Effect of Hydrocortisone and Insulin on Uptake of α-Aminoisobutyric Acid by Isolated Perfused Rat Liver

JOHN W. CHAMBERS, RALPH H. GEORG, AND ALLAN D. BASS

Department of Pharmacology, Vanderbilt University, School of Medicine, Nashville, Tennessee

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SUMMARY

In this study both hydrocortisone and insulin were shown to increase the uptake of a nonmetabolizable amino acid, α-aminoisobutyric acid (AIB), by the isolated perfused rat liver. DNA-dependent RNA synthesis was inhibited by actinomycin D to determine whether the effects of these hormones on transport were independent of their actions on the transcription of genetic information. Actinomycin D inhibited enzyme induction by hydrocortisone approximately 90% without affecting the increase in AIB transport. However, approximately half of the insulin effect on AIB transport was blocked by actinomycin D. The effect of hydrocortisone on the uptake of AIB was completely inhibited by phenoxybenzamine (PBZ), an adrenergic blocking agent. The action of insulin on AIB uptake was not affected by PBZ. Hydrocortisone and insulin together exerted an additive effect on the hepatic uptake of AIB. Both hormones act directly (but apparently at different sites) to increase the AIB uptake by the liver. Most of the steroid action and approximately half of the insulin action appears to be independent of any effect these hormones have on DNA-dependent RNA synthesis in the liver.

INTRODUCTION

Although a number of the normal metabolic functions of mammalian tissues can be modified by hormones, the complexity of the intact animal is such that it is frequently difficult to determine the exact site or nature of the action of a hormone. A case in point is the apparent stimulation of hepatic amino acid uptake by hydrocortisone or insulin. Hydrocortisone, administered *in vivo*, enhances the rate of protein catabolism in muscle (1–4) and increases the concentration of amino acids in plasma (4) and in liver (1, 5). The extrahepatic effects of the steroid have been considered to be of major importance in this transfer of amino acids from muscle to liver (1, 2). Recently, however, the increased uptake of a nonmetabolizable amino acid, α-aminoisobutyric acid (AIB), was demonstrated to be the result of a direct action of the steroid on the liver (6). The evidence did not indicate whether the increased uptake represented a direct action by hydrocortisone on the amino acid transport system or was only a secondary response to the steroid's effect on amino acid utilization. Hydrocortisone is known to increase protein synthesis in the liver (1, 3). Some of the increased protein resulting from hydrocortisone administration represents an increase in the concentration of certain enzymes, and it is possible that the steroid increases AIB transport by inducing the formation of an enzyme or enzymes involved in amino acid transport. Greengard *et al.* (7) have suggested that hydrocortisone induces enzyme formation

by stimulating the production of the messenger RNA involved in the synthesis of the enzymes. These authors have shown that actinomycin D, which blocks the formation of RNA, also blocks the induction of enzymes by hydrocortisone.

In these studies we have attempted to determine whether the steroid-enhanced transport is dependent on increased enzyme synthesis. The effects of the steroid on transport of amino acid have been separated from its effects on enzyme induction by the inhibition of that induction with actinomycin D.

Insulin has been reported to increase the concentration of cycloleucine, another nonmetabolizable amino acid, in the rat liver *in vivo* (8, 9). The data suggested, however, that this action of insulin is not directly on the liver, but is mediated largely through epinephrine released from the adrenals in response to insulin-induced hypoglycemia. We have employed the isolated perfused rat liver to study the direct effects of insulin and epinephrine on hepatic amino acid uptake.

Insulin increases the synthesis of RNA in mammalian tissue, and this action has been suggested as a possible basis for many of the known metabolic effects of this hormone (10). RNA synthesis was inhibited with actinomycin D to determine whether the action of insulin on RNA synthesis is involved in the action of the hormone on amino acid transport.

Data obtained utilizing the isolated perfused rat liver indicate that both hydrocortisone and insulin increase the uptake of AIB by direct action on the liver. Apparently, the effect of hydrocortisone on AIB uptake is independent of the action of the hormone on enzyme induction. The action of insulin on transport may be related in part to the effect of insulin on RNA synthesis, but is apparently independent of any action of epinephrine. Epinephrine alone did not significantly affect AIB transport in the isolated rat liver.

