Although heat-labile factors are responsible for the greater portion of the activity reported in these experiments a small amount is found to be due to heat-resistant substances. This brings up the question of their probable nature.

(3) In addition to the two hemolytic systems briefly described above there is evidence for the existence of a third system. Freeman and Johnson[13] and Loewy, *et al.*[14] have shown that an increase in soaps in chyle, particularly during fat absorption after a meal high in fats, can be large enough to bring about a degree of hemolysis on entering the blood. The fetal guinea pig liver, at the stages studied, has a very high fat content[15] and it is conceivable that as a result there is a sufficient increase in the soap content to account for the heat-stable hemolytic agent.

The fact that serum heated, as well as fresh, inhibits the activity of the lytic agents points to the probability that this may be part of an *in vivo* counteracting mechanism.

Bearing on the general phenomena reported here is the recent observation of Gross[16,17] to the effect that saline extracts from spontaneous mouse mammary carcinomas and human cancer frequently hemolyse red cells *in vitro*. With the former preparation hemolysis was observed after 2 to 3 hours incubation whereas with the latter it became evident only after 24 hours. Weil[10] also reported a similar study in which he observed that non-necrotic tumors behaved like normal tissue while necrotic tissue apparently produced a different kind of a lytic agent. Both Weil and Gross offer their findings as the basis for an explanation of the anemias which frequently occur during malignancies.

It should be noted that in respect to time of incubation needed to produce lysis fetal liver extracts resemble extracts of spontaneous mouse mammary carcinoma.

Summary. (1) A saline extract of the liver of fetal guinea pig possesses greater hemolytic activity than a similarly prepared extract of maternal liver, as determined after 4 hours incubation at 38°C.

(2) The activity, for the most part, is due to a heat-sensitive agent and, to a lesser extent, to a heat-resistant substance.

(3) The lytic action of such extracts is not species-specific. It will lyse red cells from the same fetus, as well as those from the mother or from another species.

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Amino Acid Composition of Morphological Fractions of Rat Livers and Induced Liver Tumors.* (17480)

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In previous studies,[1,2] comparisons were made of the chemical compositions of the nuclear, large granule (mitochondria), small granule (microsome), and supernatant fluid fractions of the livers from normal rats, the livers of rats fed the hepatic carcinogen 4-dimethylaminoazobenzene, and the liver tumors induced by this dye. Ingestion of the dye resulted in altered levels of protein, nucleic acids, and riboflavin in certain fractions. In particular, increased levels of protein in the nuclear fraction and decreased

quantities in the large and small granule fractions were observed.[1] Similar changes, but more extreme, were found in the proteins of the tumor fractions.[2] With the aid of microbiological methods developed for the determination of amino acids,[3,4] we have now determined the levels of 17 amino acids in the total proteins of the intracellular fractions from the livers of rats fed diets with or without 4-dimethylaminoazobenzene and from liver tumors. Isolation of the morphological parts of the cell permitted the detection of alterations in the composition of the cell which would have been obscured if only the whole cell proteins had been analyzed.

Experimental. Although the livers fractionated for the amino acid determinations were first perfused with saline, the degree to which contamination of the liver proteins by blood proteins might otherwise occur was determined in a preliminary study. This was calculated for each of several rats from counts of the red blood cells in the heart blood and in the perfusion fluid from the liver. The rats were killed by ether or by decapitation, and, in each case, the inferior vena cava was cannulated cephalic to the liver and ligated just caudal to the liver. The portal vein was then severed and the liver carefully excised. Isotonic NaCl was introduced through the cannula and allowed to flow out through the portal vein into a collecting vessel. The livers were weighed before and after perfusion. The livers that were fractionated were perfused in a similar manner *in situ*. For the fractionations, two groups of 4 male rats‡ weighing 180 to 200 g were fed *ad libitum*, a basal diet,[5] containing 12% of casein and 1.2 mg of riboflavin per kg. The diet for one group also contained 0.06% of 4-dimethylaminoazobenzene. After 4 weeks, the rats were killed with ether and their livers were immediately perfused. The tumor tissue was collected from the livers of 20 rats which had been fed the diet containing 0.06% of 4-dimethylaminoazobenzene for 4 to 5 months. Although only liver tumors which were less than 1 cm in diameter and which showed no gross signs of degeneration were used, histological examination showed that the tumors contained small areas of necrosis and connective tissue and numerous leucocytes.[2] The tumor-bearing livers were not perfused. These tumors were nearly white and their color was unchanged upon perfusion of the liver. This would now be expected from the recent demonstration that liver tumors are supplied only with arterial blood.[6] Furthermore, omission of this step considerably decreased the time required for the collection of the tumors. The livers or tumors were then pooled, forced through a plastic tissue mincer, homogenized in 0.88 M sucrose solution, and fractionated by differential centrifugation as described previously.[1,2,7] The perfusion, centrifugation, and all other operations prior to the precipitation of the proteins were performed at 3°C. Each fraction was suspended in sucrose solution, and aliquots from each fraction and the unfractionated homogenate were analyzed for nucleic acids.[8] The proteins in the remainder of each fraction and from an aliquot of the original homogenate were precipitated with trichloroacetic acid at a final concentration of 9% and washed once with 1 *M* acetate buffer (pH 5.0) and twice with 95% ethanol. Following extraction for 48 hours with ethanol at 60°C in a Soxhlet apparatus, the proteins were dried in weighing bottles *in*

