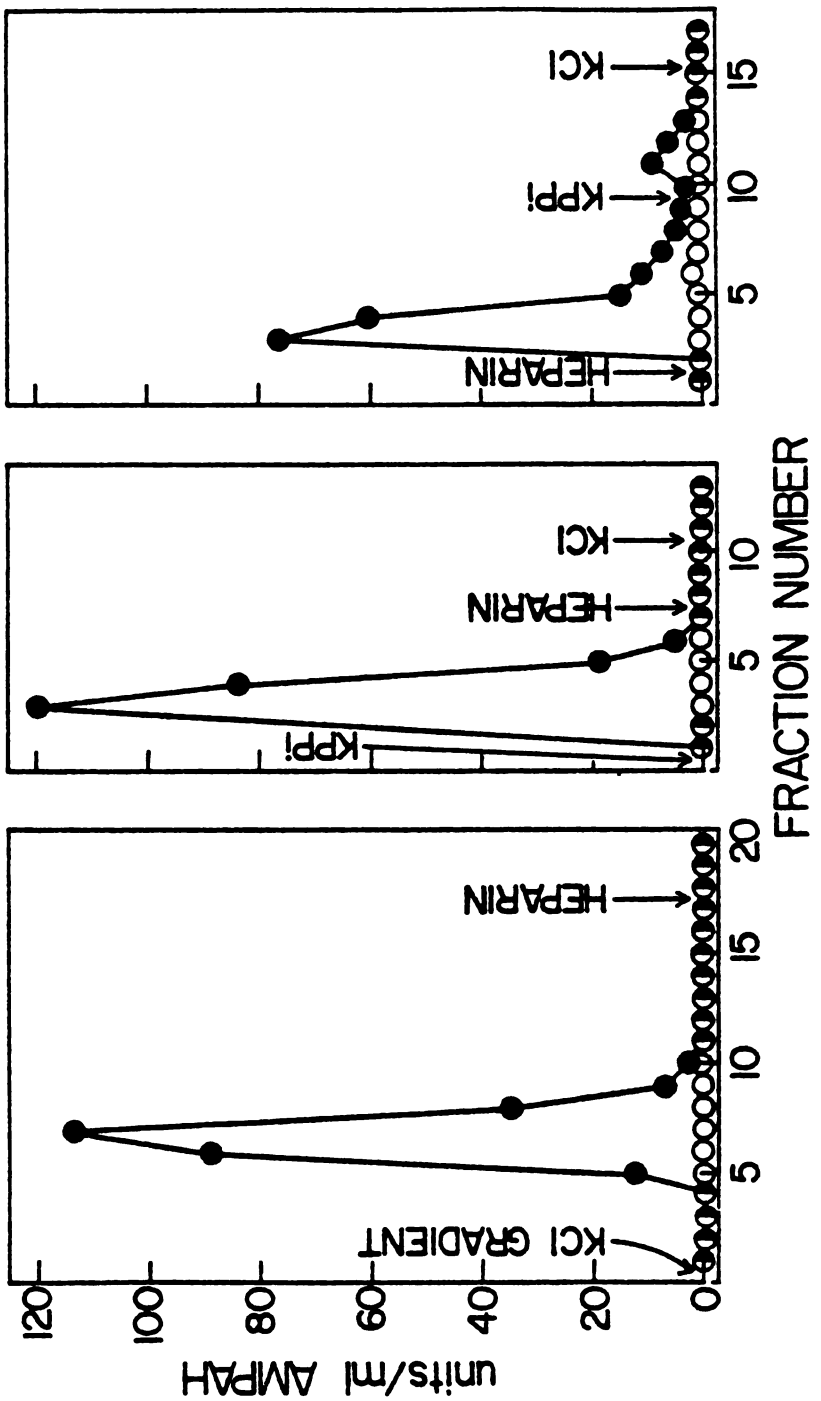
units/ml AMPAH
120
100
80
60
40
20
0
KCl GRADIENT
HEPARIN
KPPi
KCl
5
10
15
20
FRACTION NUMBER

Figure 31.