METHODS

Isolated perfused livers from mature male Sprague-Dawley rats, weighing between 250 and 300 g and fed ad libitum, were used in these studies. While the rats were under ether anesthesia, the liver was exposed by means of a large U-shaped incision in the abdomen. Two hundred units of heparin was injected into the hepatic vena cava to prevent clot formation. The portal vein was cannulated, and oxygenated saline (0.85%) was passed through the liver for the duration of the operation. An exit cannula was placed in the thoracic vena cava, and the bile duct was cannulated for collection of bile. The liver was then freed from the surrounding tissue and placed in an enclosed perfusion system (11) maintained at a constant temperature of 37°. Total time for the operation was approximately 12 min. The perfusion media consisted of 40 ml of defibrinated rat blood, 60 ml Krebs-Henseleit buffer (pH 7.4) (12), 1 ml heparin (1000 units), and 250 mg glucose. Flow of the perfusate through the liver was maintained at approximately 2.3 ml/min per gram of liver. At the end of a 30-min equilibration period, 12 mg of AIB (containing 2 μc of AIB-1-^{14}C)[1] was added to the perfusate. After thorough mixing for 3 min, a 2-ml sample was removed from the perfusion fluid and sampling was continued at 15-min intervals during the first hour of the experiment and at 30-min intervals for the next 2 hr. A 1-ml aliquot of each sample was treated with 4 ml of absolute alcohol and centrifuged for 15 min. Of the supernatant, 2 ml was then transferred to glass vials containing 10 ml of an alcohol-phosphor solution.[2] These vials were placed in a Tracerlab LSC 10 B liquid scintillation counter, and the radioactivity was determined.

The disappearance of counts from the perfusate, with appropriate corrections for the counts removed by sampling, was used as a measure of the uptake of AIB by the

[1] α-Aminoisobutyric acid, AIB-1-^{14}C; New England Nuclear Corporation, Boston, Massachusetts.

[2] "Phosphor" scintillation fluid: 7 ml toluene, 3 ml absolute ethanol, 3.5 mg *p*-bis-2(5-phenyloxazolyl) benzene, 21 mg 2,5-diphenyloxazole.

liver. AIB is not metabolized by the liver (13); therefore, the radioactivity measured accurately represents the concentration of the AIB present. To further validate the use of the disappearance of radioactivity from the perfusate as a measure of AIB-uptake by the liver, 4 livers (2 controls and 2 hydrocortisone-infused) were perfused for 120 min with media containing AIB. The livers were then carefully weighed and a 1-g portion was homogenized in ice water. The protein in the homogenate was precipitated with barium hydroxide and zinc sulfate (14). After centrifugation, a 1-ml aliquot of the supernatant was transferred to the alcohol-phosphor scintillation fluid and the radioactivity was determined. The AIB content of the liver determined from the 1-g portion of tissue was then compared with the AIB content of the liver calculated from measurement of the decrease in radioactivity of the perfusate. More than 95% of the counts that disappeared from the perfusate were recovered from the liver. The concentration of AIB in the bile was also measured. An aliquot of the total bile was dried on a metal planchett, which was then placed in a Baird Atomic proportional gas flow counter; the radioactivity was determined. The bile contained less than 1% of the total AIB.

The activity of tryptophan pyrrolase, a hepatic enzyme which can be induced by hydrocortisone (15–17), was determined by the method of Knox and Auerbach (17). Portions of the liver (1 g each) were quick-frozen by immersion in alcohol and dry ice and stored at −18° until analyzed for enzyme activity.

In the infusion experiments 5 mg of hydrocortisone[3] was added to the perfusate 30 min before addition of AIB and a constant infusion of hydrocortisone (15 mg/hr) was continued for 2 hr. Since hydrocortisone is rapidly metabolized by the isolated liver (18), infusion of relatively large amounts of the steroid was necessary to maintain an effective concentration. The insulin[4] or glucagon-free insulin[5] (1 unit per 100 ml perfusate) was added to the perfusate simultaneously with AIB. Glucagon[6] was infused into the perfusate at the rate of 2 μg/hr throughout the experiment. Epinephrine[7] was infused into the perfusate for 3 hr, either at the rate of 0.6 mg/hr in the presence of phenoxybenzamine (PBZ), or at the rate of 0.06 mg/hr without the blocking agent. The lower infusion concentration was insufficient to significantly affect the flow of the perfusate through the liver. PBZ[8] (0.5 mg) was administered to the reservoir of the perfusion system at the end of the equilibration period.