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1 Price, J. M., Miller, E. C., and Miller, J. A., *J. Biol. Chem.*, 1948, **173**, 345.

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TABLE I.
Distribution of Protein and Nucleic Acids in the Tissue Fractions.

Fraction	Livers from rats fed: Basal diet	Livers from rats fed: Basal diet + dye	Rat liver tumors
	Mg protein per g fresh tissue (to the nearest whole number)		
Whole homogenate	123	106	119
Nuclei	16	16	42
Large granules	41	29	14
Small "	18	16	12
Supernatant fluid	47	41	49
Recovery	122	102	117
	Mg of desoxypentosenucleic acid per g of fresh tissue		
Whole homogenate	1.94	2.10	5.57
Nuclei	1.89	2.01	5.00
Large granules	0.00	0.00	0.49
Small "	0.00	0.00	0.10
Supernatant fluid	0.00	0.00	0.24
Recovery	1.89	2.01	5.83
	Mg of pentosenucleic acid per g of fresh tissue		
Whole homogenate	5.70	3.57	6.89
Nuclei	0.49	0.25	1.76
Large granules	1.80	1.06	0.76
Small "	1.78	0.97	1.33
Supernatant fluid	1.11	0.96	2.47
Recovery	5.18	3.24	6.32

vacuo first over $CaCl_2$ and finally over H_2SO_4. This procedure was carried out quantitatively and the yields were determined by direct weighing. Analyses for ash, lipid, and glycogen indicate that approximately 97% of such preparations is a mixture of protein and nucleic acids.[9] Nitrogen was determined by the Dumas method.§

For the microbiological amino acid determinations, 40 mg of the dried preparations were autoclaved in 3 ml of 3 *N* HCl at 121°C for 16 hours, neutralized, diluted to 100 ml and appropriate aliquots were used for each assay. Previous studies have shown that the amino acids determined are essentially stable to this treatment. Leucine, valine, phenylalanine, and glutamic acid were determined with *Lactobacillus arabinosus* 17-5 as the test organism; arginine, threonine, and histidine with *Streptococcus faecalis* R as the test organism; lysine, methionine, aspartic acid, proline, serine, isoleucine, tyrosine, and cystine with *Leuconostoc mesenteroides* P-60; and glycine with *Leuconostoc citrovorum* 8081 as the test organism. All of the assays were carried out with a final volume of 2 ml per tube and with the assay conditions and media summarized in a recent publication.[10] Liver extract was also included in the medium for *Leuconostoc citrovorum* 8081.[11] Tryptophan was determined on separate 10-30 mg samples of the protein preparations by the Bates method as described by Graham *et al.*[12] Calculated on a fresh weight basis similar to that indicated in Table I for protein and nucleic acids, the sum of the amounts of each amino acid in the 4 fractions was in good agreement with the amount found in the unfractionated homogenate.

Results and Discussion. In the preliminary study, it was found that, when rats were killed by decapitation without ether anesthesia, the perfusion removed about 0.05 ml of blood per gram of liver. If the rats were killed

§ Clark Microanalytical Laboratories, 104½ West Main Street, Urbana, Ill.