DISCUSSION

The interaction of ^{125}I-AMPAH with hepatocyte monolayers is inhibited by sulfated polysaccharides and effectors of AMPAH enzymatic activity. The interaction is most effectively inhibited by sulfated polysaccharides with the highest sulfate content. Sulfated polysaccharides inhibit AMPAH activity, and the interaction of AMPAH with heparin is further demonstrated by the binding of AMPAH to heparin-Sepharose 4B. The binding of allosteric effectors and sulfated polysaccharides to AMPAH may inhibit the interaction of the enzyme with hepatocytes by decreasing the affinity of AMPAH for cell surface binding sites. A similar hypothesis has been suggested for the heparin-releasable binding of lipoprotein lipase to endothelial cells (252) and low density lipoprotein to fibroblasts (274). However, the phosphate-containing polyanions RNA and polyphosphate inhibit AMPAH activity (Table 21), and RNA has been shown to associate with AMPAH (275), yet these polyanions are relatively poor inhibitors of the interaction of AMPAH with hepatocytes compared to the sulfated polysaccharides. The sulfated polysaccharide binding site(s) on AMPAH may be different from that for phosphate containing polyanions, and the inhibition of the interaction of AMPAH with hepatocytes may be more marked when sulfated polysaccharides are bound to the enzyme.

The demonstration that AMPAH has a high affinity for heparin suggests that the binding of AMPAH to hepatocytes may be due to the binding of

AMPAH to cell surface glycosaminoglycans. This is similar to the mechanism proposed for the binding of lipoprotein lipase to cultured endothelial cells (254). Ninomiya et al. (276) demonstrated that heparan sulfate is the major component of cell surface glycosaminoglycans synthesized by cultured rat liver parenchymal cells. Thus, sulfated polysaccharides may compete directly for the binding of AMPAH. Furthermore, the release of AMPAH bound to heparin-Sepharose 4B by pyrophosphate and KCl suggests that effectors of AMPAH activity and salts could release AMPAH bound to glycosaminoglycans on the cell surface.

The concentration dependence for the release of AMPAH bound to hepatocytes by substrate and effectors of AMPAH activity (Figure 26), shows GTP, ADP, and ATP are more effective than pyrophosphate and AMP in releasing AMPAH bound to hepatocytes. Ashby and Frieden (277) have proposed that AMPAH has three classes of nucleotide binding sites: 1) a high affinity inhibitory site which binds nucleotide triphosphates and pyrophosphate (271); 2) an activating site which binds nucleotide diphosphates and triphosphates with medium affinity and nucleotide monophosphates with a lower affinity; and 3) a substrate binding site which binds AMP. Though speculative, the observation that pyrophosphate and AMP are less effective than ATP, ADP, and GTP at releasing bound AMPAH from hepatocytes suggests that the interaction of nucleotides with the activating site of AMPAH may be primarily responsible for the release of bound AMPAH.

The lack of a significant specific inhibition of the binding by the carbohydrates shown in Table 20 indicates that AMPAH is not bound to hepatocytes by one of the carbohydrate recognition proteins of the liver, and that a different system is involved. Because the binding of

lipoprotein lipase to endothelial cells and low density lipoprotein to fibroblasts are also markedly inhibited by heparin and other sulfated polysaccharides, it would be of interest to determine whether these enzymes purified from the chicken have an effect on the binding of AMPAH to hepatocytes.

SUMMARY AND DISCUSSION

SUMMARY AND DISCUSSION

The levels of several muscle enzymes were examined in the blood plasma from normal and dystrophic chickens. The activities of creatine kinase and muscle pyruvate kinase are markedly elevated in the circulation of dystrophic chickens compared to normal chickens. However, the activities of AMPAH and adenylate kinase are not elevated. This pattern of elevation is essentially the same as is observed in human DMD and suggests that the study of those factors which determine the levels of muscle enzymes in dystrophic chickens may be relevant to the human condition.