The antibiotic actinomycin D[9] was employed as an inhibitor of RNA synthesis in these studies (19). A saline suspension of actinomycin D (1 mg/kg) was administered intraperitoneally to the liver donor rats 4 hr prior to sacrifice.

RESULTS

The influence of hydrocortisone on the uptake of AIB by the isolated liver is shown in Fig. 1. After 90 min perfusion, control livers had taken up 18% of the total AIB in the system. The concentration of AIB in the liver was approximately twice the concentration of AIB in the perfusate. Following the infusion of hydrocortisone, the ratio of concentration of AIB in the liver to that in the perfusate was approximately 3:1.

In initial experiments, it was observed that the time of addition of hydrocortisone

[3] Hydrocortisone sodium succinate, *Solu-Cortef;* Upjohn Company, Kalamazoo, Michigan.

[4] Insulin from zinc insulin crystals; Merck, Sharpe and Dohme, Philadelphia, Pennsylvania.

[5] Glucagon-free insulin (less than 0.0003% glucagon): Gift of Dr. I. Slater and Dr. Mary Root; Eli Lilly and Co., Indianapolis, Indiana.

[6] Glucagon, crystalline: Gift of Dr. Stanley Glasser, Vanderbilt University.

[7] Epinephrine bitartrate, USP; Mann Research Laboratory, New York, New York.

[8] Phenoxybenzamine hydrochloride, *Dibenzyline* (SKF 688-A); Smith, Kline and French, Philadelphia, Pennsylvania.

[9] Actinomycin D: Gift of Dr. Karl Beyer; Merck, Sharpe and Dohme, West Point, Pennsylvania.

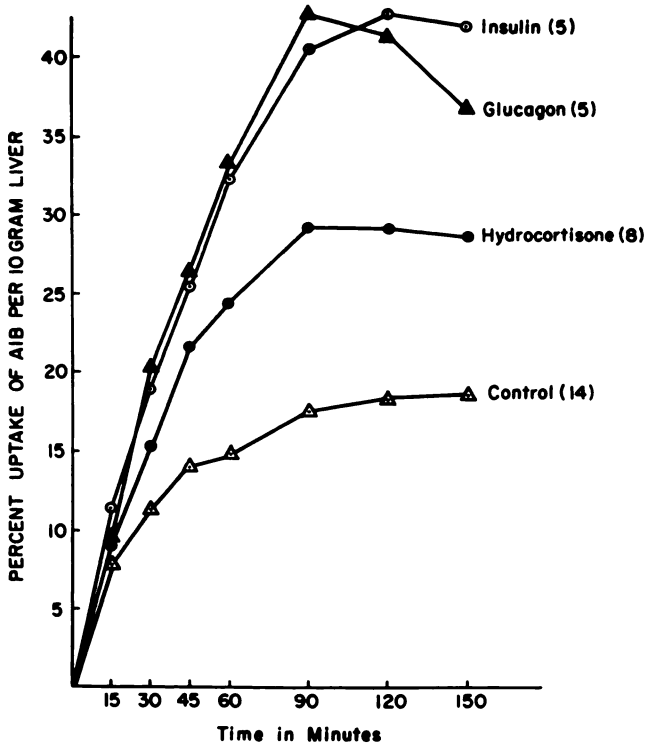

FIG. 1. *Endocrine influence on AIB uptake by isolated perfused rat livers*

The uptake of AIB is expressed as the percentage of total AIB in the system present in the liver at the time of sampling.

to the system was of considerable importance in obtaining the increased uptake of AIB. In addition to the methods used to obtain the data shown in Fig. 1, other variations in the time sequence of addition of hydrocortisone and AIB were employed. When infusion of hydrocortisone began at the same time AIB was added, there was a period of approximately 1 hr before any change in amino acid transport was detectable. However, if infusion of the steroid began 30 min before addition of AIB, an increase in the rate of uptake of the amino acid occurred within 30 min after introduction of AIB. When livers were perfused first with hydrocortisone in the media for 1 hr, and then with perfusate containing only AIB (no steroid), the uptake of AIB was comparable to that obtained with both steroid and amino acid in the perfusate (*in vitro* induction with hydrocortisone, Table 1). A minimum of 1 hr of contact with hydrocortisone was required for optimum increase in AIB uptake. When hydrocortisone had been in the system for 1 hr, its effect on AIB uptake clearly persisted even after the steroid had been removed from the perfusate. These observations suggested that the steroid was inducing the formation of some transport intermediate, perhaps even the amino acid carrier itself. Hydrocortisone is known to induce several hepatic enzymes, among them tryptophan pyrrolase (15–17). Although tryptophan pyrrolase is probably not involved in transport, it was selected as a representative induced enzyme because of the similarities in the pattern of stimulation of AIB uptake and the induction of this enzyme by hydrocortisone. The time required for optimum induction of tryptophan pyrrolase by hydrocortisone was approximately the same as that required by the steroid for the optimum

