Reineckates of ACh and components of the crude extract are shown in Fig. 2. It should be pointed out that, although some of the biological activity seems to lie outside the peak of radioactivity, this may be more apparent than real, because it is possible that all the "discrepant" biological activity is sharply localized along the inside edge of the rather broad strips at each end of the radioactivity-peak.

We believe that these findings cast doubt on the validity of the contention of Hosein and Ara,[8] especially in view of the fact that the alteration in the R_f of ACh (0·54) caused by tissue extractives, as found in our studies with Hosein's system, falls within the R_f quoted by these authors for the betaine CoA esters (0·5 to 0·7). While the existence of such esters is not disproved by this work, it is apparent from our data that ACh is responsible for the bulk of the biological activity in these extracts.

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A folic acid-active compound strongly bound by DEAE-cellulose*

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POLYGLUTAMYL conjugates of folic acid (pteroylglutamic acid) and of a number of tetrahydrofolates are widely distributed in plant and animal tissues;[1] they cannot be measured by the usual microbiological assay techniques without prior enzymatic cleavage to folic acid, 5-formyltetrahydrofolic acid, or closely related compounds. *Lactobacillus casei* cannot utilize pteroylglutamates containing more than three glutamic acid residues per molecule,[2] and *Streptococcus faecalis* cannot utilize compounds more complex than pteroyl-γ-glutamylglutamate.[3] Accordingly, a compound that permits growth of *L. casei* or *S. faecalis* maintained in a folic acid-deficient medium is generally regarded as closely related in structure to pteroylglutamate or tetrahydropteroylglutamate. All the known compounds of this group migrate readily on DEAE- or TEAE-cellulose with phosphate buffers of neutral pH; the pteroylpolyglutamates tend to migrate more slowly than do the monoglutamates.[4, 5]

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A number of commercial preparations of folic acid was purified recently by column chromatography on DEAE-cellulose prior to labeling with tritium. In many of the preparations examined, an additional compound was noted, possessing many of the properties of folic acid, including biological activity as a growth factor for *L. casei* and *S. faecalis*, but differing profoundly from folic acid and folic acid cleavage products in chromatographic behavior. Thus, in a typical run, a solution containing 150 mg of sodium folate (Folvite, Lederle, batch 4152-142 AFJ)* was brought to neutral pH with hydrochloric acid (0·2 N) and applied to a column (2 × 20 cm) of DEAE-cellulose (Eastman Kodak 7392) equilibrated by the method of Sober *et al.*[6] The effluent was passed through an ultraviolet absorptiometer (LKB Uvicord, Stockholm) and the absorption recorded on a 1-ma recording ammeter (Texas Instruments). Folic acid and its cleavage products were eluted with sodium phosphate buffer (0·4 M, pH 6·9) as previously described.[7] Fifty ml of ammonium hydroxide solution (1 N) was then perfused through the column, and this was followed by sodium hydroxide (0·5 N). The latter treatment quickly eluted the unknown compound, which could be visualized on the column as a descending yellow band; the chromatographic record is shown in Fig. 1. The smaller peak, labeled

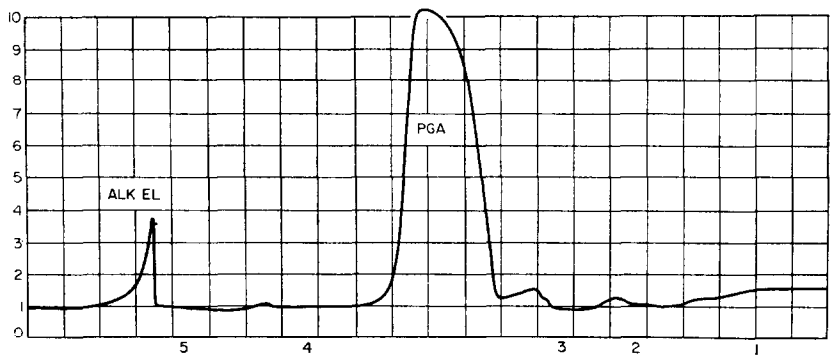

FIG. 1. Ultraviolet absorptiometer record of initial separation by column chromatography on DEAE-cellulose. At 1, a solution containing 150 mg sodium folate was applied to the column, followed by NaH_2PO_4:Na_2HPO_4 buffer (0·01 M; pH 6·9). The ultraviolet absorption of the effluent was monitored as described in the text. At 2, the strength of the buffer was increased (0·07 M) and again at 3 (0·4 M). After the elution of pteroylglutamate (PGA), a solution of ammonium hydroxide (1 N) was applied at 4, and this was followed, at 5, by a solution of sodium hydroxide (0·5 N). The latter treatment quickly eluted the fraction containing the unknown folic acid-active compound.

