Accumulation of Amino Acids by the Perfused Rat Liver in the Presence of Ethanol

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1. The rate of gluconeogenesis from alanine in the perfused rat liver is affected by the presence of other metabolizable substances, especially fatty acids, ornithine and ethanol. Gluconeogenesis is accelerated by oleate and by ornithine. When both oleate and ornithine were present the acceleration was greater than expected on the basis of mere additive effects. 2. Much NH_3 and some urea were formed from alanine when no ornithine was added. With ornithine almost all the nitrogen released from alanine appeared as urea. 3. Lactate was a major product of alanine metabolism. Addition of oleate, and especially of oleate plus ornithine, decreased lactate formation. 4. Ethanol had no major effect on gluconeogenesis from alanine when this was the sole added precursor. Gluconeogenesis was strongly inhibited (87%) when oleate was also added, but ethanol greatly accelerated gluconeogenesis when ornithine was added together with alanine. 5. In the absence of ethanol the alanine carbon and alanine nitrogen removed were essentially recovered in the form of glucose, lactate, pyruvate, NH_3 and urea. 6. In the presence of ethanol the balance of both alanine carbon and alanine nitrogen showed substantial deficits. These deficits were largely accounted for by the formation of aspartate and glutamine, the formation of which was increased two- to three-fold. 7. When alanine was replaced by lactate plus NH_4Cl , ethanol also caused a major accumulation of amino acids, especially of aspartate and alanine. 8. Earlier apparently discrepant results on the effects of ethanol on gluconeogenesis from alanine are explained by the fact that under well defined conditions ethanol can inhibit, or accelerate, or be without major effect on the rate of gluconeogenesis. 9. It is pointed out that in the synthesis of urea through the ornithine cycle half of the nitrogen must be supplied in the form of aspartate and half in the form of carbamoyl phosphate. The accumulation of aspartate and other amino acids suggests that ethanol interferes with the control mechanisms which regulate the stoichiometric formation of aspartate and carbamoyl phosphate.

Findings on the effects of ethanol on gluconeogenesis from alanine in the isolated perfused rat liver are conflicting. Kreisberg (1967) and Krebs *et al.* (1969a) found inhibitions by ethanol whereas Williamson *et al.* (1969a,b) reported an acceleration. To explore the reasons for these discrepancies we studied the fate of alanine in the perfused liver systematically in the presence and absence of ethanol.

As a gluconeogenic precursor alanine differs from lactate and pyruvate in that it provides not only the carbon skeleton for gluconeogenesis, but also produces NH_3 . When accumulating, NH_3 shifts the redox state of the liver (primarily in the mitochondria) in the direction of oxidation because NH_3 is an oxidant in the glutamate dehydrogenase system, one of the major mitochondrial redox systems of the liver. If not accumulating, because of prompt conversion into urea, NH_3 gives rise to an ATP consumption of four molecules per molecule of urea formed and this may

affect the phosphorylation state of the adenine nucleotides. Thus gluconeogenesis from alanine and from lactate, and the manner in which it is affected by ethanol, may be expected to have different characteristics. To test this, removal of alanine, the formation of glucose, lactate, pyruvate, NH_3 and urea, and the consumption of O_2 in the perfused liver were measured under a variety of conditions. These include the addition of ornithine (which accelerates the conversion of NH_3 into urea) and of oleate (which supplies the main substrate of respiration).

In the published work on the fate of alanine in the perfused liver the disappearance of alanine has not been measured because no convenient method for the determination of alanine was available. In the present experiments alanine was determined spectrophotometrically with the help of the NAD-linked alanine dehydrogenase of *Bacillus subtilis* prepared by the method of Yoshida & Freese (1964), so that balance

sheets of the alanine removed and the products formed could be drawn up.

Methods

The method of perfusion was essentially that described by Hems *et al.* (1966) and Krebs *et al.* (1969b). Female Wistar rats weighing 160–200g and starved for 48h were used unless otherwise stated. The substrates were added as 0.20M of the neutral solutions, except for ethanol (which was 1.0M) and oleate [which was 0.1M and was added together with albumin as described by Krebs *et al.* (1969b)]. A period of 38min perfusion was allowed before the addition of substrates and further additions of substrates were as indicated in the Tables or the text.

L-Alanine (chromatographically pure) was obtained from British Drug Houses Ltd., Poole, Dorset, U.K. The test for D-alanine with D-amino acid oxidase indicated the presence of less than 0.1% of the D-isomer.