TABLE 1

Effect of hormones on AIB uptake, bile production, and tryptophan pyrrolase activity[a]

Type of experiment[b]	% AIB uptake per 10 g liver		Bile production (ml/2 hr/10 g liver)	Relative tryptophan pyrrolase activity
	90 min	120 min		
Normal control (14)[c]	17.6 ± 0.8	18.3 ± 0.7	1.13 ± 0.19	1.00
Hydrocortisone (8)	29.2* ± 1.3	29.2* ± 0.8	2.10* ± 0.22	2.10*
Insulin (5)	40.3* ± 4.7	42.5* ± 4.1	1.38 ± 0.11	1.14
Glucagon-free insulin (3)	43.8* ± 1.4	44.8* ± 3.7	1.78* ± 0.18	0.94
Glucagon (5)	42.1* ± 4.5	41.2* ± 4.1	1.20 ± 0.13	0.75
Hydrocortisone + insulin (4)	47.7* ± 0.7	50.5* ± 1.1	2.64* ± 0.17	—
Actinomycin D control (4)	19.0 ± 1.9	21.7 ± 2.2	1.18 ± 0.16	0.44
Actinomycin D + hydrocortisone (4)	29.6* ± 3.2	30.1* ± 3.0	1.85* ± 0.30	0.57
Actinomycin D + insulin (4)	27.2* ± 3.3	29.4* ± 3.9	1.34 ± 0.19	0.42
Phenoxybenzamine control (7)	19.6 ± 0.7	19.4 ± 0.6	1.21 ± 0.18	1.42
Phenoxybenzamine + insulin (5)	37.9* ± 2.1	39.8* ± 2.5	1.52* ± 0.18	—
Phenoxybenzamine + hydrocortisone (6)	19.8 ± 1.4	19.6 ± 0.6	2.17* ± 0.22	2.23*
Ergotamine control (5)	19.0 ± 0.6	21.5 ± 1.0	0.95 ± 0.05	—
Ergotamine + hydrocortisone (4)	26.9* ± 2.1	29.1* ± 2.2	1.95* ± 0.22	—
Adrenalectomized control (4)	17.2 ± 1.4	18.8 ± 2.9	1.05 ± 0.14	—
Adrenalectomized + insulin (4)	33.2* ± 3.2	38.5* ± 5.1	1.04 ± 0.01	—
Epinephrine control (4)	18.9 ± 3.6	18.5 ± 3.3	0.88 ± 0.14	—
Epinephrine + phenoxybenzamine (6)	21.9 ± 1.0	21.8 ± 0.8	0.94 ± 0.06	—
In vitro induction control (3)	20.5 ± 0.8	19.3 ± 1.3	1.19 ± 0.06	—
In vitro induction with hydrocortisone (7)	23.9 ± 1.4	26.8* ± 2.0	1.52* ± 0.05	—

[a] All values are means ± S.E. The differences between experimental and control values that are significant at the 5% level are indicated by an asterisk.

[b] For drug doses, preparations, and methods of administration see Methods.

[c] Figures in parentheses represent the number of experiments.

effect on AIB transport (1–2 hr). The increased enzyme activity and the increased AIB uptake continued after hydrocortisone had been removed from the perfusate. If actinomycin D (which inhibits the steroid induction of tryptophan pyrrolase) could be shown to inhibit the stimulation of AIB uptake, this would be further evidence to support the concept that hydrocortisone acts through the induction of a transport intermediate of a protein nature. However, when the effects of hydrocortisone were studied in livers from actinomycin D-pretreated animals, the induction of tryptophan pyrrolase was significantly inhibited (Table 1), but there was no evidence that actinomycin D modified the increased AIB uptake (Fig. 2). It appeared from these observations that there was no direct correlation between the effect of hydrocortisone on enzyme induction and the effect of the hormone on amino acid transport.

