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# STUDIES OF ISOLATED PERFUSED RAT LIVERS—I
## EFFECT OF ARGININE AND GLUTAMATE ON AMMONIA METABOLISM IN NORMAL, FATTY, AND PRECANCEROUS LIVERS

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**Abstract**—Arginine or arginine–glutamate was shown to assist isolated perfused normal rat livers and experimentally produced pathological livers in detoxifying administered ammonia. This detoxification was reflected both in removal of ammonia from the perfusate and in the stimulation of urea production.

In general, fatty livers and azo dye-fed livers were not as efficient as normal livers in producing urea from ammonium salts, amino acids, or combinations of these supplements placed in the blood perfusate. Glutamate when added to the perfusate of fatty livers did not elevate urea as in normal and precancerous livers.

AMINO acids have been used extensively as a therapeutic measure to combat ammoniagenic coma in patients with liver disease. Najarian and Harper[1] successfully treated patients in hepatic failure with parenteral arginine administration and Walshe[2] and others have shown beneficial results with parenteral administration of sodium glutamate in patients with severe liver disease.

In experimental animals, Greenstein and co-workers[3] showed that arginine is specific among the amino acids in protecting against lethal doses of injected ammonia.

Barak *et al.*[4] have shown that parenteral infusion of glutamate or arginine increases the efficiency of the ammonia detoxifying systems in experimentally induced hyperammonemia in dogs.

All the above reports have merely demonstrated what generally transpires in the intact organism and leave unanswered the question whether the amino acids produce their effect in the liver or in extrahepatic organs or tissues. In this study the effect of arginine and glutamate on ammonia metabolism in the normal isolated perfused rat liver was investigated. It was felt that the isolated perfused liver technique applied in this case would not only establish the site of action of these amino acids as existing in normal liver but could also determine whether these same amino acids are beneficial to the ammonia-detoxifying mechanisms in fatty and precancerous livers.

## METHODS AND MATERIALS

The surgical and liver perfusion procedures used in this study were essentially those of Miller *et al.*[5] All perfusions were carried out for 4-hr periods with freshly drawn heparinized rat blood that was diluted 1:1 with sterile saline. A volume of 130 ml

diluted blood was used in the perfusions, and 2-ml aliquots were drawn serially from the perfusate for pH, blood ammonia, and urea nitrogen determinations. Perfusate withdrawn for serial analyses was replaced with saline in order to keep the volume of perfusate constant throughout the experiment. Weights were obtained on livers immediately after 4-hr perfusion.

Adequacy of liver perfusions in this study were indicated by both bile flow and blood flow rates. In each experiment the bile flow was noted, but the rate of flow was not measured. Between 2 and 3 ml bile was collected in each 4-hr experiment. Blood flow rates varied in all types of livers studied from 25 ml to 34 ml per min. It was necessary to fortify the perfusates of the fatty livers with an extra 10,000 units of heparin at the 2-hr period in order to prevent formation of clots on the perfusate filters.

Ammonia metabolism studies were conducted in normal, fatty, and azo dye-fed livers by the addition of various metabolites to the perfusates. Control experiments in which no supplement was added to the perfusate were performed on each type of liver. All supplement infusions into the perfusion system were made instantaneously by adding the compounds in solution directly to the blood reservoir. Ammonium acetate, arginine–glutamate (A–G), arginine, and glutamate when infused were added in the amounts of 6 m-moles/kg body weight of the rat donating the rat liver. In those experiments in which two different compounds were added to the perfusion medium, they were dissolved together in the same single solution and added concomitantly. The perfusing medium was maintained between pH 7·1 and 7·2 at all times because this is the pH range of portal vein blood in the rat. Because of the continuous outflow of organic acids by the liver during perfusion, it was necessary to add small amounts of sodium bicarbonate to the perfusion medium periodically to maintain the pH. Only Sprague-Dawley rats were used in the study. Normal rats were fed a commercial diet (Rockland rat pellets) and water *ad libitum*. Dietary fatty livers were produced in rats by placing them for 21 days or longer on a high-fat, low-choline diet similar to that used by Humoller and Zimmerman.[6] Azo dye-fed rats were prepared according to the method described by Burke and Miller.[7] Rats that were liver donors for perfusion were all fasted 16 hr before the experiment. Rats sacrificed as blood donors were not fasted.