"Alk El," marks the passage of the unknown compound through the absorptiometer cell; the eluate was collected, quickly neutralized with hydrochloric acid (5 N) and brought to a volume of 20 ml. A 2-ml aliquot of the yellow solution was taken for microbial assay, for determination of the ultraviolet absorption spectrum, and for measurement of diazotizable amine by the direct and indirect Bratton-Marshall procedure.[8] Both *L. casei* and *S. faecalis* were used for determination of folic acid activity. The *L. casei* assay† was carried out by the technique of Usdin *et al.*,[9] as modified by Baker *et al.*[10] and Herbert.[11] Assay with *S. faecalis* was by the method of Teply and Elvehjem,[12] as described by Chanarin *et al.*[13] The total yield, as indicated by the indirect Bratton-Marshall method, was 460 μg (expressed as folic acid). The pH of the remaining 18 ml was then adjusted to 2·8 with hydrochloric acid (5 N). A yellow gel-like precipitate formed and was recovered by centrifugation; the supernatant fraction was discarded. The centrifugate was redissolved in 0·2% sodium bicarbonate solution and rechromatographed on DEAE-cellulose. The chromatographic record, which is reproduced in Fig. 2, disclosed no folic acid peak. The alkaline eluate was again collected, neutralized, and brought

* Similar results were obtained with Folic Acid Batch KM2, Charles E. Frosst & Co., Montreal.

† The medium used for this assay was obtained from Baltimore Biological Corp.

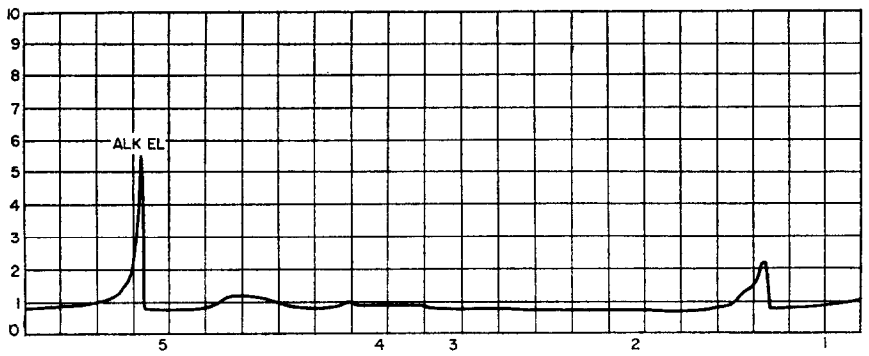

FIG. 2. Rechromatography of alkaline eluate. The alkaline eluate of Fig. 1 was brought to pH 2·8 with hydrochloric acid; the precipitate was collected, redissolved in a solution of sodium bicarbonate (0·2%), and rechromatographed on DEAE-cellulose. The elution sequence was the same as that used for the initial separation.

to a volume of 20 ml. The yield by the indirect Bratton-Marshall procedure was 384 μg (expressed as folic acid). The chemical and microbiological assay data are summarized in Table 1.

The ultraviolet absorption spectrum, in 0·1 N sodium hydroxide solution, of the material which had been rechromatographed, is shown in Fig. 3. Molecular extinction coefficients are plotted on the provisional assumptions that reductive cleavage by the indirect Bratton-Marshall method releases only one arylamino group per molecule and that only a single molecular species is present. It can be seen that while maxima are present at 280 and 365 mμ, as in the folic acid spectrum, the characteristic maximum of folic acid at 255 mμ appears to be represented here only by a shoulder at 259 mμ on the main 280-mμ peak.

TABLE 1. MICROBIAL ACTIVITY OF UNKNOWN FOLIC ACID-ACTIVE FACTOR AFTER ELUTION FROM DEAE-CELLULOSE

	(a) Concentration (expressed as folic acid) by indirect Bratton-Marshall (μg/ml)	(b) Concentration (expressed as folic acid) by *L. casei* assay (μg/ml)	Ratio (a)/(b)	(c) Concentration (expressed as folic acid) by *S. faecalis* assay (μg/ml)	Ratio (a)/(b)
Alkaline eluate, first chromatog.	23·0	3·3	100/14	3·5	100/15
Alkaline eluate, second chromatog.	19·2	2·7	100/14	2·7	100/14

Exposure of a solution containing 40 mg of folic acid from the same batch to ultraviolet light (GE 275W sunlamp) for 3 hr at a distance of 30 cm greatly increased the total yield of UV-absorbing compounds in the alkaline eluate, but failed to increase the total microbial activity. The absorption spectrum was altered as a result of the UV treatment; a well-defined maximum developed at the site of the former shoulder at 259 mμ; i.e. the strongly adsorbed compound or compounds resulting from ultraviolet irradiation was not identical with the unknown folic acid-active compound, although both were eluted from DEAE-cellulose by sodium hydroxide (0·5 N). It is therefore suggested that the substance with microbial activity did not result from the decomposition of folic acid by exposure to light during or after manufacture.

