antibodies against ICH virus; ICH antibodies in a 1:5 titre were found in 1 of 23 sera from wild raccoons. 3. ICH virus was isolated from dog kidney tissue cultures derived from 2 apparently healthy dogs. In one instance, specific ICH neutralizing antibody was present in the blood.

1. Cabasso, V. J., Stebbins, M. R., Norton, T. W., Cox, H. R., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 239.

2. Muller, J. Thordal-Christensen, A., *Nord. Vet. Med.*, 1954, v6, 767.

3. Fieldsteel, H. A., Emery, J. B., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 819.

4. Yoshihara, G. M., *ibid.*, 1957, v95, 683.

5. Green, R. G., Evana, C. A., Yanamura, H. T., *ibid.*, 1943, v53, 186.

6. Siedentopf, H. A., Carlson, W. E., *J. Am. Vet. Med. Assn.*, 1949, v115, 109.

7. Younger, J. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 202.

8. Morgan, J. F., Morton, H. J., Parker, R. C., *ibid.*, 1950, v73, 1.

9. Reed, L. J., Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

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Antagonistic Action of Liver Amino Acids and 2,4-Dinitrophenol on Growth of *Escherichia coli*. (24063)

C. COCITO* (Introduced by V. Bryson)

Institute of Microbiology, Rutgers, The State University, New Brunswick, N. J.

It is known that extracts and fractions of liver protect animals from toxic effects of poisons and hormones. Ability of liver extracts to counteract the toxic activity of thyroid powder and 2,4-dinitrophenol (DNP) on various living systems is an example of this type of antagonism and is of particular interest because DNP and thyroxin are both inhibitors of oxidative phosphorylation. Crude liver preparations and components of liver extracts reverse thyroid stress occurring in rats fed with purified diets containing thyroid powder or iodized protein(1). Attempts have been made to identify the unknown factors (2,3) responsible for this anti-thyrotoxic activity, or to refer such activity to known components of the liver(4,5). An antagonism of liver extracts against inhibitory action of DNP has been described in studies with mammalian tissue homogenates(6), as well as with bacteria(7). Analysis of growth-stimulating and anti-DNP activity of liver extracts in bacterial systems shows that several fractions are involved: one is composed of bile salts; another of liposoluble vitamins; a third of steroids; a fourth of unsaturated fatty acids and possibly hydroxy-acids; and a fifth of amino acids and oligopeptides. The hypothesis has been expressed that these substances represent physiological antagonists of thyroid hormones in mammalian tissues, at a cellular level. Similar results were obtained by other authors(4,5) using metabolic-rate test of rats fed with iodized protein for titrating the anti-thyrotoxic activity of liver components. The present work is concerned with the amino acid fraction of liver extracts.

Technics. Liver-amino acid preparation was obtained as follows: Cow's liver, homogenized in Waring Blendor and autolyzed under toluene at 37°C, was dried, pulverized, and extracted by refluxing twice each with boiling ethanol followed by hot water. The residue of coagulated proteins was washed several times with H_2O and hydrolyzed with 6*N* HCl at 100°C for 18 hours. HCl was removed by vacuum distillation and the residue brought several times to dryness after addition of water. The dry material was then dissolved in H_2O. The resulting preparation had a nitrogen content of 13 mg/ml and was used without neutralization for chromatographic analysis, or was neutralized with KOH and sterilized for microbial assays. Medium A used for *E. coli* strain B had the following

* Lepetit postdoctoral fellow in Microbiology.

TABLE I. Action of DNP, Liver Amino Acids, and Mixture A on Growth of *E. coli*.