Glucose, lactate, pyruvate and urea were determined as described by Hems *et al.* (1966). The NH_3 was measured by the method of Kirsten *et al.* (1963). Alanine was determined by a modification of the method of Williamson *et al.* (1967) with alanine dehydrogenase prepared from *B. subtilis* strain 60127 by the method of Yoshida & Freese (1964) as modified by Williamson *et al.* (1967). The preparation was purified as far as the stage of chromatography on DEAE-cellulose and the final product was dialysed to remove NH_3 and checked for the absence of lactate dehydrogenase and malate dehydrogenase activities. The reaction mixture contained 1ml of 0.05M-Tris-hydrazine buffer, pH 10.0, 0.6ml of 0.5% (w/v) NAD^+ , 0.2ml of 0.1M-EDTA and up to 1.0ml of sample containing about 0.2 μmol of alanine. The final volume was 3.1ml. The blank contained all of the above additions except for the sample. The reaction, which was followed at 340nm and was started by the addition of 20 μl of the enzyme, took about 60min to reach completion.

The O_2 uptake was calculated from the difference in the concentrations in the medium entering and leaving the liver and the flow rate through the liver. The method of Van Slyke & Neill (1924) was used to determine the O_2 concentration and the flow rate was measured by timing the filling of a siphon of known volume by the blood leaving the liver. In the course of the work the method of Van Slyke & Neill (1924) was replaced by that of Mayers & Forster (1966), Tucker (1967) and Solymar *et al.* (1971). This is based on the use of an oxygen electrode for the determination of the O_2 content of the blood after treatment with potassium ferricyanide.

In the calculation of the carbon balances it was assumed that the formation of aspartate from alanine requires one molecule per molecule and the formation

of glutamate and glutamine two molecules per molecule.

Results

Effects of oleate and ornithine on the fate of alanine in the perfused rat liver

Effects of oleate and ornithine on glucose formation and alanine removal. The results of a series of experiments on the fate of alanine in the perfused liver of rats starved for 48h are given in Table 1. The results refer to the total changes in the perfusion medium which occurred during 90min after the addition of the substrate. Oleate increased gluconeogenesis from alanine, as noted by Williamson *et al.* (1969a). Ornithine also increased gluconeogenesis from alanine, especially in the presence of oleate. The effects of oleate and ornithine were not merely additive but much greater: had they been additive, the amount of glucose formed in the presence of alanine, oleate and ornithine would have been 477 μmol ; in fact it was 640 μmol . As ornithine itself is glucogenic, some increase on addition of ornithine is not unexpected, but the effect of ornithine in the presence of oleate was much greater than expected on the basis of gluconeogenesis from ornithine. The added ornithine (150 μmol) could have accounted for 75 μmol of extra glucose (as was actually formed when ornithine was the sole substrate), whereas the extra glucose formed on the addition of ornithine to oleate and alanine was 218 μmol . Thus most of the extra glucose formed on addition of ornithine was derived from alanine, the removal of which was in fact increased by ornithine (from 825 to 1261 μmol in the presence of oleate). Alanine removal was not affected by oleate alone; it was slightly increased by ornithine.

Formation of urea and NH_3 . Large amounts of NH_3 (540 μmol) were formed when alanine was the sole substrate. Addition of oleate decreased the yield of NH_3 somewhat and increased urea production to about the same extent. In the presence of ornithine, as may have been expected, NH_3 production was almost negligible, whereas the yield of urea was greatly raised.

Formation of lactate and pyruvate. As reported by others (see Williamson *et al.*, 1969a,b) substantial amounts of lactate are formed on addition of alanine. Ornithine alone had no significant effect on the yield of lactate, but oleate alone decreased the lactate production (from 313 to 74 μmol) and in the presence of both ornithine and oleate lactate production became negligible (22 μmol). The formation of pyruvate was substantial (about 90 μmol) when oleate was absent, but became very small in the presence of oleate. The [lactate]/[pyruvate] ratio at the end of the perfusion (at 90min after addition of substrate) was rather low (3.5) when alanine was the sole substrate.