Following the demonstration of an action by hydrocortisone on AIB uptake, it was of interest to observe the effect of adrenalectomy on AIB transport in the liver. For this purpose, livers from rats which had been adrenalectomized 12 days previously were employed. The data in Table 1 demonstrate that there is no modification of AIB uptake in livers from these rats. Moreover, infusion of epinephrine into the perfusate at a rate which did not affect the hepatic vasculature (0.06 mg/hr) had no effect on AIB transport (Table 1). When higher concentrations of epinephrine (0.6 mg/hr) were employed, it was necessary to add phenoxybenzamine to

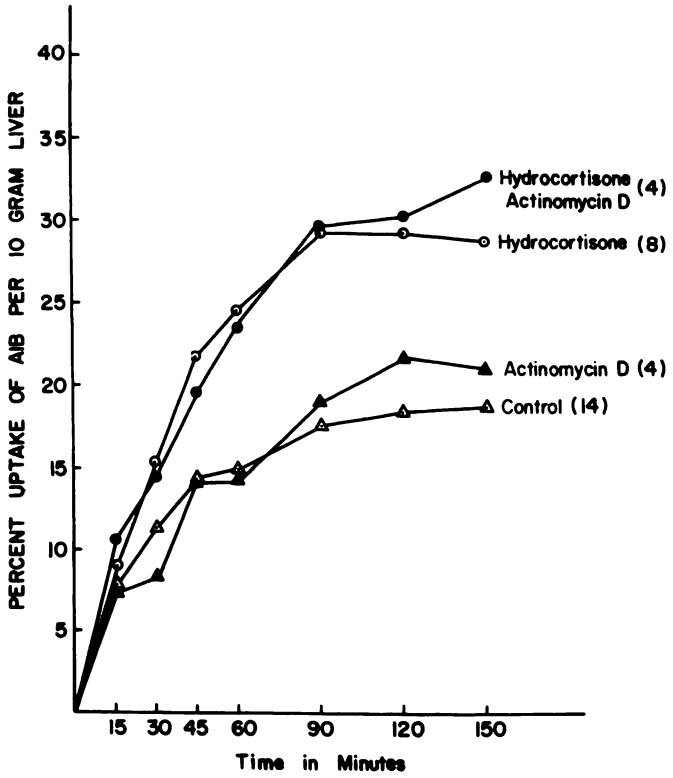

FIG. 2. *Hydrocortisone influence on AIB uptake by the isolated perfused liver from actinomycin D-pretreated rats*

The uptake of AIB is expressed as the percentage of total AIB in the system present in the liver at the time of sampling.

prevent vasoconstriction in the liver. Under these conditions epinephrine did not affect AIB uptake (Table 1). PBZ was found to block the increase in AIB uptake caused by hydrocortisone (Fig. 3) whereas PBZ alone had no effect on AIB transport. Apparently, this inhibition of the steroid effect on AIB transport is not a general property of adrenergic blockers; ergotamine tartrate[10] (0.5 mg), another adrenergic blocker, had no effect on the stimulation of AIB uptake by hydrocortisone (Table 1).

The effects of insulin administration on AIB uptake by the isolated perfused liver are seen in Fig. 1. The addition of 1 unit of insulin to the perfusate increased the uptake of AIB by the isolated liver more than 100%. Two hours after the addition of insulin the concentration of AIB in the liver was approximately 6 times as great as the concentration in the perfusate.

The crystalline insulin used in initial experiments was a commercial preparation contaminated with small quantities of glucagon. To eliminate the possibility that the effect of the insulin was due to the glucagon present, studies were made on AIB transport employing glucagon and glucagon-free insulin. Glucagon-free insulin proved to be as effective as the commercial crystalline insulin (Table 1). Glucagon (2 μg/hr) when infused into the isolated liver also increased AIB uptake to the same degree as did insulin (Fig. 1).

Sanders and Riggs reported that adrenal-

[10] Ergotamine tartrate, *Gynergen;* Sandoz, New York.