Blood ammonia determinations were carried out by the method of Humoller *et al.*,[8] and blood urea nitrogen was determined by the urease-iodophenol method of Searcy *et al.*[9] Liver fat determinations were carried out by the method of Humoller *et al.*,[10] and percentage of dried fat-free tissue (FFT) was determined by drying to constant weight the tissue residue left after extraction of the lipid material. Percentage of dried fat-free tissue was determined for each individual liver.

For the sake of comparing the results obtained in normal livers, fatty livers, and azo-dye livers, the data have been expressed in terms of μmoles ammonia removed in 1 hr or urea nitrogen produced in 4 hr per total livers, per g liver wet weight and per g of dried fat-free tissue. Results of ammonia removed were reported on a 1-hr basis, because the majority of added ammonium ion is taken up by all types of livers studied between the first and second hour of perfusion. Efficiencies of livers were compared on this hourly basis.

The statistical method used in this study was the *t* test for determining the difference between means from normal populations.

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sigma \sqrt{1/N_1 + 1/N_2}}$$

$$\text{where } \sigma = \sqrt{\frac{N_1 S_1^2 + N_2 S_2^2}{N_1 + N_2 - 2}}$$

A 90 per cent confidence interval was selected as ordinarily being appropriate for biological data of this nature. The same statistical method was applied in both the ammonia removal parameter and the urea production parameter of this study. As is common with liver perfusion experiments, time was the limiting factor in planning the number of observations which were to be made in each category. Perfusion failures in both the normal and azo dye-fed livers with the supplement of arginine-glutamate and ammonium acetate limited the observations to two in each case. The ammonia removal data in these experiments were deemed to be of value, however. These individual observations when compared to the distributions of their counterpart (the ammonium acetate supplement) were seen to fall at approximately the mean plus 2 standard deviations. If, in fact, the observations were a part of the ammonium acetate supplement population, then one should expect to obtain such observations only 5 per cent of the time. It therefore seems appropriate to conclude that the A–G- and ammonium acetate-treated populations are different from the ammonium acetate-treated population even though it cannot be so concluded at the preselected 90 per cent confidence interval.

## RESULTS

Fatty livers in this study ranged from 10 to 46 per cent total fat on a wet weight basis. After 3 weeks on the high-fat diet those rats on the diet the longest period of time showed the least amount of fat in the liver. This is in accord with the findings of Zaki *et al.*,[11] who reported a decrease in liver fat and an increasing cirrhosis in rats after a 3-week period on a high-fat, choline-deficient diet. The dried fat-free tissue of normal livers comprises 28·4 per cent of the wet weight of the liver. Fatty livers averaged 14·3% FFT, and azo dye-fed livers averaged 24·5%.

### 1. *Ammonia removal*

It can be seen from Table 1 that when the quantity of ammonia removed from the perfusate by the liver is expressed in terms of total liver or per liver wet weight, the normal liver appears to be about twice as efficient as fatty livers and precancerous livers. However, when the removal of ammonia is recorded in terms of per g FFT, entirely different results are obtained. In this case the fatty livers were as efficient as normal livers, but the dye-fed livers were not. In terms of ammonia removed per g FFT, all three types of livers utilized arginine to remove ammonia from the blood perfusate, but the normal liver was more capable than the other livers.

Although only two observations were made, it appeared that ammonia removed with arginine–glutamate was equivalent to that obtained with arginine in normal livers. Likewise, although only two experiments were obtained with the azo dye-fed livers, it appeared that these livers were more capable of utilizing A–G to remove added ammonium ion than were fatty livers, which demonstrated only a borderline ability in this regard.

In studying the effect of infusion of sodium glutamate on ammonia removal, it was shown (Table 1) that this amino acid was not as effective on the isolated livers as were arginine or arginine–glutamate. On a statistical basis, sodium glutamate did not increase the ability of any of the livers to remove added ammonium ion from the perfusate.

