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SOME CHARACTERISTICS OF A THYMIC HUMORAL FACTOR DETERMINED BY ASSAY *IN VIVO* OF DNA SYNTHESIS IN LYMPH NODES OF THYMECTOMIZED MICE*

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Abstract—An improved method for determining the activity of the thymic humoral factor (THF), by means of its enhancement of ^{3}H-thymidine incorporation into lymph node DNA, is described, using thymectomized mice for greater sensitivity.

The active component of THF was found to be a heat-labile macromolecule, susceptible to pronase digestion. Fractionation of calf thymus extract by ammonium sulphate resulted in a partial separation and concentration of THF activity from several other inactive proteins in the extract.

IT HAS previously been shown that most of the pathological effects of neonatal thymectomy in mice can be prevented by intraperitoneal implantation of syngeneic thymus tissue, enclosed in Millipore diffusion chambers which are permeable to fluids but not to cells. Implanted animals did not suffer the weight loss and lymphoid depletion evinced in the thymus deprived controls[1] and the immune response was restored in many of the mice.[2, 3] Similar restorative effects were elicited in various species of laboratory animals thymectomized at birth by syngeneic or allogeneic thymus tissue contained in Millipore diffusion chambers[4–7] and by subcutaneous xenogeneic thymic grafts[8] in mice.

Evaluation of these data suggested the existence of a non-species-specific diffusible product (or products) from thymus tissue which enabled thymectomized animals to produce new lymphocytes and to develop immunological competence. In fact, a thymic humoral factor (THF), capable of inducing lymphocytosis in the peripheral blood, and of increasing the lymphocyte population in the spleen of normal and thymectomized mice, has been obtained from thymuses of sheep, calves and rabbits,[9] that of the calf thymus being subsequently shown to repair also the damage to the immune response induced in newborn mice by thymectomy, or in adult mice by thymectomy followed by total-body irradiation.[10]

Klein *et al.*[11] have found that extracts of thymus (in contrast to other organs of mice, rats or calves) enhanced ^{3}H-thymidine incorporation into DNA of lymph nodes of adult intact mice, and therefore suggested the presence of a thymic lymphocytopoietic factor in these extracts.

In the present communication, an improved method of assay, using thymectomized

* This work is presented by members of Professor I. Berenblum's department, who wishes to associate himself warmly with this tribute to Sir Alexander Haddow.

mice, was employed for the determination of some of the biological properties of THF, and as a guide towards the purification and chemical characterization of the active component.

MATERIALS AND METHODS

Preparation of THF

Extracts were prepared from fresh thymus and kidney of 3–4 months-old C57Bl/6 mice, and from fresh calf thymus, kidney, liver and salivary gland. All procedures were carried out at 0–5° with aseptic precautions. Calf organs were obtained from a local abattoir and transported to the laboratory on ice. The capsule of the organ was removed and the pulp, diluted with two parts of buffer solution, pH 5·7 (Na phosphate 0·1 M, di Na EDTA 10^{-3} M) unless otherwise stated was disintegrated in a Virtis homogenizer for 2 min at 45,000 rpm (unless otherwise stated). The crude homogenates were centrifuged at 2500 *g* for 20 min and the resulting supernate centrifuged at 105,000 *g* for 1 hr. The supernatant was strained through gauze to remove fat, and sterilized by passing through Millipore filters of 0·8 μ pore size. Protein concentration was determined by the Biuret reaction, and the extracts diluted to the desired concentration by additional phosphate-EDTA buffer. The preparations were stored at −20° for a maximum period of 3 weeks.

For ammonium sulphate fractionation, ground crystals (Purissima grade, Fluka A. G., Basel) were slowly stirred into the solution to reach a level of saturation calculated for a system at 0°. Centrifugation was at 14,500 *g* for 30 min, and precipitates were dissolved in phosphate buffer.

