Tryptophan and the Biosynthesis of Nicotinamide*

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There has been much discussion as to how tryptophan affects nicotinamide metabolism. It is generally believed that a direct conversion of tryptophan into nicotinamide takes place and many experiments have been carried out to elucidate the route of this conversion. Ellinger & Abdel Kader (1949*c*) observed that in *Escherichia coli* the biosynthesis of nicotinamide from ammonium lactate and ornithine was completely inhibited by DL-2-, 4-, 5- and 7-methyltryptophans in 2 mM-concentrations, growth not being affected. This inhibition suggested, in the light of Fildes's (1940) theory of metabolite antagonism, that tryptophan is actively involved in the biosynthesis of nicotinamide. Heidelberger, Gullberg, Morgan & Lepkowsky (1948) found recently that after administration of DL-tryptophan labelled in the β-position of the side chain with ^{14}C to dogs, rabbits and rats, kynurenine and kynurenic acid eliminated in the urine contained the labelled carbon atom, but that the urinary nicotinamide methochloride was free from radioactivity. These results suggested the possibility of tryptophan not being directly converted into nicotinamide, but having a catalytic coenzyme-like action in the biosynthesis of nicotinamide. The present paper deals with experiments to test this hypothesis.

EXPERIMENTAL

Experiments were carried out *in vitro* with mixed cultures from rat caecum content and with pure cultures of *E. coli*, and *in vivo* with rats.

Material used. The mixed cultures of rat caecum content were prepared in the following way: rats which had been found to have a high nicotinamide methochloride elimination were gassed, and immediately afterwards the content of the caecum was placed in weighing bottles under sterile conditions. One portion (about 200 mg. wet weight) was ground in a mortar with 10 ml. peptone water to form a homogeneous suspension and the rest was dried to constant weight in order to calculate the dry weight of the suspended material. From the suspension and from dilutions prepared from it agar plates were inoculated and smears were made in order to obtain qualitative and quantitative information about the organism present. There was a great variety of organisms contained in the mixed cultures. The most frequent types were hitherto unidentified cocci growing on agar in minute colonies, various strains of streptococci, staphylococci and coliform bacilli. Their relative frequency in the caecum content was about 8000:160:80:1, respectively. In addition, a number of other organisms (acidophilus, *Proteus*, Gram-positive cocci and diplococci, Gram-negative oval cocci and Gram-positive bacilli) were occasionally found. The *E. coli* used were the strains 3*c* and 4*c* isolated from human and rat faeces respectively, which were classified as type I faecal (Ministry of Health, 1939). The bacteria were grown on agar slopes and fresh cultures were made in ammonium lactate for inoculation.

Six rats used in the *in vivo* experiments were cross breds from the hooded Lister stock and P. Ellinger's albino stock, of both sexes, about 4 months old and weighing 280–320 g. They were kept on the mixed diet described previously (Ellinger, Fraenkel & Abdel Kader, 1947). Their average daily nicotinamide methochloride output and their response to nicotinamide had been examined for several months.

Salt solutions. The following solutions were used for incubation: (1) saline solution: 0·89% (w/v) NaCl; (2) ammonium lactate solution (Fildes, 1938); (3) saline phosphate solution: KH_2PO_4, 4·5 g., NaCl, 1·0 g.; $MgSO_4$, 0·02 g.; $FeSO_4$, 0·02 g., in 400 ml. water, adjusted with NaOH to pH 7·6; 4 ml. of this solution were made up to 10 ml. with water; (4) saline phosphate glucose solution: glucose was added to solution (3) to a final concentration of 1 mM.; (5) casein hydrolysate solution (Barton-Wright, 1944).

Analytical methods. Estimations were carried out in duplicate. The growth of bacteria was measured by comparing the opacity of a suspension with that of a standard (Brown, 1919). Nicotinamide was assayed microbiologically (Barton-Wright, 1944) either in the medium alone after removing the cells on the centrifuge, or in the medium and organisms after autoclaving. Nicotinamide methochloride was measured by the acetone method (Huff & Perlzweig, 1947). Free tryptophan (without indole) was estimated by the method of Horn & Jones (1945). DL-Tryptophan from different sources was used: for the rat experiments, commercial samples from Glaxo Laboratories Ltd. and from Roche Products Ltd., unpurified; for bacterial experiments the same samples, unpurified and purified by repeated recrystallization, and one sample prepared and kindly supplied by Dr R. L. M. Synge. No difference was observed in their action.