9 Miller, E. C., and Miller, J. A., *Cancer Research*, 1947, **7**, 468.

10 Schweigert, B. S., Guthneck, B. T., Kraybill, H. R., and Greenwood, D. A., *J. Biol. Chem.*, 1949, **180**, 1077.

11 Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, 1948, **176**, 165.

12 Graham, C. E., Smith, E. P., Hier, S. W., and Klein, D., *J. Biol. Chem.*, 1947, **168**, 711.

with ether and not decapitated, approximately 0.2 ml of blood was removed per gram of liver. The livers from the rats killed with ether had the same weight before and after perfusion while the livers from decapitated rats gained approximately 15% in weight; thus in either case the saline remaining in the portal vessels accounted for about 20% of the fresh weight of the livers. Therefore, the total proteins from the unperfused livers of rats killed by decapitation or by ether anesthesia would contain approximately 6 or 22%, respectively, of blood proteins, even assuming that the blood contained as little as 20% of protein and the perfused liver as much as 15% of protein. These results are in agreement with those of Grund[13] on the perfusion of dog liver. The levels of total protein, desoxypentosenucleic acid, and pentosenucleic acid in the whole homogenates and in each of the fractions (Table I) are in agreement with previous results.[1,2] Subsequent studies have shown that increasing the casein content of the basal diet from 12 to 24% does not alter the level or distribution of protein in the livers of adult rats.[14] In order to express the amino acid data on a protein basis, the "nucleic acid-free protein" has been calculated by subtracting the quantities of the two nucleic acids from the amount of protein in the same fraction.

The levels of arginine, histidine, lysine, leucine, valine, isoleucine, threonine, tryptophan, phenylalanine, and methionine in the nucleic acid-free proteins from the whole homogenates and from each of the fractions are presented in Table II. Similar data for proline, glycine, aspartic acid, glutamic acid, cystine, tyrosine, and serine are shown in Table III. By Dumas analysis, the protein-nucleoprotein preparation of the whole homogenate from the livers of rats fed the basal diet contained 14.5% nitrogen and the whole homogenate from the liver tumors contained 15.1% nitrogen. By calculation, the nucleic acid-free proteins from these

TABLE II. Amino Acid Composition of the Proteins of the Tissue Fractions. (Expressed as % of the nucleic acid-free proteins).

Fraction	Arginine	Histidine	Lysine	Leucine	Valine	Isoleucine	Threonine	Tryptophan	Phenylalanine	Methionine
Livers from rats fed basal diet for 4 wk										
Whole homogenate	6.44	3.20	8.84	10.66	5.76	4.92	4.26	1.49	4.97	2.91
Nuclei	7.15	2.68	9.56	10.10	5.22	4.62	4.17	1.22	3.92	2.60
Large granules	5.57	2.69	8.35	10.45	5.50	5.50	3.97	1.54	4.82	2.98
Small "	6.22	2.97	8.20	9.80	5.00	4.36	4.09	1.85	4.70	2.48
Supernatant fluid	5.59	2.92	8.12	10.10	5.15	4.66	3.76	1.05	4.42	2.66
Livers from rats fed basal diet + dye for 4 wk										
Whole homogenate	6.10	2.75	8.66	10.40	5.14	4.63	4.07	1.52	4.51	2.74
Nuclei	7.57	2.51	8.68	9.62	4.93	4.38	3.90	1.36	3.79	2.55
Large granules	6.71	2.55	7.94	10.65	5.64	4.77	4.15	1.42	4.48	2.71
Small "	6.14	2.79	7.86	9.90	5.13	4.40	3.79	2.15	4.50	2.07
Supernatant fluid	6.12	2.97	7.89	10.73	5.35	4.85	3.99	1.40	4.36	2.28
Liver tumors induced by feeding dye 4 to 5 mo.										
Whole homogenate	6.32	2.75	8.45	9.45	4.80	4.53	3.92	1.39	3.74	1.95
Nuclei	6.98	2.26	8.06	8.52	4.59	3.91	3.64	1.09	3.30	1.54
Large granules	5.90	2.20	7.94	9.80	5.15	4.53	3.27	1.68	3.90	2.14
Small "	5.38	2.40	7.53	9.15	4.80	4.25	3.64	1.62	3.87	1.89
Supernatant fluid	5.83	2.71	8.15	9.58	5.25	4.30	4.30	1.03	3.89	2.00

[13] Grund, G., *Z. Biol.*, 1910, **54**, 173.