These redissolved precipitates were each refractionated at least twice by the same procedure until no further precipitate formed at an ammonium sulphate concentration corresponding to the neighboring lower fraction (e.g. all material precipitating at 0–20 per cent saturation was discarded from the 20–40 per cent fraction by means of two or more refractionations).

Assay of THF

C57Bl/6 mice, bred at the Weizmann Institute of Science by sibling mating, or obtained from Cumberland Farms, were used as the test system in the present experiments. Animals of similar age, weight and sex were used in each experiment. The animals were housed in metal cages in air-conditioned rooms kept at 22–26°, and fed Purina Laboratory Chow pellets, supplemented by barley, sunflower seeds and tap water *ad libitum*.

Thymectomy was performed at 5–6 weeks after birth. (Any animal found to contain a thymic remnant at autopsy, at the end of the experiment, was discarded.) The mice were 8–12 weeks old at the time of the first injection. Eight to twelve animals were used in each control or experimental group.

The extracts were injected intraperitoneally into the animals three times, on consecutive days, in a volume of 0·5 ml per injection. From the time of the first injection until the end of the experiment, the animals were kept in individual cages, to avoid mutual disturbance and consequent stress. Twenty-four hours after the last injection, and 2 hr before sacrifice, ^{3}H-thymidine, from 0·36 to 16 mc/m-mole (The Radiochemical Centre, Amersham), 1 μc/g body weight, was injected intraperitoneally. The mice were killed by cervical fracture. The axillary and inguinal lymph nodes

were removed (along with kidney and liver in some cases), the nodes from each mouse were weighed on a torsion balance, and placed in cold 5 per cent TCA. The extraction of nucleic acids was carried out as described by Klein *et al.*,[11] with the exception that TCA (5 and 1 per cent) was used instead of perchloric acid. Aliquots (0·5 ml) of the final TCA extract were placed in 15 ml of Bray's solution, and counted in a Packard Tri-Carb scintillation counter. DNA content was analysed according to Burton.[12] Since incorporation of ^{3}H-thymidine was measured in individual mice, 95 per cent confidence intervals for the mean of each group were calculated (using a table of "*t*" values) Disk electrophoresis on polyacrylamide gel was carried out according to Davies,[13] with the exception that the order of the layering of the gel solutions was reversed.

RESULTS

Specificity of THF in 105,000 g *supernatant extracts*

Preliminary experiments were carried out to confirm the finding by Klein *et al.*[11] that extracts isolated from the thymus specifically enhance ^{3}H-thymidine incorporation into the DNA of peripheral lymph nodes in normal mice. Extracts from various calf organs, in addition to thymus, were prepared and injected into intact mice. As can be seen from Fig. 1, calf, kidney, lung and salivary gland extracts stimulated ^{3}H-thymidine incorporation almost to the same extent as did calf thymus extract.

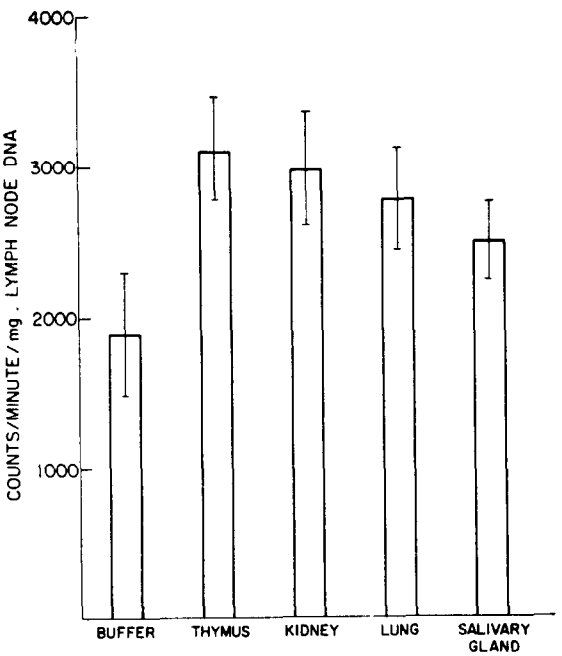

FIG. 1. Effect of 105,000 *g* extracts of calf organs on incorporation of ^{3}H-thymidine into lymph node DNA. Amount of protein per injection = 5·8 mg.