DNP, moles		Liver amino acid prep. (.1 ml)	Mixture A (.5 ml)	Growth (K.U.)*	
2×10^{-4}	4×10^{-4}			4 hr	8 hr
—	—	—	—	23	57
—	—	+	—	45	60
—	—	—	+	43	57
+	—	—	—	7	38
—	+	—	—	3	13
+	—	+	—	24	54
—	+	+	—	15	51
+	—	—	+	27	58
—	+	—	+	17	56

* Klett-Summerson Colorimeter, red filter, readings at time 0 subtracted from the subsequent readings, values of growth expressed in Klett Units.

Medium: Synthetic medium A, 4.3 ml; DNP 10^{-2} solution, .1 and .2 ml; liver amino acid prep., .1 ml; amino acid mixture A, .5 ml; dist. H_2O, up to 5 ml in each tube.

Inoculum: .1 ml *E. coli* strain B in synthetic medium A (2×10^9 cells/ml).

Growth: Static culture in sterile Klett tubes placed in water bath at 37°C.

composition: K_2HPO_4, 7 g; KH_2PO_4, 3 g; Na citrate, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $(NH_4)_2SO_4$, 1 g; glucose, 2 g; H_2O to 1 liter. DNP, added to this medium at levels of 2 x 10^{-4} M, gave respectively an inhibition of 80 and 90% in growth of *E. coli* strain B (Table I). Within 4 hours after inoculation, turbidity of the 5 ml culture had doubled in synthetic medium A containing 0.1 ml of liver-amino acid preparation, an amount which almost completely removed the inhibition of the 2 x 10^{-4} and 4 x 10^{-4} M levels of DNP (Table I).

A chromatographic analysis of liver-amino acid preparation has been accomplished with Whatman No. 1 filter paper and a bidimensional system consisting of butanol:acetic acid:water (6:25:25) and phenol:water (4:1) in an NH_3 atmosphere. Several chromatograms were run simultaneously by this technic, some being reserved for biological assays and others for development with ninhydrin (Fig. 1).

Anti-DNP activity of the spots was tested by placing dry undeveloped chromatograms on the surface of DNP-minimal agar seeded with the indicator organism. To prepare agar for the assay, 5 ml of *E. coli* strain B (10^9 cells/ml) was added to 500 ml of synthetic medium A containing 1.8% agar and 10^{-4} M DNP; the preparation was poured immediately onto an 11 by 17 inch sterile plate and allowed to solidify. After 30 minutes at room temperature, the chromatogram sheet was removed and the plate incubated at 37°C for 24 hours. At the level of 10^{-4} M, DNP inhibited completely the growth of *E. coli* strain B in solid medium, but some growth appeared on the surface of the agar, in locations corresponding to the position of migrated amino acids. Growth of the indicator strain, however, was not restricted to well-defined spots corresponding to those found in a ninhydrin-developed chromatogram; confluent zones of growth were seen, presumably arising from additive or synergistic action produced by diffusion and mixture of single eluted components.

In other chromatograms, the position of each amino acid was identified by means of ultraviolet lamp, and the spots were eluted and tested for growth-stimulating and anti-DNP activities in the synthetic medium A. Most of the eluted components showed no activity, a few (glutamic and aspartic acids, arginine, isoleucine, and cysteine) exhibited a slight degree of antagonism to DNP; the same negative results were obtained by testing activity of commercial, pure amino acids. It was concluded, therefore, that no single amino acid, but only a mixture of amino acids,

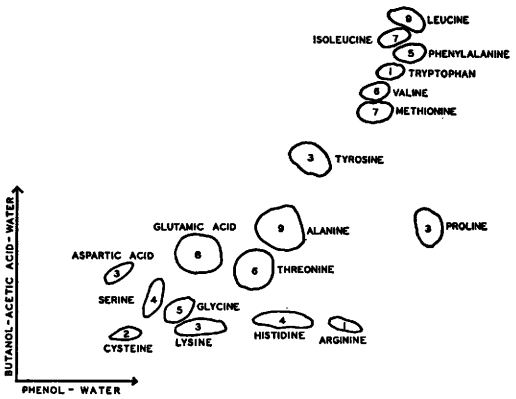

FIG. 1. Ratio of amino acids in chromatograms of liver preparation. In Mixture A, the amino acids have the above ratios, on a molecular basis. In Mixture B, 2 moles of the following amino acids have been added to Mixture A, giving a total of 100 moles: methionine, tyrosine, histidine, cysteine, tryptophan, ornithine, asparagine.

has growth-stimulating and anti-DNP activity: this is in accord with results obtained with undeveloped chromatograms on bacteria-seeded agar.