Table 1. *Changes in metabolites during alanine metabolism in perfused rat liver (medium only)*

The values are μmol (means $\pm$ s.e.m., with number of observations in parentheses). The initial concentrations of L-alanine were 10mM, of oleate 2mM and of L-ornithine 1mM. The liver weights were between 5.8 and 7.5 g and the results are expressed in terms of a mean weight of 6.41 g. The substrates were added 38 min after the start of the perfusion. The changes refer to the 90 min period beginning 2 min after the addition of the substrates. The values for the O_2 uptake are based on three samples of hepatic portal-hepatic vein differences taken in each experiment at 15, 45 and 75 min after addition of substrates. 'Corrections for blank' means that the values obtained without alanine (i.e. without any added substrate or with ornithine) have been deducted. Controls with oleate as the sole substrate and with oleate plus ornithine give the same values as those without oleate. Minus signs indicate disappearances of metabolites or a negative balance, and plus signs the reverse.

Substrates added ...	Alanine	Alanine+oleate	Alanine+ornithine	Alanine+oleate+ornithine	None	Ornithine
Changes in metabolites						
Glucose	189 $\pm$ 35 (5)	422 $\pm$ 32 (5)	244 $\pm$ 16 (4)	640 $\pm$ 36 (5)	10	79.7 $\pm$ 10.6 (4)
Lactate	313 $\pm$ 32 (5)	73.6 $\pm$ 6.8 (5)	376 $\pm$ 60 (4)	22 $\pm$ 13 (5)		47.3 $\pm$ 20 (4)
Pyruvate	98 $\pm$ 26 (5)	13.6 $\pm$ 3.2 (5)	87 $\pm$ 5.2 (4)	11.2 $\pm$ 6.5 (5)		
Urea	213 $\pm$ 40 (5)	312 $\pm$ 37 (5)	651 $\pm$ 45 (4)	764 $\pm$ 38 (5)	76.6 $\pm$ 2.9 (3)	195 $\pm$ 20 (4)
NH_3	540 $\pm$ 55 (5)	394 $\pm$ 73 (5)	-5 $\pm$ 6.8 (4)	12.4 $\pm$ 9 (5)	28 $\pm$ 3.8 (3)	10.3 $\pm$ 7.5 (4)
Alanine	-860 $\pm$ 25 (5)	-825 $\pm$ 72 (5)	-945 $\pm$ 80 (4)	-1261 $\pm$ 37 (5)		
O_2	-1587 $\pm$ 83 (3)	-2530 $\pm$ 154 (3)	-1969 $\pm$ 35 (3)	-2022 $\pm$ 107 (3)	-1363 $\pm$ 111 (3)	-1335 $\pm$ 102 (3)
Sum of glucose, lactate plus pyruvate (as C_3)	789	932	951	1313	20	206
Sum of urea plus NH_3 (as NH_3)	966	1018	1297	1540	181	400
Excess of urea plus NH_3 formed (as NH_3) over alanine removed	+106	+193	+352	+279	+181	+400
Excess of glucose plus lactate plus pyruvate formed (as C_3) over alanine removed	-71	+107	+6	+52	+20	+206
Lactate formed/pyruvate formed	3.2	5.4	4.3	1.9		
[Lactate]/[pyruvate] at 90 min	3.5	6.3	5.3	16.5		
Alanine carbon recovered, corrected for blank	769	912	745	1107		
Carbon balance, corrected for blank	-91	+87	-200	-154		
Alanine nitrogen recovered, corrected	785	837	897	1140		
Nitrogen balance, corrected	-75	+12	-48	-121		

This is not unexpected because the two immediate products of alanine degradation (pyruvate and NH_3) affect the redox systems as oxidants, the former in the cytoplasm, the latter in the mitochondria. Either oleate or ornithine raised the [lactate]/[pyruvate] ratio, the former presumably by introducing reducing equivalents, the latter by removing NH_3 . In the presence of both oleate and ornithine the ratio was highest.

O₂ consumption. The O_2 consumption was slightly raised by alanine. Addition of either oleate or ornithine caused substantial further increases, the effect of oleate being greater than that of ornithine. Added together oleate and ornithine gave intermediate increases. The extra O_2 consumption caused by the additions was roughly paralleled by the increased rates of the two main energy consuming processes, gluconeogenesis and urea synthesis.