Four further experiments, in which the effect of calf thymus extract was compared to that of kidney extract (which showed the highest activity after thymus) are summarized in Fig. 2. Significant differences were not always found, in contrast to the

results of Klein *et al.*, in which extracts of rat spleens or lymph nodes did not increase ^{3}H-thymidine incorporation when injected into mice. In order to avoid effects attributable to the antigenic properties of foreign proteins, which could explain the above results, the activity of syngeneic mouse thymus and kidney extracts were

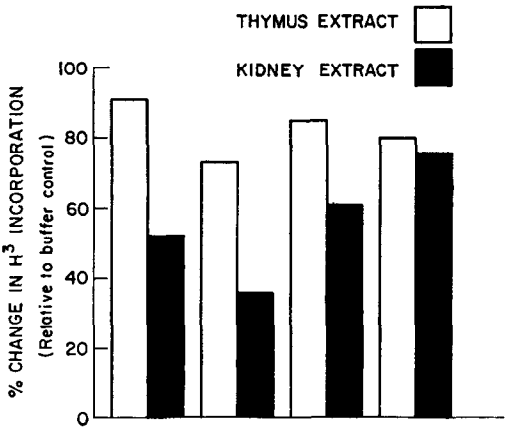

FIG. 2. Comparison of activity of 105,000 *g* extracts of calf thymus and kidney on the enhancement of ^{3}H-thymidine incorporation into lymph node DNA. The amount of protein per injection varied from 5·2 to 7 mg in the four independent experiments.

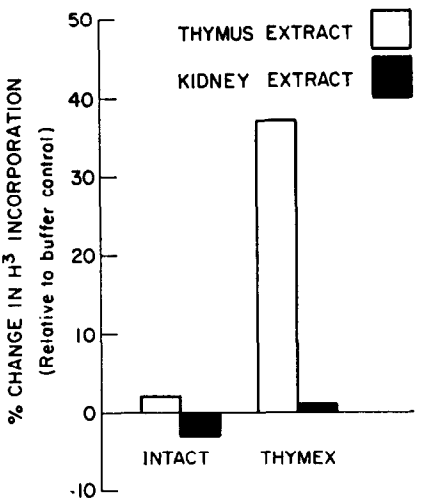

FIG. 3. Effect of syngeneic thymus and kidney 105,000 *g* extracts on the incorporation of ^{3}H-thymidine into lymph node DNA in intact and thymectomized mice. Amount of protein per injection = 5·3 mg.

compared. Neither substance enhanced ^{3}H-thymidine incorporation in intact mice (see Fig. 3, left side).

It appeared, therefore, that the characterization of THF by means of enhanced ^{3}H-thymidine incorporation required the finding of a biological assay system more sensitive than intact mice. Theoretically, thymectomized animals deprived of their

own source of hormones ought to be more responsive to an exogenous hormone. In fact, as shown on the right of Fig. 3, syngeneic thymic extract showed a stimulatory effect on ^{3}H-thymidine incorporation when injected into thymectomized mice, while the effect of kidney extract was negligible.

Significant differences between the effects of thymus and kidney extracts were also observed when xenogeneic (calf) material was injected into thymectomized mice, whereas intact animals showed less contrast (Fig. 4).