The results in Chapter II demonstrate that AMPAH and adenylate kinase activity are rapidly lost from the circulation with half-lives of only a few minutes after the intravenous injection of the enzymes purified from normal chicken breast muscle. In contrast, the activities of pyruvate kinase and creatine kinase are lost with half-lives of several hours. Based on the estimated rate of efflux of pyruvate kinase from dystrophic muscle tissue, it was determined that the rapid circulatory clearance of AMPAH and adenylate kinase is sufficient to reduce the circulatory levels of these enzymes so that the activities are not significantly elevated in the circulation of dystrophic chickens as are the more slowly cleared proteins creatine kinase and pyruvate kinase. These results suggest that the circulatory clearance rates of muscle proteins in the serum may determine the levels of these proteins in the serum of dystrophic

chickens. It would be of interest to extend these experiments to determine whether there is a correlation between the circulatory clearance rates and the extent of elevation of some other muscle enzymes in avian muscular dystrophy.

Though these experiments demonstrate the ability of chickens to rapidly remove AMPAH and adenylate kinase from the circulation, they do not prove that these enzymes are released into the circulation from dystrophic muscle tissue as are the enzymes pyruvate kinase and creatine kinase. The examination of the activities of these enzymes in muscle press juices compared to muscle homogenates suggests that AMPAH, but not adenylate kinase, is associated with intracellular components to a significant extent. This extensive association could result in the retention of AMPAH within the dystrophic tissue and therefore retard the release of AMPAH into the circulation. Furthermore, the possibility can not be eliminated that adenylate kinase and AMPAH are retained in the muscle tissue because the sarcolemma is not permeable to these two proteins.

There are several experimental approaches that might verify the release of AMPAH and adenylate kinase into the circulation of dystrophic chickens. Using a sensitive AMPAH assay it may be possible to demonstrate arteriovenous differences in AMPAH activity across the breast muscle. AMPAH that is cleared from the circulation after intravenous injection is slowly internalized by the liver and spleen and can be released from the liver and spleen by heparin injection before internalization. It may be possible to demonstrate increased AMPAH activity in the circulation of dystrophic chickens after heparin injection that is due to enzyme which has been released from the dystrophic chicken muscle

and has been cleared but not yet internalized. Preliminary experiments demonstrated no apparent release of AMPAH into the circulation of normal or dystrophic chickens after heparin injection. However, the high ultraviolet absorbance of blood plasma at high sample concentrations interferes with the assay of AMPAH which monitors the increase in ultraviolet absorption at 290 nm or decrease in absorption at 265 nm as AMP is deaminated to form IMP. A more sensitive assay for AMPAH using radioactive substrate as described by Maguire and Aronson (278) may be advantageous to further studies of this type.

In the case of adenylate kinase, the examination of the isoenzyme profiles of the low levels of adenylate kinase activity in normal and dystrophic serum might reveal an increase in the level of the muscle isoenzyme in the serum of dystrophic chickens. The muscle isoenzyme can be distinguished from the isoenzymes from other tissues on the basis of inactivation of the muscle isoenzyme by sulfhydryl modifying reagents (222-224), electrophoretic mobility (68,222), or immune precipitation (68,279).

The rapid circulatory clearance of adenylate kinase activity may be due to enzyme inactivation caused by the oxidation of essential sulfhydryl residues of the enzyme. Adenylate kinase activity is rapidly lost when the enzyme is incubated in serum in vitro and the inactivation is prevented by adding dithiothreitol to the serum. Enzyme activity was not recovered in the primary tissue sites of clearance of ^{125}I-adenylate kinase shortly after clearance. The rate of clearance of ^{125}I-adenylate kinase was also rapid, indicating that the enzyme probably does not remain in the circulation in an inactive form. It may be possible to demonstrate a prolonged circulatory

clearance rate of adenylate kinase by the coinjection of a sulfhydryl compound such as penicillamine.