Balances. The calculation of the balance of the amounts of alanine removed and products formed is not free from uncertainties because it is not known to what extent corrections should be made for the reactions which occur in the absence of alanine. It is possible, and even likely, that the presence of alanine modifies, in particular suppresses, the 'blank' reactions. Because of these uncertainties the balances are presented both uncorrected and corrected. The carbon balance, i.e. the difference between the alanine carbon removed and carbon recovered in the form of glucose, lactate and pyruvate, matches very well in the presence of ornithine when the uncorrected values are used. In the absence of ornithine there are some discrepancies, but they are relatively small (see Table 1). Thus most of the alanine carbon removed can be accounted for by glucose, lactate and pyruvate. In the case of the nitrogen balance, i.e. the comparison of the amounts of alanine removed and the nitrogen recovered as urea and NH_3 , the best balances are obtained when a correction is made. This is to be expected, because the formation of urea and NH_3 in the blank is rather large. Except in the presence of alanine and oleate only, there is a relatively small deficit in the nitrogen recovered which, however, is less than 10% of alanine removed.

Effects of ethanol on the metabolism of alanine

The effects of ethanol were very much modified by the addition of oleate and/or ornithine (compare Tables 1 and 2). When alanine was the sole added substrate, ethanol had no effect on the rate of removal of alanine, but the metabolic products of alanine (glucose, lactate, pyruvate, urea and NH_3) all appeared in smaller quantities. This implies that the formation of the expected products was inhibited and that some alanine carbon and alanine nitrogen were diverted to other products. When oleate was also present, ethanol caused a slight inhibition of

alanine removal and a great inhibition of glucose formation; instead of glucose much lactate was formed. Thus pyruvate arising from alanine was diverted from gluconeogenesis to lactate formation. Pyruvate itself was not detectable in the presence of ethanol. Oleate greatly decreased the formation of both urea and NH_3 in the presence of ethanol and again the alanine removed was not fully accounted for by the formation of glucose, lactate, urea and NH_3 . The decrease in NH_3 formation caused by ethanol was particularly large in the presence of oleate. It fell from 394 to 39 μmol .

Ornithine, in the presence of ethanol, greatly increased gluconeogenesis from alanine, from 164 μmol without ornithine to 461 μmol with ornithine. The result can also be expressed by the statement that in the presence of ornithine ethanol increased the rate of gluconeogenesis (from 244 μmol to 461 μmol). Ornithine partially reversed the inhibition by ethanol of gluconeogenesis from alanine in the presence of oleate, but ethanol still caused a strong inhibition (from 640 to 194 μmol) when both oleate and ornithine were present. This inhibition was mainly due to a decrease in alanine utilization (from 1261 to 564 μmol). An additional factor was the increased formation of lactate from 22 to 277 μmol . The formation of urea decreased somewhat, parallel with the decreased alanine removal. The values for the O_2 consumption indicate a slight inhibition when oleate was present.

As stated above, production of urea and NH_3 was decreased by ethanol even when the disappearance of alanine was not decreased. The fall in NH_3 production was particularly large in the presence of alanine plus oleate (from 394 to 39 μmol). A peculiar effect of ethanol is the replacement of glucose formation from ornithine by a disappearance of glucose that is not accompanied by a formation of extra lactate. Thus the presence of ornithine and ethanol causes an unexplained loss of endogenous glucose. A general and important feature of the effects of ethanol was the substantial deficit in the carbon and nitrogen balance of alanine (see lower part of Table 2). The deficit was greater in the absence of ornithine than in its presence and was most pronounced when alanine and ethanol were the sole added substrates. This result prompted a search for other products of alanine metabolism. As the deficits were greatest with alanine plus ethanol this condition was selected for detailed study.

Additional products of alanine metabolism in the presence of ethanol

Since the deficits of carbon and nitrogen were about equal and since pathways leading from alanine to non-essential amino acids are known to be available in the liver it seemed likely that the missing

Table 2. *Effect of ethanol on alanine metabolism in perfused rat liver (medium only)*

Ethanol (1500 μ mol) was added twice at 38 and 85 min. The values are μ mol (means $\pm$ s.e.m., with number of observations in parentheses). The initial concentrations of L-alanine were 10 mM, of oleate 2 mM and of L-ornithine 1 mM. Pyruvate was not detectable; this means that less than 10 μ mol was present. For other details see Table 1.