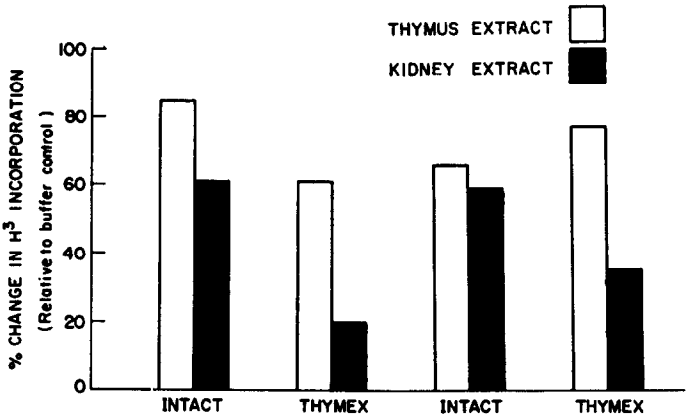

FIG. 4. Effect of thymectomy on the enhancement of ^{3}H-thymidine incorporation by calf thymus and kidney extracts. Two independent experiments, comparing thymectomized with intact mice in their response to calf thymus and kidney 105,000 *g* extracts, are presented. Amount of protein per injection in each experiment = 5·2 and 5·8 mg respectively.

To determine the specificity of THF in relation to its site of action, incorporation of ^{3}H-thymidine into thymectomized animals was measured in liver and kidney, and compared to that in lymph nodes. After treatment of mice with calf thymus extracts, a significant change was apparent only in lymph nodes (Fig. 5). This was, therefore, the test system adopted in the subsequent experiments.

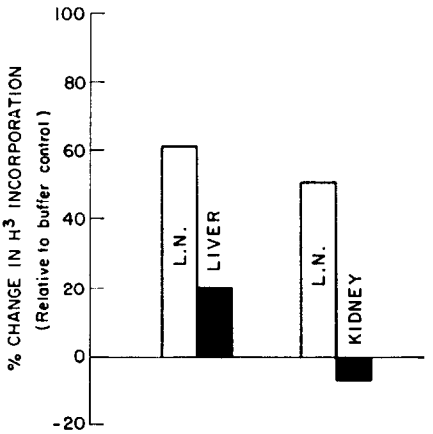

FIG. 5. Effect of calf thymus 105,000 *g* extract on the incorporation of ^{3}H-thymidine into different organs of recipient mice. Amount of protein per injection in the two experiments = 5·2 and 4·4 mg respectively. L.N. = Lymph nodes.

Characterization of THF

The active component appeared to be a heat-labile macromolecule since it was destroyed by boiling for 5 min, and was non-dialyzable (Table 1). The formation of a precipitate during dialysis could explain the reduced activity of the thymic extract after this step, and suggested that fractionation be carried out at other pH values (see below).

TABLE 1. DISTRIBUTION OF THF IN CALF THYMUS EXTRACT AFTER DIALYSIS*

Extract	cpm/μg lymph node DNA		% Change
	Mean	95% Confidence interval	
Phosphate EDTA buffer pH 5·7	3·8	3·4–4·2	—
Thymus extract	6·0	5·0–7·0	+57
Thymus extract diluted in half	4·9	4·3–5·5	+29
Thymus extract post equilibrium dialysis	4·8	4·3–5·3	+27
Equilibrium dialyzate	3·1	2·8–3·3	−18
Thymus extract post exhaustive dialysis	5·1	4·5–5·7	+34
Thymus extract, fraction soluble after 5 min boiling	3·6	3·1–4·1	− 5

* Calf thymus extract (100 ml of 105,000 *g* fraction) was dialyzed 24 hr with stirring at 5° against three successive 2 l. portions of phosphate-EDTA buffer pH 5·7 (exhaustive dialysis). Another portion of the same extract was dialyzed 24 hr against an equal volume of buffer (equilibrium dialysis). Concentrations of dialyzed extracts were adjusted to match the concentration of 8·8 mg protein/ml in the original extract and 0·5 ml portions were injected. In the equilibrium dialyzate and in the soluble fraction after boiling, only negligible amounts of protein were detected.