[14] Price, J. M., Miller, E. C., Miller, J. A., and Weber, G. M., *Cancer Research*, in press.

TABLE III.
Amino Acid Composition of the Proteins of the Tissue Fractions
(Expressed as % of the nucleic acid-free proteins)

Fraction	Proline	Glycine	Aspartic acid	Glutamic acid	Cystine	Tyrosine	Serine
Livers from rats fed basal diet 4 wk							
Whole homogenate	4.00	4.01	8.38	9.87	.58	3.99	4.05
Nuclei	4.37	5.62	8.06	8.85	.46	3.71	3.89
Large granules	3.90	4.16	8.17	9.20	.52	3.87	4.08
Small "	3.78	3.37	7.79	9.20	.55	4.09	3.98
Supernatant fluid	4.20	3.71	7.99	10.28	.71	3.70	3.76
Livers from rats fed basal diet + dye 4 wk							
Whole homogenate	4.21	3.97	7.61	9.81	.64	3.93	3.95
Nuclei	4.24	5.83	6.87	11.35	.46	3.45	4.19
Large granules	4.05	3.90	7.13	10.44	.49	3.86	3.94
Small "	3.64	3.45	7.54	9.20	.59	4.19	4.08
Supernatant fluid	3.81	3.74	8.20	11.10	.85	3.84	3.87
Liver tumors induced by feeding dye 4 to 5 mo.							
Whole homogenate	4.07	4.86	7.72	11.11	.74	3.66	4.30
Nuclei	4.24	6.93	7.26	10.83	.60	3.63	4.28
Large granules	3.61	4.10	7.79	9.68	.75	4.02	4.29
Small "	3.41	3.56	7.80	9.79	.63	3.68	4.34
Supernatant fluid	3.66	3.58	8.35	11.41	.89	3.99	5.00

samples contained 14.5 and 14.9% of nitrogen, respectively. Through an oversight, none of the protein sample from the whole homogenate of the livers of the rats fed the dye was reserved for nitrogen analysis. The 17 amino acids determined accounted for 85 and 79% of the nitrogen in the nucleic acid-free proteins from the whole homogenates of the livers of the rats fed the basal diet and of the liver tumors. Analyses for alanine with *Leuconostoc citrovorum* indicated that the liver proteins from the rats fed the basal or dye-containing diets and the tumors contained approximately 6% of alanine. The inclusion of alanine increased the nitrogen recoveries to 91 and 86%, respectively, for the nucleic acid-free proteins from the livers of the rats fed the basal diet and the liver tumors.

Table IV lists those amino acids whose concentrations in the nucleic acid-free proteins from the various fractions differed from their concentrations in the proteins from the unfractionated tissues by 15% or more. In general, the proteins from the nuclear fractions contained less histidine, tryptophan, cystine, and phenylalanine and about 40% more glycine than the proteins from the unfractionated tissues. The proteins from the large granules usually had a relatively low concentration of histidine, while those from the small granules generally had a lower concentration of methionine, proline, and glycine and a higher concentration of tryptophan than the whole homogenate proteins.

In each case the supernatant fluid proteins contained more cystine than whole liver protein. Melampy[15] has shown that the whole nuclei of chicken erythrocytes contain less histidine, tryptophan, and phenylalanine than the cytoplasm; no analyses for cystine or glycine were reported. The livers of the rats which had been fed the dye for 4 weeks contained 20 to 33% higher concentrations of glutamic acid in the nuclear proteins, arginine in the large granule proteins, and cystine and tryptophan in the supernatant fluid proteins than were found in the corresponding fractions from the normal livers, Table V. The tumor proteins differed from the normal liver proteins even more than did those from the livers of the rats fed the dye. The methionine contents of the proteins from each fraction were decreased by 25 to 41% while the cystine contents were increased by 15 to 44%. However, the levels of cystine in these proteins

[15] Melampy, R. M., *J. Biol. Chem.*, 1948, **175**, 589.

TABLE IV.
Summary of Differences Between Amino Acid Composition of Proteins from Fractions and the Whole Homogenates Which Exceeded 15%.
(Expressed as % higher (+) or lower (—) in concentration in the fraction).