Substrates added ...	Alanine	Alanine + oleate	Alanine + ornithine	Alanine + oleate + ornithine	None	Ornithine
Changes in metabolites						
Glucose	164 $\pm$ 99 (4)	57 $\pm$ 15 (4)	461 $\pm$ 36 (6)	194 $\pm$ 52 (5)	15.5 $\pm$ 13 (4)	-40.5 $\pm$ 9.7 (4)
Lactate	177 $\pm$ 33 (4)	422 $\pm$ 52 (4)	127 $\pm$ 23 (6)	277 $\pm$ 69 (5)	168 $\pm$ 36 (4)	110 $\pm$ 10.5 (4)
Urea	128 $\pm$ 45 (4)	186 $\pm$ 26 (4)	525 $\pm$ 38 (6)	316 $\pm$ 27 (5)	95.5 $\pm$ 16 (4)	148 $\pm$ 21 (4)
NH ₃	256 $\pm$ 121 (4)	39 $\pm$ 11 (4)	23 $\pm$ 8 (6)	34 $\pm$ 9 (5)	<5	<5
Alanine	-881 $\pm$ 183 (4)	-652 $\pm$ 126 (4)	-1080 $\pm$ 116 (6)	-564 $\pm$ 32 (5)		
O ₂	-1877 $\pm$ 127 (3)	-1985 $\pm$ 218 (3)	-1962 $\pm$ 79 (3)	-2625 $\pm$ 136 (3)	-1365 $\pm$ 114 (4)	-1618 $\pm$ 120 (3)
Sum of glucose plus lactate (as C ₃)	505	536	1049	665	199	29
Sum of urea plus NH ₃ (as NH ₃)	512	411	1073	666	191	296
Excess of urea plus NH ₃ formed over alanine removed	-369	-241	-7	+102	+191	+296
Excess of glucose plus lactate formed (as C ₃) over alanine removed	-376	-116	-31	+101	+199	+29
Alanine carbon recovered, corrected for blank	-306	337	1020	636		
Carbon balance, corrected for blank (as C ₃)	-575	-315	-60	+72		
Alanine nitrogen recovered, corrected	321	220	777	370		
Nitrogen balance, corrected	-560	-432	-303	-194		

Table 3. *Balance of metabolites found in perfusion medium*

Substrates were added after a preliminary period of 40 min and perfusion was continued for another 90 min after the addition 90 min incubation after addition of substrate; they refer to the total amounts in the medium. The results for the freeze-clamped analysed at the end of the preliminary period. Some of the livers were those of the experiments recorded in Table 2. Carbon glucose, glutamate, glutamine and oxoglutarate. Values prefaced by the symbol < have been neglected for the calculation of the

Metabolite	Liver wt. ... Substrates added ...	Liver 1 7.4 g		Liver 2 9.1 g		Liver 3 6.5 g	
		Alanine+ethanol		Alanine+ethanol		Alanine+ethanol	
		Medium (μ mol)	Tissue (μ mol)	Medium (μ mol)	Tissue (μ mol)	Medium (μ mol)	Tissue (μ mol)
Glucose		84	5	194	11	215	9
Aspartate		66	63	114	105	92	75
Glutamate		30	13.4	66	22	39	16.4
Glutamine		120	16.4	120	26	92	16.4
α -Oxoglutarate		<7	<3	<7	<3	<7	<3
Malate		<7	<3	<7	<3	<7	
Lactate		218	11.5	111	9.2	158	9.5
Pyruvate		<7	<0.4	<7	<0.4	<7	0.4
Alanine		-776	20.2	-938	70	-846	83
Urea		235	11	257	16	261	12
NH ₃		48	9.2	63	11.4	158	18.1
Sum of nitrogen formed		1014		1289		1243	
Sum of carbon formed (as C ₃)		915		1287		1192	

products of alanine metabolism were amino acids. The presence of aspartate, glutamate and glutamine in the perfusion medium and in the liver tissue was investigated. α -Oxoglutarate and malate were also measured. Glycogen was not determined because any glucose synthesized by the starved liver is, under test conditions, discharged into the medium and not deposited as glycogen. Control experiments in which alanine, or ethanol, or no substrate was added were also carried out, as well as metabolite measurements after 40 min perfusion, i.e. before the addition of substrates. The results are given in Table 3. As the study of metabolite balances was the main aim, individual experiments rather than means are shown.