When fractionation by ammonium sulphate was performed with phosphate buffer at 3 different pH values (Table 2) the highest activity was recovered in the fraction precipitated at 20–40 per cent saturation. During subsequent dialysis of the 20–40

TABLE 2. DISTRIBUTION OF THE ACTIVE COMPONENT OF CALF THYMUS EXTRACTS IN AMMONIUM SULPHATE FRACTIONS*

Fraction	Processed at pH 5·7			Processed at pH 7·4			Processed at pH 8·4		
	cpm/μg lymph node DNA		% Change	cpm/μg lymph node DNA		% Change	cpm/μg lymph node DNA		% Change
	Mean	95% confidence interval		Mean	95% confidence interval		Mean	95% confidence interval	
Control buffer	4·3	3·6–5·0	—	3·8	3·1–4·5	—	3·9	3·2–4·6	—
Original extract	—	—	—	4·6	4·0–5·2	+21	4·9	4·2–5·6	+25
0–20% ammonium sulphate fraction	3·8	3·0–4·6	−12	†	—	—	4·8	4·3–5·3	+23
20–40% ammonium sulphate fraction	4·9	4·4–5·4	+14	5·9	5·2–6·6	+55	5·9	5·0–6·8	+51
40–65% ammonium sulphate fraction	4·6	3·9–5·3	+ 7	5·3	5·0–5·6	+39	4·9	4·2–5·6	+26
65–80% ammonium sulphate fraction	3·9	3·3–4·5	− 9	4·1	3·7–4·5	+ 8	3·9	3·3–4·5	0

* Amount of protein per injection = 5·8 mg.
† Negligible precipitate found during fractionation.

per cent ammonium sulphate fraction obtained from the pH 5·7 extract, a precipitate formed which could not be redissolved. This was accompanied by a significant loss in activity of the remaining fraction. Higher activity was recovered after dialysis of extracts prepared and fractionated at higher pH values (in the absence of EDTA). Therefore, further preparations were extracted and fractionated at pH 7·4.

Dose response curves (Fig. 6A) demonstrate the concentration of THF in the 20–40 per cent ammonium sulphate fraction as compared with either the 40–65 per cent fraction or the unfractionated extract. Saturation of activity of the most active fraction was reached with less than 1 mg of protein per injection, whereas the same protein concentration of the complete calf thymus extract was far from reaching this activity. Furthermore, three repeated injections of the most active fraction (20–40 per cent) resulted in a progressive increase of activity; this was not the case with the less effective 40–65 per cent fraction (Fig. 6B).

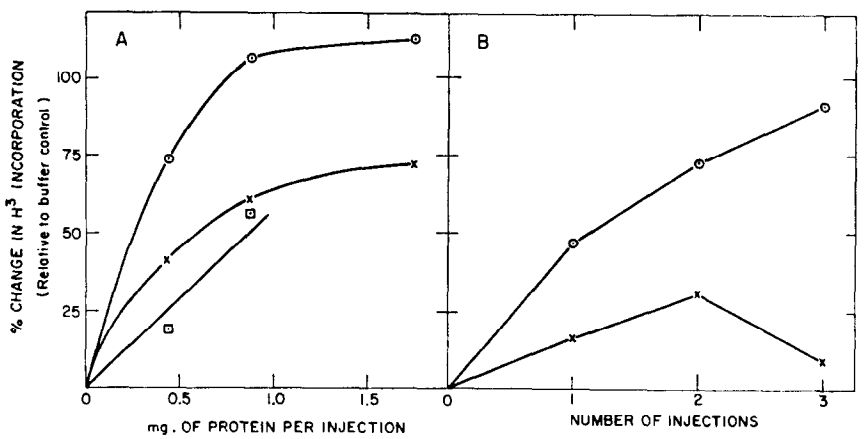

FIG. 6. Effect of ammonium sulphate fractions of 105,000 *g* calf thymus extract on the incorporation of ^{3}H-thymidine into lymph node DNA. A. Effect of increasing amounts of protein. B. Effect of increasing number of injections.

⊙ = 20–40 per cent ammonium sulphate fraction.
× = 40–65 per cent ammonium sulphate fraction.
⊡ = Unfractionated 105,000 *g* thymus extract.