Quantitative composition of the liver hydrolyzate was established by evaluating intensities of the ninhydrin-developed spots of amino acids in bidimensional chromatograms: ratios of the component amino acids are shown in Fig. 1. A mixture of amino acids, having the reported ratios, was prepared with commercial products (Mixture A). The level of amino acids and the nitrogen content in the mixture were equal to one-fifth of corresponding values of liver hydrolyzate. A 0.5 ml quantity of this mixture showed almost the same growth-stimulating and anti-DNP activity as the corresponding amount (0.1 ml) of liver hydrolyzate (Table I).

Addition of several components of liver extracts (bile salts, lipo-soluble vitamins, steroids) to Mixture A did not enhance the anti-DNP activity of the amino acid combination on growth of *E. coli* strain B. Some increase was obtained by adding to Mixture A a supplement of amino acids destroyed during the process of acid-hydrolysis (tryptophan, tyrosine, histidine, cysteine, methionine) (Mixture B).

Discussion. Several questions arise in assessing the significance of these findings. First it should be observed that the ratio and quantitative relationship of amino acids shown to have anti-DNP activity may have no particular specificity as opposed to other ratios. Furthermore, there is no reason to suppose at present that liver as an amino acid source is superior to any other mammalian tissue. Since it is well known that many poisons and inhibitory agents act at lower concentrations in the absence of organic substances, there is the possibility that anti-DNP activity in bacterial systems is merely another example of this widely recognized phenomenon. Basic amino acids would be expected to combine extra-cellularly with DNP and reduce its effective concentration. However, additional experiments, not reported here, show that the amino acid preparation will allow induced-enzyme synthesis in DNP-inhibited cells after removal of extra-cellular DNP. Glycine or single amino acids do not have this property. Furthermore, by using cysteine-resistant *E. coli* mutants as test organism, it has been shown that anti-DNP activity obtained with small amounts of cysteine does not increase with amount of amino acid added. Such an increase would be expected if cysteine were acting solely by extra-cellular binding of DNP in proportion to the amount of reactants present.

Summary. 1) Liver hydrolyzates counteract the inhibitory action of 2,4-dinitrophenol (DNP) on both mammalian cells and bacteria. Of the several fractions responsible for activity of liver extracts, one is composed of amino acids. 2) Growth-stimulating and anti-DNP activity of this fraction has been tested on *Escherichia coli* strain B, and its composition established by chromatographic analysis. No one of the single components has activity, but a mixture of amino acids stimulates growth-rate of *E. coli* strain B in synthetic medium and counteracts inhibition by high concentrations of DNP. 3) A mixture of commercial amino acids has been prepared, having similar composition and activity to liver-amino preparation.

1. Ershoff, B. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 391.

2. Schwarz, K., *Ann. N. Y. Acad. Sci.*, 1954, v57, 878.

3. Tria, E., Barnabei, O., *Dicerca Scient.*, 1958.

4. Emerson, G. A., Esser, B., Page, A. C., *Fed. Proc.*, 1956, v15, 549.

5. Page, A. C., Koninszy, F. R., Wolf, D. E., Aldrich, P., Folkers, P., *ibid.*, 1956, v15, 568.

6. Barnabei, O., Cocito, C., *Minerva Med.*, 1955, v46, 1858.

7. Barnabei, O., Brighenti, L., *Ricerca Scient.*, 1954, v24, 1841.

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