When ethanol was the only added substrate (control liver 3) small amounts of aspartate and glutamate and some glutamine were present in tissue and medium. These were of the same order as in control liver 4 (no substrate added). Addition of alanine alone (control livers 1 and 2) increased the amounts of aspartate and glutamate in both tissue and medium, whereas the amounts of glutamine in the medium did not change much. For reasons which are not yet understood it caused a depletion of the tissue glutamine. Addition of ethanol plus alanine (livers 1-3) caused large increases in the amounts of aspartate and glutamine in both medium and tissue. Surprisingly this addition prevents the loss of tissue glutamine which occurs on addition of alanine alone. The amounts of aspartate were increased by ethanol about

five- to ten-fold. The increases in the amounts of glutamine were smaller on a percentage basis, but in absolute terms they were also substantial. The amounts of glutamate were not much affected by ethanol. Ethanol also increased the yield of urea from alanine and decreased that of NH₃.

The calculations of these balances (bottom of Table 3) show that the sum of nitrogen and carbon recovered in all conditions was rather greater than the amounts of alanine removed, as expected because some nitrogen and carbon were released without the addition of alanine (see results for control livers). A reliable correction for the 'blank' metabolite release is not practical, as mentioned above, because the addition of alanine may affect the endogenous metabolism in a major way, but it may be concluded that the negative balances in the presence of ethanol (recorded in Table 2) were largely caused by the formation of aspartate and glutamine.

Effect of ethanol on the nitrogen balance in the presence of ammonium salts and lactate

When alanine was replaced as substrate by lactate plus NH₄Cl, ethanol likewise increased the formation of amino acids, especially of aspartate (Table 4). In the absence of ethanol, addition of NH₄Cl plus lactate caused a significant increase in the formation of alanine and a small increase in the formation of glutamate, but the yields of aspartate and glutamine

and liver after perfusion with alanine and ethanol

of substrates. Ethanol (10mM) was added twice at 40 and 85min. The results given for the medium are the changes during the liver refer to the amounts found in the total liver at the end of the incubation period, except in control livers 5 and 6, which were balances were calculated on the assumption that two molecules of alanine are needed for the synthesis of one molecule each of balance.

Control liver 1 5.9g Alanine		Control liver 2 6.2g Alanine		Control liver 3 4.8g Ethanol		Control liver 4 7.3g None		Control liver 5 6.6g (Analysed at end of pre- liminary period)	Control liver 6 6.7g (Analysed at end of pre- liminary period)
Medium (μmol)	Tissue (μmol)	Medium (μmol)	Tissue (μmol)	Medium (μmol)	Tissue (μmol)	Medium (μmol)	Tissue (μmol)	Tissue (μmol)	Tissue (μmol)
261	6	129	5	-59		16			
17.5	8.9	8.1	13.9	2	1.2	2	3.2	3.2	2.0
52	14.6	25.5	16.0	3.8	7.4	5	7.3	7.3	7.8
56	1.95	55	2.73	76	19.8	50	16.3	16.3	10.5
<2	0.35	<2	0.40						
	6.8		5.8						
276	9.7	285	9.9	118	4	6	10		
137	2.9	191	3.9	7	0.4		0.4		
-891	40.5	-659	53.6	7.1	1.8		2.8		
172	4	129	3	89	2	144	5	6	6
490	8	598	11	2	7		1	2	2
1099		1106		404		451			
1284		1038		235		210			

were not much affected. Ethanol (together with lactate and NH_4Cl) increased the amounts of aspartate in the medium about fivefold and in the tissue about tenfold (compared with the amounts formed in the presence of lactate and NH_4Cl only). The increase of alanine formation caused by ethanol was about threefold in both medium and tissue. Glutamate increased about 50% in the medium and in the liver about fourfold. Glutamine showed only small increases.

The carbon balance with lactate alone, or with lactate plus NH_4Cl , showed a slight surplus of products formed over lactate removed, but these were probably within the limits of error. In the presence of ethanol the surplus of carbon was much larger (144 μmol). There was some release of nitrogenous substances into the medium in the presence of lactate alone, mainly glutamine. This was partly pre-formed and partly formed from endogenous precursors. As expected, the yield of nitrogenous material was much increased by the addition of NH_4Cl . When the yield of nitrogenous substances in the presence of lactate plus NH_4Cl was corrected for the control, the amounts of NH_4Cl removed and of nitrogenous materials formed were almost the same. On addition of ethanol there was a substantial surplus of nitrogen, which was of the same order as the carbon surplus. This result indicates that ethanol under the test conditions causes a release of nitro-

genous material from the liver, a finding which requires further study. The amino acids contributed 38 μmol to the corrected balance in the absence of ethanol and 159 μmol in the presence of ethanol.