The above experiments supported the belief that the active component of the thymus extract was protein in nature. More direct evidence for this was found when the activity of the 20–40 per cent ammonium sulphate fraction was almost completely destroyed by hydrolysis with pronase (Table 3).

To see whether THF activity could be assigned to a specific protein or group of proteins, gel electrophoresis of both active and inactive ammonium sulphate fractions was applied.

The electrophoretic pattern on acrylamide gel of the different ammonium sulphate fractions is shown in Fig. 7, as compared with a commercial sample of crystalline human serum albumin. A significant separation of some of the groups was obtained by ammonium sulphate precipitation. Of the large number of bands found, three were present in large amounts in the active 20–40 per cent ammonium sulphate fraction. One of these bands is located in the spacer gel (close to the sample gel), another at the boundary of the spacer and separation gels, and the third is located

nearby, in the separation gel. A large amount of protein from this fraction also remained in the sample gel. The other weak bands seen in the separation gel are those which appear in greater concentration in the less active fractions of the thymus extract.*

TABLE 3. EFFECT OF PRONASE ON THF ACTIVITY OF THE 20–40 PER CENT AMMONIUM SULPHATE FRACTION ISOLATED FROM CALF THYMUS*

Extract	cpm/μg lymph node DNA		% increase
	Mean	95% confidence interval	
Control phosphate buffer	4·6	4·1–5·1	—
Control pronase in buffer	5·0	4·5–5·5	+8
20–40% fraction incubated without pronase	7·8	6·6–9·0	+70
20–40% fraction untreated diluted in half	6·5	6·0–7·0	+41
20–40% fraction after incubation with pronase	5·3	4·7–5·9	+15
Pronase treated fraction post equilibrium dialysis	5·7	4·8–6·6	+24
Pronase treated fraction equilibrium dialyzate	5·2	4·8–5·8	+13

* Amount of protein per injection = 1·15 mg.
Sixty-six millilitres of the 20–40 per cent ammonium sulphate fraction in 0·1 M phosphate buffer, pH 7·4, containing 2·3 mg of protein per ml was incubated with pronase (40 μg/ml) (Calbiochem, Los Angeles) for 5 hr at 30°, along with a control portion without pronase, and pronase itself in buffer. An aliquot of the pronase treated fraction was dialyzed as described in Table 1 against Na phosphate buffer (0·1 M pH 7·4). Following pronase treatment only 14 per cent of the original protein remained precipitable by TCA.

DISCUSSION

The results presented here emphasize the problem of separating the specific effects of the thymic hormone factor from the general stimulating effects of foreign protein in lymphoid organs.

The demonstration of enhanced responses to a thymic factor in thymectomized mice (Fig. 3) can be related most probably to a reduced level of endogenous THF which permits the augmented response to exogenous THF. This explanation could also apply to the increased reproducibility of response to THF using thymus-deprived animals.

A further advantage of the use of thymectomized mice was a reduced response to the antigenic effect of foreign proteins which resulted in a relative increase in the effects of thymus extracts when compared with extracts from any other organ (Fig. 4). While such a reduced antibody response is only demonstrable several months after thymectomy in adult mice[14] the present results indicate the possibility of such a phenomenon occurring within weeks after thymus deprivation. If this is confirmed, the present technique may become a valuable tool for the measurement of reduced response to antigens.

Characterization of THF

Since THF proved to be heat-labile and non-dialyzable (Table 1), fractionation of calf thymus extract by ammonium sulphate could be used to enrich the active factor. The fact that THF activity was destroyed by pronase (Table 3) gave further evidence

* The electrophoresis was performed in a TRIS-Glycine buffer of pH 8·3. The sample and spacer gel containing 2·5% acrylamide had a pH of 6·7 and the separation gel with 7% acrylamide was at pH 8·9.