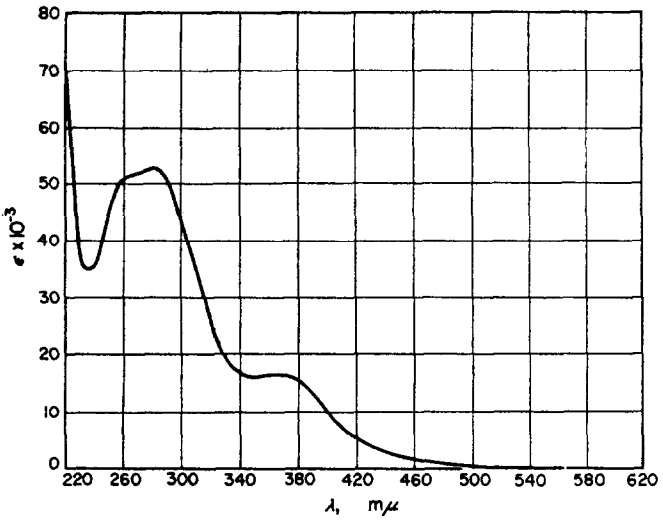

FIG. 3. Ultraviolet absorption spectrum of unknown folic acid-active factor in sodium hydroxide (0·1 N).

Until more data are available, any proposed structure for the unkown folic acid-active compound can only be speculative; it should be noted, however, that dimerization and other condensation reactions are known to occur in the case of xanthopterin and other pteridines.[14, 15] If such dimerization occurred in the case of pteroylglutamate, the resulting adduct could be expected to possess chromatographic properties differing profoundly from those of the parent compound without necessarily having a greatly modified ultraviolet absorption spectrum. Furthermore, if the coupling were partially reversible under the conditions of assay, microbial activity for *L. casei* and *S. faecalis* would still be present.

The investigation is being extended to determine whether similarities other than behavior on DEAE-cellulose exist between the unknown compound and a tritium-labeled compound present in human plasma[16] and urine[7] after the intravenous administration of chromatographically pure tritium-labeled folic acid.

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The action of norepinephrine on the transport of fatty acids and triglycerides by the isolated perfused rat liver

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IN RECENT years it has become apparent that the catecholamines, epinephrine and norepinephrine, stimulate release of nonesterified fatty acids (NEFA) from fat depots[1, 2] and raise the levels of the plasma NEFA.[3–6] Furthermore, stimulation of the nerve supply to adipose tissue was observed to increase the release of NEFA,[7] whereas denervation increased the lipid content of the depot.[8] The nervous system was further implicated as a regulator of NEFA transport since it was reported that psychological and physical stimulation increase plasma levels of NEFA in man.[9, 10] It has been tacitly assumed that the effects of the catecholamines on plasma NEFA levels have been primarily a result of stimulation of lipolysis of adipose tissue triglycerides. In order to ascertain whether the transport and metabolism of lipids by the liver was affected by catecholamines, we investigated the action of norepinephrine on the uptake of fatty acids and the release of triglycerides by the isolated, perfused rat liver. We observed that both fatty acid uptake and triglyceride release were significantly inhibited by the addition *in vitro* of norepinephrine to the perfusion medium.

EXPERIMENTAL

Male rats,* weighing 250 to 400 g, maintained on a balanced ration and water *ad libitum*, were used as liver donors. Details of the perfusion procedure, apparatus, and medium have been reported previously.[11, 12] The liver was removed from the donor and placed in the perfusion apparatus.[11] A constant infusion of *l*-norepinephrine bitartrate in 0·9 per cent NaCl was started after maximum flow rates were obtained (20 to 30 min). The norepinephrine had a marked vasconstrictor effect on the liver, and flow of perfusate through the liver was sharply reduced. This vascular effect of norepinephrine, however, was inhibited by microgram quantities of phenoxybenzamine: 500μg phenoxybenzamine HCl in 0·9 per cent NaCl, injected directly into the portal vein cannula, were sufficient to maintain the normal maximum flow rates through the liver during the constant infusion of norepinephrine. The phenoxybenzamine, moreover, appeared to have no effect on the metabolic actions of norepinephrine. The phenoxybenzamine was injected routinely within a few minutes after the start of the norepinephrine infusion; 20 mg of palmitic acid, as the fatty acid-serum complex,[12] were added to the perfusate 10 min after the phenoxybenzamine addition. At this time, maximal flow rates had been reattained. After an additional 3 min, samples were taken for initial analytical measurements. The triglyceride and glucose data represent net changes in perfusate concentration during the following 3 hr. The fatty acid uptake, recorded as disappearance from the medium, was measured 10 min after the initial sample was taken. The rate of uptake was linear during this period. The concentration

* Obtained from the Holtzman Co., Madison, Wis.