TABLE 1. AVERAGE AMMONIA REMOVED BY PERFUSED RATE LIVERS IN ONE HOUR[a]

| Supplement | No. of expt. | Per total liver (μmoles/130 ml) | Per g liver wet weight (μmoles/130 ml) | Per g FFT (μmoles/130 ml) | Significance of difference of removal over $NH_4Ac$. expt., $t$ test[b] | | |
|---|---|---|---|---|---|---|---|
| | | | | | $t_{90}$ | $t$ | |
| **Normal livers** | | | | | | | |
| $NH_4$ Acetate[c] | 4 | 1418[d] (1040–2057) | 133 (100–182) | 469 (350–646) | | | |
| Arginine[e] + $NH_4$ acetate[c] | 4 | 2054 (1861–2312) | 220 (194–248) | 769 (673–836) | 1·44 | 3·71 | sig. |
| Arginine glut.[f] + $NH_4$ acetate[c] | 2 | 2597 (2353–2841) | 244 (220–253) | 859 (831–887) | too few observations | | |
| Na Glut.[g] + $NH_4$ acetate[c] | 3 | 1388 (1244–1508) | 143 (141–145) | 506 (498–512) | 1·48 | 0·43 | not sig. |
| **Fatty livers** | | | | | | | |
| $NH_4$ Acetate[c] | 6 | 702 (361–1202) | 60 (32–89) | 405 (99–853) | | | |
| Arginine[e] + $NH_4$ acetate[c] | 4 | 1242 (915–1472) | 103 (74–148) | 662 (548–760) | 1·40 | 1·94 | sig. |
| Arginine glut.[f] + $NH_4$ acetate[c] | 6 | 830 (729–1266) | 77 (50–121) | 580 (383–842) | 1·37 | 1·33 | bord. |
| Na Glut.[g] + $NH_4$ acetate[c] | | 744 (607–923) | 72 (64–80) | 594 (564–645) | 1·42 | 1.25 | not sig. |
| **Azo diet livers** | | | | | | | |
| $NH_4$ Acetate[c] | 3 | 895 (440–1480) | 80 (54–130) | 318 (216–516) | | | |
| Arginine[e] + $NH_4$ acetate[c] | 3 | 1523 (951–2042) | 144 (86–221) | 613 (400–875) | 1·53 | 1·73 | sig. |
| Arginine glut.[f] + $NH_4$ acetate[c] | 2 | 2135 (1983–2288) | 158 (153–163) | 625 (605–645) | too few observations | | |
| Na Glut.[g] + $NH_4$ acetate[c] | 5 | 1232 (478–2203) | 109 (63–154) | 420 (235–608) | 1·44 | 0·84 | not sig. |

[a] Expressed in μmoles $NH_3N$.
[b] $t$-Test refers to comparison of values per gram FFT; "sig." indicates significant difference at the 90% confidence interval; "bord." indicates borderline, cannot be called significant at the 90% confidence interval.
[c] Ammonium acetate, 6 m-moles/kg body weight of donor rat.
[d] Figures in parentheses give the range of values obtained.
[e] Arginine base 6 m-moles/kg body weight of donor rat.
[f] Arginine glutamate 6 m-moles/kg body weight of donor rat.
[g] Glutamate 6 m-moles/kg body weight of donor rat.

2. *Urea production*

Removal of ammonia from the circulating blood in itself is not a valid test for liver metabolism of ammonia; consequently, the ability of these livers to produce urea was also studied. Under the conditions of the study about 80 per cent of added ammonia nitrogen was recovered as urea nitrogen in 4-hr perfusion with normal livers, 71 per cent with fatty livers, and 78 per cent with precancerous livers.