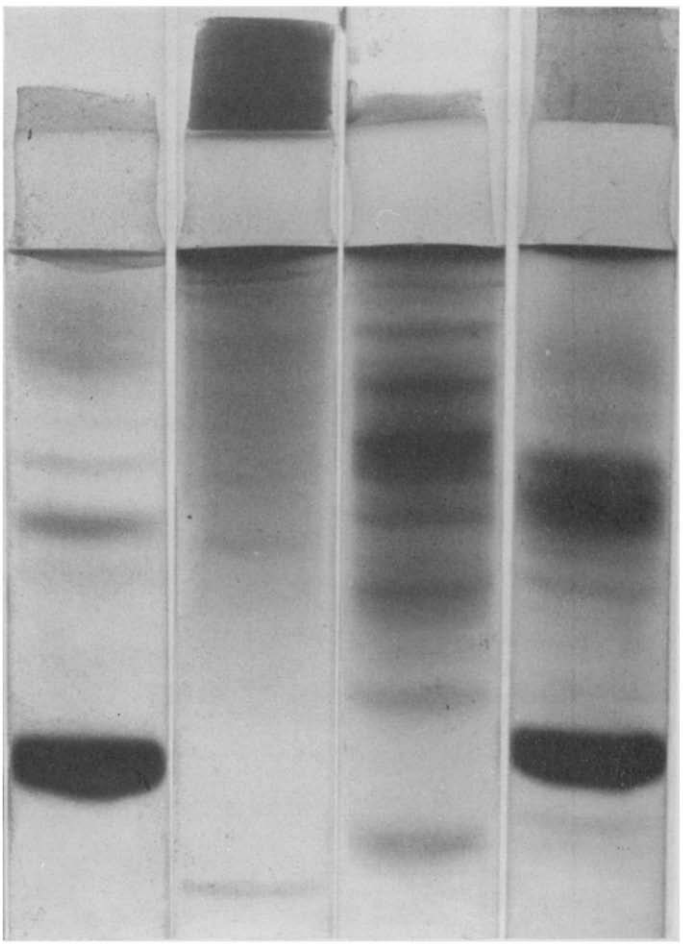

FIG. 7. Polyacrylamide gel electrophoresis patterns of ammonium sulphate fractions isolated from 105,000 *g* calf thymus extracts. Reading from the left: Crystallized human albumin (Mann Research Lab. Inc., New York), 20–40, 40–65, and 65–80 per cent ammonium sulphate fractions.

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that this factor is a protein or protein conjugate, though the possibility still exists that the active component is a smaller molecule, firmly bound to a protein, the latter acting as a stabilizer.

The patterns obtained by polyacrylamide gel electrophoresis (Fig. 7) of the active (20–40 per cent), partly active (40–65 per cent), and inactive (65–80 per cent) ammonium sulphate fractions provide further information on the problem of the specificity of THF. The finding that a significant amount of protein occurring in several different bands (which are absent in the active fractions), appears in a totally inactive fraction, rules out the possibility that THF activity is simply an antigenic response to foreign protein. Inspection of the gel patterns reveals a single band which appears to be present in both the active and partly active fractions which is absent from the inactive fraction. The separation, however, is performed under conditions in which a significant amount of protein is retained by the sample gel—which necessitates further characterization before it can be proposed as a candidate for THF activity. The other bands are less likely candidates, either because they are completely absent from the partially active fraction, or because of their presence in the inactive fraction in considerable amounts. The possibility that THF is present in such low concentration that it does not result in a visible band in any of the gels, is however not altogether excluded.

It is interesting to compare the properties of THF reported here to those of other lymphocytosis stimulating extracts. Goldstein *et al.*[15] (who also cite previous reports of thymic extracts having the property of stimulating lymphocytes) have recently described the partial purification of a carbohydrate-containing protein called thymosin, whose activity was assayed on lymphocyte suspensions *in vitro*. Since this protein was found to be relatively heat-stable and dialyzable it appears to be distinct from THF as characterized in this report. On the other hand, the lymphocytosis stimulating factor described by Metcalf,[16] which is heat-labile and non-dialyzable and therefore distinguishable from thymosin, may be related to THF.

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