Fraction	Livers from rats fed: Basal diet		Basal diet + dye		Rat liver tumors	
Nuclei			Arginine	+24		
	Histidine	—16			Histidine	—18
	Tryptophan	—18			Tryptophan	—22
	Phenylalanine	—21	Phenylalanine	—16		
					Methionine	—21
	Glycine	+40	Glycine	+47	Glycine	+43
			Glutamic acid	+17		
	Cystine	—21	Cystine	—28	Cystine	—19
Large granules	Histidine	—16			Histidine	—20
					Threonine	—17
					Tryptophan	+21
					Glycine	—16
			Cystine	—23		
Small granules					Arginine	—15
	Tryptophan	+24	Tryptophan	+41	Tryptophan	+17
	Methionine	—15	Methionine	—24		
			Proline	—16	Proline	—16
	Glycine	—16			Glycine	—27
					Cystine	—15
Supernatant fluid	Tryptophan	—30			Tryptophan	—26
			Methionine	—17		
					Glycine	—26
	Cystine	+22	Cystine	+33	Cystine	+20
					Serine	+16

were relatively low and the cystine assay is less reliable than the other microbiological assays used. The concentrations of glycine and glutamic acid in the nuclear proteins and of serine in the supernatant fluid proteins were also increased by 23 to 33%, respectively. Whether the alterations found following the ingestion of the carcinogen were due to changes in the relative amounts of the various proteins or to alterations in the composition of specific proteins will require much further study.

Summary. 1. The perfused livers from adult rats fed either a basal diet containing 12% of casein or the same diet plus 4-dimethylaminoazobenzene for 4 weeks, and the liver tumors induced by this dye were fractionated into nuclear, large granule (mitochondria), small granule (microsome), and supernatant fluid fractions. The amounts of 17 amino acids were determined in the total proteins from the original homogenate and in each of the fractions. The importance of using perfused livers for these analyses was demonstrated experimentally.

2. When the basal diet was fed, the proteins from the nuclear fraction contained lower concentrations of histidine, tryptophan, phenylalanine, and cystine and a higher concentration of glycine than the whole liver proteins. The large granule proteins contained less histidine, the small granule proteins less methionine and glycine and more tryptophan, and the supernatant fluid proteins less tryptophan and more cystine than the proteins of the original homogenate.

3. The livers of rats fed the dye contained higher concentrations of glutamic acid in the nuclear proteins, arginine in the large granule proteins, and cystine and tryptophan in the supernatant fluid proteins than normal livers.

4. The proteins from each fraction of liver tumor contained less methionine and more cystine than the proteins in the corresponding

TABLE V.
Summary of Amino Acids Which Occurred in Livers of Rats Fed Dye and in Tumors at Levels That Differed from Normal Liver by 15% or More.
(Expressed as % higher (+) or lower (—) in concentration than in normal liver).

Fraction	Livers from rats fed basal diet + dye		Rat liver tumors			
Whole homogenate			Methionine	—33		
			Cystine	+28		
			Glycine	+21		
			Phenylalanine	—25		
			Valine	—17		
Nuclei	Aspartic acid	—15				
			Methionine	—41	Histidine	—16
			Cystine	+30	Isoleucine	—15
			Glycine	+23	Lysine	—16
			Phenylalanine	—16	Leucine	—16
	Glutamic acid	+28	Glutamic acid	+22		
Large granules	Arginine	+20				
			Methionine	—28	Histidine	—18
			Cystine	+44	Isoleucine	—18
			Phenylalanine	—19	Threonine	—18
Small granules	Tryptophan	+16				
	Methionine	—17	Methionine	—24	Histidine	—19
			Cystine	+15		
			Phenylalanine	—18		
Supernatant fluid	Tryptophan	+33				
			Methionine	—25		
	Cystine	+20	Cystine	+25		
			Serine	+33		

fractions of normal liver. In addition, the concentrations of glutamic acid and glycine in the nuclear proteins and of serine in the supernatant fluid proteins were higher in the tumor tissue than in normal liver.

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Utilization of Guanine by the C57 Black Mouse Bearing Adenocarcinoma Eo771.* (17481)

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The effectiveness of the triazole analogue of guanine, azaguanine, in inhibiting the growth of subcutaneous transplants of mammary adenocarcinoma Eo771[1] in mice of the C57 black strain has been reported by Kidder, Dewey, Parks and Woodside[2] and confirmed for this tumor.[3] It was suggested by Kidder and co-workers[2] that their results indicated that the tumor tissue utilizes guanine for nucleic acid synthesis while normal tissue does not.

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