TABLE 2. AVERAGE UREA NITROGEN PRODUCTION BY PERFUSED RAT LIVERS IN FOUR HOURS*

| Supplement | No. of expt. | Per total liver (μmoles/130 ml) | Per g liver wet weight (μmoles/130 ml) | Per g FFT (μmoles/130 ml) |
|---|---|---|---|---|
| **Normal livers** | | | | |
| Control | 3 | 679 (396–947) | 78 (59–109) | 270 (208–379) |
| $NH_4$ acetate | 4 | 2689 (2544–2817) | 287 (231–396) | 889 (806–943) |
| Arginine | 3 | 5939 (5033–6535) | 677 (579–819) | 2370 (2112–2871) |
| Arginine + $NH_4$ acetate | 5 | 6509 (5417–7576) | 681 (637–750) | 2388 (2217–2585) |
| Arginine glutamate | 5 | 6033 (4512–7865) | 608 (270–811) | 2138 (1631–2874) |
| Arg. glut. + $NH_4$ acetate | 2 | 7937 (7521–8356) | 752 (696–808) | 2639 (2442–2836) |
| Na glutamate | 4 | 1241 (964–1885) | 155 (115–237) | 418 (310–642) |
| Na glut. + $NH_4$ acetate | 3 | 2386 (2249–2525) | 247 (215–270) | 870 (761–955) |
| **Fatty livers** | | | | |
| Control | 4 | 219 (56–371) | 22 (5–35) | 110 (32-157) |
| $NH_4$ acetate | 6 | 995 (715–1392) | 89 (54–149) | 607 (400–697) |
| Arginine | 3 | 1519 (1281–1903) | 149 (104–180) | 872 (603–1306) |
| Arginine + $NH_4$ acetate | 5 | 3432 (2581–4680) | 296 (189–383) | 1647 (1139–1908) |
| Arginine glutamate | 3 | 1435 (965–1968) | 107 (72–127) | 839 (588–1112) |
| Arg. glut. + $NH_4$ acetate | 6 | 2883 (2228–4031) | 253 (200–340) | 1886 (1613–2507) |
| Na glutamate | 3 | 132 (83–204) | 12 (8–19) | 110 (68–166) |
| Na glut. + $NH_4$ acetate | 4 | 746 (585–891) | 71 (55–100) | 587 (417–800) |
| **Azo-diet livers** | | | | |
| Control | 3 | 408 (334–538) | 46 (30–63) | 211 (143–294) |
| $NH_4$ Acetate | 4 | 1955 (1615–2228) | 190 (168–207) | 752 (665–815) |
| Arginine | 3 | 3528 (2971–3900) | 257 (212–309) | 1171 (981–1361) |
| Arginine $NH_4$ | 3 | 5359 (4364–6110) | 505 (351–609) | 2179 (1561–2573) |
| Arginine glutamate | 2 | 6532 (6380–6685) | 547 (526–570) | 2158 (2073–2243) |
| Arginine glutamate + $NH_4$ acetate | 2 | 6856 (6406–7307) | 509 (490–529) | 2006 (1932–2080) |
| Na glutamate | 3 | 944 (798–1141) | 84 (75–95) | 331 (297–375) |
| Na glutamate + $NH_4$ acetate | 5 | 2498 (1170–3933) | 227 (153–332) | 930 (578–1541) |

* The amounts of supplements added are the same as those given in Table 1. Figures in parentheses represent the range of values obtained.

*Normal vs. fatty livers.* Of further interest are the data listed in Table 2. In these experiments, the amount of urea produced in 4 hr from added amino acids and ammonium salts was measured. Comparing the data on the basis of urea production per g FFT, it is seen that in control experiments in 4-hr perfusion, more urea was produced by normal livers than fatty livers ($t_{90}$ 1·40, $t$ 2·86). Also in the face of an ammonia challenge, normal liver produced more urea than the fatty tissue ($t_{90}$ 1·40, $t$ 4·38). Normal tissue displayed a greater ability to form urea in the experiments where arginine alone was added as a supplement for perfusion ($t_{90}$ 1·53, $t$ 4·51). Under these conditions, normal tissue produced an average of 2370 μmole urea nitrogen and fatty livers only 872. When arginine and ammonia were added concomitantly, the normal liver produced no more urea than with arginine alone. In fatty livers the story is somewhat different, With arginine and ammonia added together, the urea produced is on a lower level than that produced in normals ($t_{90}$ 1·42, $t$ 3·95) but is greater than that produced by single additions of ammonium acetate ($t_{90}$ 1·38, $t$ 7·71) and arginine ($t_{90}$ 1·44, $t$ 3·18). With arginine–glutamate added, the normal liver again produced a high level of urea, and the fatty liver was able to synthesize only about one third of this ($t_{90}$ 1·44, $t$ 4·14). After the addition of A–G and ammonia together, the urea level was increased to high levels by the normal livers, but these levels are not of the order of magnitude to be expected from the sum of what was attained in the experiments in which single additions were made. As in the arginine series the fatty livers were able to produce higher levels of urea with ammonia and A–G than the sum of urea produced in the experiments with the single additions.

Normal livers were quite capable of converting glutamate nitrogen to urea nitrogen. When glutamate was added with ammonia to normal livers, the urea produced did not come up to the sum produced by single supplement experiments. Fatty livers appeared to be incapable of converting glutamate nitrogen to urea nitrogen.