The process responsible for the rapid circulatory clearance of AMPAH was studied in detail. AMPAH activity is cleared with a half-life of only about 5 min and is recovered primarily by the liver and the spleen after intravenous injection of the purified enzyme. Using radioactively labeled AMPAH it was determined that cleared AMPAH is internalized and degraded in lysosomes in the liver and the spleen. Clearance is inhibited by heparin, but not by inhibitors of carbohydrate recognition systems of the liver which might recognize carbohydrate residues on the enzyme. Perhaps relevant is the observation that these same characteristics were observed for the circulatory clearance of intravenously injected lipoprotein lipase in rats (251). It would be of interest to purify lipoprotein lipase from chickens and to determine if AMPAH and lipoprotein lipase compete with one another for clearance in vivo and/or the binding to hepatocyte monolayers.

I have demonstrated that AMPAH binds to hepatocyte monolayers with a high affinity. As is observed for the in vivo clearance of AMPAH, the enzyme is internalized, is degraded, and the binding is inhibited by sulfated polysaccharides. These results, however, are indirect evidence that the binding of AMPAH to cultured hepatocytes is by the same process as that responsible for the rapid clearance of the enzyme in vivo. Further evidence might be obtained by the purification of the hepatic component which binds AMPAH at the cell surface. It would be of interest to determine the effect of the purified hepatic component, or antibodies against the component if it is a protein, on the binding of AMPAH to hepatocyte monolayers in vitro. However, initial attempts to

purify such a component from chicken liver were not successful. Crude membrane fractions from liver did not specifically bind AMPAH, even when thiol proteinase inhibitors were included during the preparation of the crude membrane fraction. Further trials under different conditions may be more successful.

The interaction of AMPAH with hepatocytes is markedly inhibited by molecules which bind AMPAH including effectors of AMPAH activity and sulfated polysaccharides. However, some molecules which bind AMPAH are less effective inhibitors of the interaction of AMPAH with hepatocytes. Heparin binds AMPAH tightly, as judged by a K_i for the inhibitor of AMPAH activity by heparin of 20 ng ml^{-1}, the association of AMPAH with heparin-Sepharose 4B, and the release of AMPAH bound to hepatocytes at heparin concentrations of 10 $\mu g\ ml^{-1}$ or less. It is possible that AMPAH binds a heparin-like molecule at the cell surface. It has been demonstrated that heparan sulfate is the major glycosaminoglycan on the cell surface of cultured rat hepatocytes (276). Recently, Cheng et al. (254) prepared an enzyme from human platelets which specifically degrades heparin and heparan sulfate. Treatment of endothelial cells with this preparation abolished the binding of lipoprotein lipase to these cells. An analogous experiment to determine whether treatment of hepatocytes with heparinase reduces the binding of AMPAH might help establish the basis for the binding of AMPAH. A preliminary experiment with a crude platelet heparinase purified as described by Oldberg et al. (280), resulted in a 35% reduction in the binding of ^{125}I-AMPAH to hepatocyte monolayers. However, the heparinase activity of this preparation was low, and some protease activity was apparent in the preparation.

The physiological significance of the rapid circulatory clearance is not clear. The rapid clearance of AMPAH from the circulation may be necessary to remove the enzyme from the circulation so that high circulatory levels of AMP are maintained. Though the circulatory levels of AMP and other nucleotides are low, it has been suggested that circulatory AMP may be an important source of purines in lymphocytes (281). Furthermore, nucleotides are potent vasodilators at physiological concentrations (282) and the relative levels of different nucleotides in circulation may be important in the regulation of blood flow. It would be of interest to determine with sensitive radioactive AMPAH assays, whether there is a steady-state level of AMPAH bound extracellularly that is released into the circulation by heparin injection that may play a role in the regulation of circulatory levels of AMP.