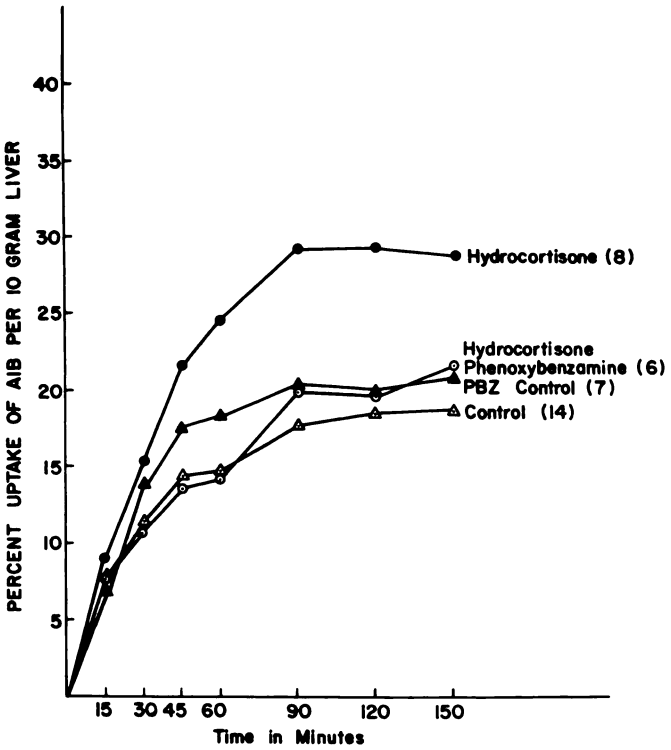

FIG. 3. *Hydrocortisone influence on AIB uptake by the isolated perfused rat liver in the presence of phenoxybenzamine*

The uptake of AIB is expressed as the percentage of total AIB in the system present in the liver at the time of sampling.

ectomy abolished 90% of the action of insulin on cycloleucine uptake by the liver (9). These investigators suggested that the effects of insulin on cycloleucine uptake are not directly on the liver, but mediated largely through epinephrine released from the adrenal gland. The isolated liver eliminates the adrenals as a source of epinephrine; however, blood and livers from adrenalectomized rats were employed to further reduce the levels of catecholamines in the system. The effect of insulin on AIB uptake by the isolated liver was not altered by adrenalectomy of the donor rats (Table 1). Although epinephrine may be essential for the action of insulin on cycloleucine uptake by the liver *in vivo,* in the isolated liver insulin alone increased the uptake of AIB.

To determine whether the action of insulin on AIB transport was dependent upon a modification of RNA synthesis, the effects of insulin were studied using livers from actinomycin D-pretreated rats. Under these conditions, the increase in AIB uptake was partially inhibited (Fig. 4). This observation suggests that the action of insulin on AIB transport is, in part, dependent on the action of the hormone on RNA production in the liver. However, a significant increase in AIB transport was still obtained with insulin in the presence of inhibited RNA synthesis.

The actions of insulin and hydrocortisone in this system appear to be qualitatively similar. However, these hormones probably act at different sites or by different mechanisms. This is suggested by the observations that PBZ will block the action of hydrocortisone but not that of insulin (Fig. 5), and that actinomycin D pretreatment partially modifies the action

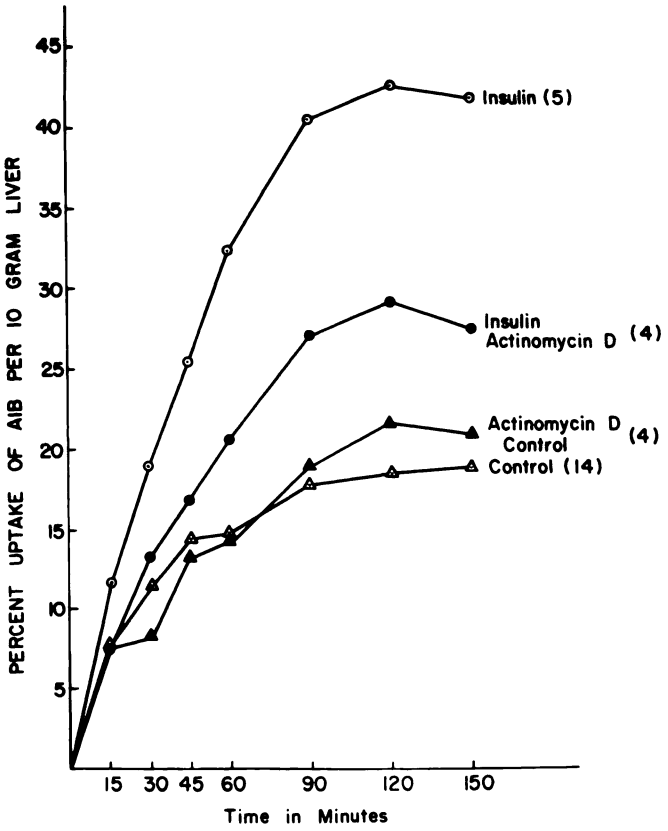

FIG. 4. *Insulin influence on AIB uptake by the isolated perfused rat liver from actinomycin D pretreated rats*