Time-course of the ethanol effect in the presence of oleate and ornithine

Inhibition of gluconeogenesis by ethanol in the presence of oleate and ornithine was complete during the first 60min and then rapidly decreased (Table 5, Expt. I). Further ethanol added after 45min (because ethanol is known to disappear within 45–60min by oxidation in the liver and evaporation in the oxygenator) made no difference to the inhibition, whereas addition of oleate after 45min (Expt. II) maintained the inhibition. The quantities of ketone-bodies formed indicate that at 60min about two-thirds of the added oleate had been used up, as one molecule of oleate can form 4.5 molecules of ketone bodies. The ratio of [β -hydroxybutyrate]/[acetoacetate] changed parallel with the rate of gluconeogenesis. The ratio was high (4–5) when gluconeogenesis was inhibited and fell to the normal range of about 2 in Expt. I when inhibition disappeared. Thus the effect of oleate in sensitizing gluconeogenesis towards ethanol appears to be dependent on a relatively high concentration of oleate and/or a highly reduced mitochondrial redox state of the NAD couple.

Table 4. *Effect of ethanol on amino acid formation by perfused livers in the presence of lactate and NH₄Cl*

The general conditions were as described in Table 3, except that alanine was replaced by lactate and NH₄Cl, and that the duration of the perfusion was 45 min after addition of the substrates. The values are μmol and are means of two (when lactate was the only substrate added) or three experiments $\pm$ S.E.M.; they refer to a standard liver weight of 5.93 g. Those for the medium represent changes during the perfusion; those for the liver are the total amounts found in the freeze-clamped liver at the end of the perfusion. 'Blank', refers to livers to which lactate only was added. Substantial amounts of nitrogenous substances were present in medium and liver when no NH₄Cl had been added. In the calculation of the nitrogen balance a correction has been made for the endogenous sources of nitrogen.

Metabolites	Substrates added (mm) ...		Lactate (10)		Lactate (10)+NH ₄ Cl (5)		Lactate (10)+NH ₄ Cl (5)+ ethanol (10)	
	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue
Glucose	+317 $\pm$ 28		+279 $\pm$ 48		+225 $\pm$ 25		+225 $\pm$ 25	
Lactate	-813 $\pm$ 66	24 $\pm$ 2.0	-855 $\pm$ 52	26 $\pm$ 1.9	-786 $\pm$ 73	18 $\pm$ 1.6	-786 $\pm$ 73	18 $\pm$ 1.6
Pyruvate	+74 $\pm$ 2	2.1 $\pm$ 0.2	+130 $\pm$ 20	1.7 $\pm$ 0.2	+3 $\pm$ 0.17	0.4 $\pm$ 0.1	+3 $\pm$ 0.17	0.4 $\pm$ 0.1
Urea	+48 $\pm$ 2		+186 $\pm$ 45		+169 $\pm$ 22		+169 $\pm$ 22	
NH ₃	-6 $\pm$ 4		+354 $\pm$ 38		-331 $\pm$ 62		-331 $\pm$ 62	
Aspartate	+4.4 $\pm$ 0.1	2.7 $\pm$ 0.1	+5.2 $\pm$ 0.35	5.7 $\pm$ 0.8	+29 $\pm$ 5.3	53 $\pm$ 0.2	+29 $\pm$ 5.3	53 $\pm$ 0.2
Alanine	+4.4 $\pm$ 1.0	4.4 $\pm$ 1.2	+36 $\pm$ 4.9	10.8 $\pm$ 1.6	+103 $\pm$ 41	36 $\pm$ 6.3	+103 $\pm$ 41	36 $\pm$ 6.3
Glutamine	+39 $\pm$ 2.6	15 $\pm$ 1.5	+38 $\pm$ 10.4	6.7 $\pm$ 2.2	+48 $\pm$ 9	8.0 $\pm$ 2.5	+48 $\pm$ 9	8.0 $\pm$ 2.5
Glutamate	+6.1 $\pm$ 0.6	7.5 $\pm$ 0.15	+17.4 $\pm$ 1.9	9.4 $\pm$ 2.0	+28 $\pm$ 0.8	35 $\pm$ 4.5	+28 $\pm$ 0.8	35 $\pm$ 4.5
Total carbon (as C ₃)		884		905		930		930
Carbon balance		+71		+50		+144		+144
Total nitrogen		228		547		734		734
Nitrogen corrected for blank				319		506		506
Nitrogen balance corrected for blank				+35		+175		+175