*Normal vs. azo dye-fed livers.* Azo dye-fed livers were able to produce urea nitrogen to the same degree as normal livers in control experiments, but when challenged with an ammonia load azo livers had a lesser ability than normals to synthesize urea nitrogen ($t_{90}$ 1·48, $t$ 2·65). As with fatty livers, the azo dye-fed livers did not convert arginine to urea to the same extent as normal livers ($t_{90}$ 1·53, $t$ 4·39). When arginine was added along with ammonia to the azo-diet livers, urea production attained about the same level as that seen in similar experiments with normal livers but to a point which is greater than the added results of single experiments.

The azo dye-fed livers produce about as much urea from A–G as do normal livers. This level was not increased when ammonium ion was added along with the A–G. The precancerous livers were almost as capable as normal livers in converting amino nitrogen of glutamate to urea. In precancerous livers as in normal livers, when both glutamate and ammonia were added, the urea production was not equivalent to the sum of the urea produced in the single experiments.

## DISCUSSION

Examination of the data shows very clearly that the isolated perfused rat liver is capable of removing added ammonia from the perfusate and converting it to urea. This not only serves as a good means of testing viability of the liver but gives the investigator an excellent tool for studying ammonia metabolism in the organism.

In an effort to study the effect of the amino acids arginine and glutamate on the

metabolism of added ammonia, the control experiments have duplicated and essentially confirmed the earilier work of Burke and Miller.[12, 13] Although different sources of ammonium ion were used in the two laboratories, both agree that dye-fed livers are similar to normal livers in their ability to remove added ammonia from the blood perfusate. Both reports show that in precancerous livers, a disturbance in amino acid metabolism exists. For example, the urea production from the added substrate arginine has been shown by both groups to be about half that produced by the normal liver.

This study has shown that the amino acids arginine and arginine–glutamate assist the normal liver in removing ammonia from the perfusate, but the results give no clear indication concerning the mechanism by which this effect is produced. It could be due to a permeability change that causes a greater diffusion of ammonia into the cell, or it could be a more efficient conversion of ammonia to urea. Not only do these two amino acids assist the normal liver in removing added ammonium ion from the perfusate but it appears that they also aid fatty livers and azo dye-fed livers in this same mechanism. Ammonia removal with arginine–glutamate was no more efficient than with arginine in any of the livers studied. This demonstrated the absence of synergistic action between arginine and glutamate in the isolated liver. None of the liver types was capable of utilizing glutamate to good advantage in lowering blood ammonia.

The fact that glutamate was not effective against administered ammonia in the isolated perfused liver is in accord with the report of Barak *et al.*[4] that in the intact animal glutamate assists the ammonia-detoxifying system of extrahepatic tissue. Burke and Miller,[12] and Burke[14] have reported that glutamic acid when added to the blood perfusate of normal livers produces a lesser amount of urea than most other amino acids. This study had demonstrated not only that normal livers and precancerous livers produce less urea with glutamate than with arginine but also that deamination of glutamate does not occur in the fatty liver.

Little is known of the ammonia removal mechanism in the liver; however, an interesting observation not portrayed in the tables is that added ammonium ion is prompt in disappearing from the blood, but there is a lag of 4 hr or better for maximal urea production. How the ammonia is held within the liver until it is converted to urea is not understood. In this connection, data presented by Duda and Handler[15] indicate that $N^{15}$ fron $N^{15}$-labeled ammonia is converted first to glutamine by the liver before appearing as urea. This step may possibly account for this observed lag.

Dye-induced hepatomas were reported to be low in arginase by Greenstein.[16] The fact that arginine-supplemented fatty livers and axo dye-fed livers yielded low levels of urea in this study, coupled with the findings of Burke and Miller,[13] allows speculation. It is possible that in both cirrhosis and carcinogenesis, biochemical lesions are produced at the arginase level.

In normal livers, the ureas produced with arginine alone and ammonium acetate alone were not additive when the two supplements were infused together. In the fatty livers and precancerous livers the experiments show more than an additive effect, indicating that arginine promoted urea production from the infused ammonia nitrogen. This implies that arginine was beneficial at least to the pathological livers.

In the experiments on the normal livers, where the results are not additive, it is seen that the urea levels reach much higher levels than in the pathological livers. This could mean that the high levels of urea in the normal livers may cause a mass action effect,

producing a feedback in the ornithine cycle and resulting in a type of inhibition of ureogenesis. Conceivably, if the experiments had been carried out in conditions under which urea was continuously removed from the perfusate, entirely different results would have been obtained. In the fatty and azo-dye livers the high levels of urea did not occur, and thus it was possible to produce an additive effect or better.

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