The uptake of AIB is expressed as the percentage of total AIB in the system present in the liver at the time of sampling.

of insulin on AIB transport but does not affect the action of hydrocortisone. Further correlative support for the concept of separate sites of action might be obtained by determining the increase in AIB transport when the two hormones are present simultaneously in the system. Since hydrocortisone is present in its maximally effective concentration, insulin would be expected to exert an additional effect on amino acid transport if separate sites or mechanisms are involved in the action of the two hormones. Data presented in Fig. 6 indicate that the effects of the two hormones on AIB uptake are additive.

Bile production was increased almost 100% by hydrocortisone infusion (Table 1). In these experiments, neither insulin nor hydrocortisone significantly altered the rate of flow of the perfusate through the liver.

DISCUSSION

Hydrocortisone has been shown to increase the uptake of AIB by the isolated perfused rat liver under conditions where the extraneous influence of other hormones and the extrahepatic actions of hydrocortisone were minimal (6). In the present investigation secondary effects on hepatic amino acid transport, resulting from the action of hydrocortisone on intracellular metabolism, were reduced by the use of an inhibitor of enzyme induction and a nonmetabolizable amino acid, AIB. Increased amino acid uptake in the presence

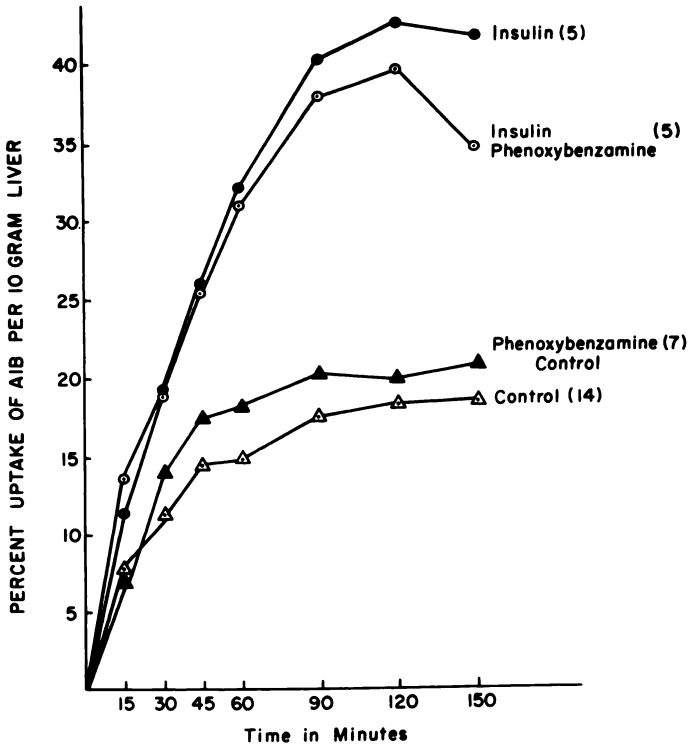

FIG. 5. *Insulin influence on AIB uptake by the isolated perfused rat liver in the presence of phenoxybenzamine*

The uptake of AIB is expressed as the percentage of total AIB in the system present in the liver at the time of sampling.

of inhibited synthesis of enzyme implies that hydrocortisone does not enhance AIB uptake by inducing the formation of an enzyme involved in amino acid transport. Therefore, the increased uptake of AIB may be the result of a direct action of the steroid on the transport system for amino acids. The exact nature of this transport system is not known, and it is difficult to postulate a precise mode of action for the steroid in the stimulation of hepatic amino acid uptake.

Phenoxybenzamine, an adrenergic blocking agent, inhibits the effect of the steroid on transport, but does not block the increased induction of tryptophan pyrrolase by hydrocortisone. This is further evidence that the increased amino acid transport and enzyme induction are independent actions of the steroid. The inhibition by PBZ of the action of hydrocortisone on transport probably indicates that the steroid effect does not depend on an action of epinephrine because another adrenergic blocking agent, ergotamine, did not inhibit this effect and hydrocortisone is effective in adrenalectomized rats (6).