Table 5. *Time-course of the inhibition of gluconeogenesis from alanine by ethanol in the presence of oleate and ornithine*

The rats were starved for 48 h. At the start of the experimental period the medium contained 10 mm-alanine, 1 mm-ornithine, 2 mm-oleate and 10 mm-ethanol. In both experiments further 10 mm-ethanol was added after 45 min. In Expt. II further 2 mm-oleate was added after 45 min. The results are the total amounts in μmol in the perfusion medium.

Time (min)	Expt. I (liver wt. 6.6 g)				Expt. II (liver wt. 7.0 g)			
	β -Hydroxy- butyrate		Acetoacetate		β -Hydroxy- butyrate		Acetoacetate	
	Glucose			[β -Hydroxybutyrate] [acetoacetate]	Glucose			[β -Hydroxybutyrate] [acetoacetate]
0	190	71	59	1.20	132	33	143	0.23
15	173	218	59	3.70	148	297	132	2.25
30	180	403	76	5.27	143	448	136	3.30
45	156	543	105	5.14	125	582	130	4.46
60	168	660	149	4.43	126	707	181	3.91
75	280	741	193	3.85	125	838	214	3.90
90	355	702	250	2.80	130	939	238	3.97

Discussion

The experiments demonstrate that gluconeogenesis from alanine is much affected by the presence of other metabolizable substances, in particular oleate, ornithine and ethanol. Acceleration of gluconeogenesis by oleate and other fatty acids (see also Williamson *et al.*, 1969*a,b*), already known to occur with lactate as precursor (Krebs *et al.*, 1965; Ross *et al.*, 1967), could be due to an increased steady-state concentration of acetyl-CoA, which would accelerate the pyruvate carboxylase reaction. In accordance with this assumption is the fact that increased gluconeogenesis is accompanied by decreased formation of lactate. This correlation between glucose and lactate formation may be a matter of maintenance of the redox balance of the NAD couple. The formation of pyruvate and NH_3 from alanine is brought about by the combined action of glutamate-pyruvate aminotransferase and glutamate dehydrogenase, the overall effect being:

Thus the deamination of alanine would come to a standstill unless the NADH formed is reconverted into NAD^+ . This could be achieved by the respiratory chain, but the fact that the deamination of alanine is accompanied by an approximate stoichiometric formation of either lactate or glucose suggests that the oxidation of NADH is brought about either by the lactate dehydrogenase reaction or by the reduction of 3-phosphoglycerate to glyceraldehyde phosphate in the course of gluconeogenesis. Hence if the carboxylation of pyruvate is limited by lack of acetyl-CoA, pyruvate would form lactate in place of glucose.

A remarkable phenomenon is the acceleration of gluconeogenesis from alanine by ornithine in the presence of ethanol or, expressed in a different way, the acceleration of gluconeogenesis by ethanol in the presence of ornithine. Before attempting to discuss explanations we prefer to await further experimental analysis of the phenomenon. The increased rate of alanine removal in the presence of ornithine could be due to acceleration of reaction (1) through the removal of the NH_4^+ formed. Another set of observations that cannot yet be fruitfully discussed is the disappearance of preformed glucose in the presence of ethanol and ornithine (Table 2).

The fact that ethanol under definable conditions can inhibit, or accelerate, or have no major effect on

gluconeogenesis from alanine accounts for earlier apparently discrepant results described in the literature (Kreisberg, 1967; Krebs *et al.*, 1969*a*; Williamson *et al.*, 1969*a,b*).

One of the main findings is the diversion by ethanol of NH_3 formed from alanine, or of added NH_3 in the presence of lactate, from the synthesis of urea to the synthesis of aspartate, glutamate and glutamine. This is probably related to the fact that in urea formation through the ornithine cycle half the nitrogen must be supplied in the form of aspartate and half in the form of carbamoyl phosphate. Thus control mechanisms must exist which regulate the stoichiometric formation of carbamoyl phosphate and aspartate. Ethanol appears to interfere with these control mechanisms (see Krebs *et al.*, 1973).

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