The results of the press juice experiments in Chapter II demonstrate that a large percentage of the AMPAH in muscle tissue exists in a bound form intracellularly and is not free in the cytoplasm of the muscle cell. If it is assumed that pyruvate kinase exists entirely as a soluble enzyme in the muscle cell, then by comparison of the ratios of the activities of these enzymes in the press juice to that in the crude homogenate, 78% of the AMPAH in the muscle exists in an intracellularly bound form. Ashby and Frieden (124) estimated that only 5-10% on the AMPAH in muscle exists in a complex with contractile elements in isolated myofibrils. It is possible that AMPAH is bound to cellular components other than myofibrillar proteins. Preliminary experiments demonstrated that AMPAH binds not only to hepatocytes in cell culture, but also with chick embryo fibroblasts and muscle cells. It would be of interest to determine whether there are binding sites for AMPAH on membranes within the muscle

cell analogous to the binding sites responsible for the binding of AMPAH to the cell surface. This binding might have a marked effect on the regulation of the activity of the enzyme intracellularly and on the role of AMPAH in the regulation of metabolism within muscle (283). The association of AMPAH with membranes within the muscle cell might explain the high extent of intracellular association of AMPAH. Similarly, Pipoly et al. (284) have demonstrated that human erythrocyte AMPAH binds to the cytoplasmic side of erythrocyte membrane ghosts, and that the binding is inhibited by nucleotide effectors of the enzyme and by salts.

In summary, these results provide for the first time an explanation based on experimental evidence, for the observations that some muscle proteins are not elevated in the serum of dystrophic animals. Based on the rates of circulatory clearance of muscle proteins, it was also possible to estimate the rate of efflux of creatine kinase and pyruvate kinase from dystrophic chicken muscle. These results are important in terms of describing the character of the proposed membrane defect in muscular dystrophy, and the role of this membrane defect in determining the serum and muscle levels of enzymes in muscular dystrophy.

The study of the rapid clearance of AMPAH was actively pursued because it has characteristics which differ from other mechanisms for the uptake of proteins from the circulation. This may describe a general mechanism for the specific binding and uptake of some proteins. The development of an in vitro method to study the binding and uptake of AMPAH will allow the elucidation of further details of the interaction of AMPAH with cells.

APPENDIX

APPENDIX: Papers, Abstracts, and Manuscripts in Preparation.

1. Suelter, C.H., Thompson, D., Oakley, G., Pearce, M., Husic, H.D., and Brody, M.S. (1979) Comparative Enzymology of 5'-AMP Aminohydrolase from Normal and Genetically Dystrophic Chicken Muscle. Biochem. Med. 21, 352-365.

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3. Husic, H.D. and Suelter, C.H. (1980) The Rapid Disappearance of Muscle AMP Aminohydrolase from the Blood Plasma of Normal and Genetically Dystrophic Chickens. Biochem. Biophys. Res. Commun. 95, 228-235.

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5. Husic, H.D. and Suelter, C.H. (1982) Circulatory Clearance of Muscle Enzymes from Normal and Dystrophic Chickens. Vth International Congress on Neuromuscular Diseases, Marseille, France (Abstract).

6. Husic, H.D. and Suelter, C.H. (1982) The Levels of Adenylate Kinase and Creatine Kinase in the Plasma of Dystrophic Chickens Reflect the Rates of Loss of these Enzymes from the Circulation. Biochem. Med., submitted for publication.

7. Husic, H.D. and Suelter, C.H. (1982) Circulatory Clearance, Uptake and Degradation of Muscle AMP Aminohydrolase. Manuscript in Preparation.

8. Husic, H.D. and Suelter, C.H. (1982) Internalization and Degradation of AMP Aminohydrolase Bound to Hepatocyte Monolayers. Manuscript in Preparation.

9. Husic, H.D., Baxter, J.H., Pearce, M., and Suelter, C.H. (1982) Comparative Enzymology Throughout the Development of Normal and Genetically Dystrophic Chickens. Manuscript in Preparation.

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