Insulin has been shown in this study to increase AIB uptake by the isolated perfused rat liver. Sanders and Riggs (9) have suggested that the effect of insulin *in vivo* on amino acid uptake by the liver is indirect, mediated to a large extent by epinephrine. Epinephrine, in our system, did not significantly alter AIB uptake. Insulin, however, increased the uptake more than 100%, indicating that this hormone does act directly on the liver. Apparently, a part of the increased uptake was dependent on the action of insulin on RNA synthesis, since 50% of the insulin effect on hepatic AIB uptake was inhibited by blockade of

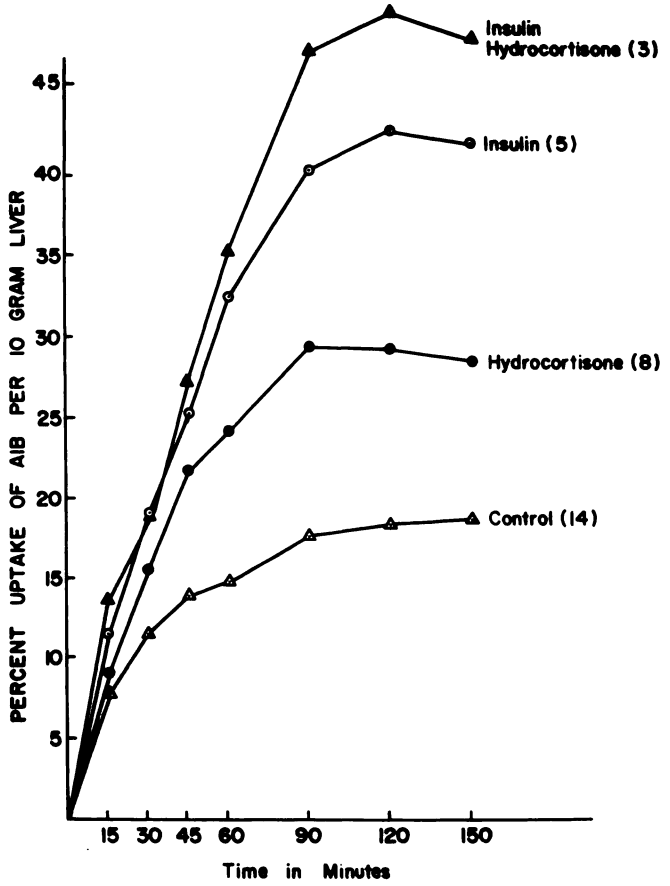

FIG. 6. *Insulin and hydrocortisone influence on AIB uptake by the isolated perfused rat liver*

The uptake of AIB is expressed as the percentage of total AIB in the system present in the liver at the time of sampling.

RNA production. However, in the presence of inhibited RNA synthesis there remained a significant increase in transport, suggesting that possibly there are two mechanisms by which insulin increases the hepatic uptake of AIB. Akedo and Christensen (20) presented evidence that, in the isolated diaphragm, insulin increases the apparent affinity of the transport site for AIB. An action of insulin which modifies the efficiency of the transport system in this manner would account for that portion of the increased AIB transport not affected by the blockade of RNA synthesis.

Both hydrocortisone and insulin appear to act in some degree on the amino acid transport system. The action of insulin differs from the action of hydrocortisone in that it is not blocked by PBZ, suggesting that hydrocortisone and insulin modify AIB uptake at different sites or by different mechanisms. This concept is further supported by the demonstration that the effects of the two hormones on AIB transport are additive.

The concentrations of hormones used in our system were somewhat greater than those encountered physiologically (21). However, this may not be significant in the case of hydrocortisone, as the metabolism of that hormone is much more rapid in the *in vitro* system than in the intact animal (18), and the effective levels of the steroid represent only a small percentage of the total hormone added.

Under the conditions of this study, hy-

drocortisone and insulin modify amino acid transport by direct action on the liver. Apparently, the effects of hydrocortisone on enzyme induction by means of increased RNA synthesis are not involved in the stimulation of amino acid transport. However, with insulin both a direct effect on the transport system and an indirect effect resulting from the action of the insulin on RNA synthesis may be involved.

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