General arrangement of the experiments

In order to measure the consumption of tryptophan during the synthesis of nicotinamide, casein hydrolysate and ammonium lactate solutions without or with the addition of 2 mM-DL-tryptophan, and 2 mM-DL-ornithine dihydrochloride were inoculated with a heavy inoculum of pure cultures of *E. coli* 4*c*, the saline washings of broken *E. coli* 4*c* or a mixed culture from the rat caecum and incubated at

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* Some of the results presented in this paper were communicated to the Biochemical Society on 4 December 1948 (Ellinger & Abdel Kader, 1948).

37° for 48 hr. Nicotinamide and free tryptophan in the solution were measured before and after incubation.

The effect of washing the cells on their ability to synthesize nicotinamide from ammonium lactate and on the effect of tryptophan on this synthesis was studied. A 48 hr. culture of *E. coli* in casein hydrolysate was centrifuged and the organisms were washed with water. Samples of the suspension (75×10^6 organisms/ml.) were examined for nicotinamide production in an ammonium lactate medium in the absence and presence of 2 mM-DL-tryptophan, of the unwashed cells and 3, 6 and 9 times washed cells. The samples were prepared by adding 0·5 ml. of a culture containing 15×10^8 organisms/ml. to 9·5 ml. of salt solution.

The dependence of the nicotinamide formation by washed *E. coli* on the concentration of the tryptophan was examined in the following way: *E. coli* 4*c*, washed 3 times with water, were suspended in ammonium lactate solution containing various concentrations of DL-tryptophan, incubated at 37° for 48 hr. and the nicotinamide content was measured. In order to obtain more detailed information on the biosynthesis of nicotinamide when the enzyme system involved was freed from all possible substrates, fresh *E. coli* cells washed 4 times with water and then suspended in saline were disintegrated mechanically (Curran & Evans, 1942). A portion of the broken cells was washed several times with saline, part of the washings was dialysed through cellophan against saline under sterile conditions at room temperature for 48 hr. The outside solution was changed 3 times and continuously stirred mechanically during the dialysis. The completeness of the disintegration was examined by plating and taking viable counts at the various steps. The washed cells, the broken cells, the saline washings before and after dialysis and the exhaustively washed cell fragments were used for one or other of the following experiments. They were incubated in either saline phosphate, saline glucose phosphate or ammonium lactate solutions in the absence or presence of DL-tryptophan, DL-ornithine dihydrochloride or both at 37° for 48 hr. The nicotinamide content of the solution was estimated after incubation.

Rat experiments. The rats were kept in metabolism cages, the urine was collected daily and nicotinamide methochloride elimination was determined. At intervals of 3 or 4 days the rats were given either DL-tryptophan or nicotinamide in doses rising from 20 to 400 mg. and from 2·5 to 50 mg. respectively. These compounds were administered either orally by mixing them thoroughly with the food and controlling the completeness of the consumption, or intraperitoneally.

RESULTS

Experiments with bacteria

In all microbiological experiments two to three parallel experiments were carried out which gave similar results to those reported in detail. Experiments to relate the consumption of tryptophan to the synthesis of nicotinamide during incubation are given in Table 1. Although the growth of the pure cultures of *E. coli* used was good, no marked diminution of tryptophan was observed with the method used, which is rather insensitive but is probably the best existing procedure. 20 μg./ml. is the lower limit of assay and the standard deviation is about $\pm 5\%$. By concentrating the salt solutions to be examined *in vacuo*, amounts of 1 μg./ml. might be measured with a standard deviation of about $\pm 10\%$. Similar experiments carried out with washed cells and washings of broken cells also showed no detectable consumption of tryptophan during incubation, while measurable amounts of nicotinamide were formed. However, the amounts of nicotinamide formed are very small compared with those of the tryptophan present in the medium and the method used for the former is about ten thousand times more sensitive than that employed for tryptophan. The results with pure *E. coli* are therefore not conclusive. By contrast the tryptophan consumption of mixed cultures (Table 1) during nicotinamide synthesis in both ammonium lactate-ornithine-tryptophan and in casein hydrolysate solution is definite and outside the limit of error of the method. Growth was also considerable in these experiments.

When *E. coli* cells were washed repeatedly with water they gradually produced less nicotinamide (Table 2). The nicotinamide synthesis, however, was always restored to the level of the unwashed cells when 2 mM-tryptophan was added; with the washed cells no growth was observed. The relative effect of tryptophan on the synthesis of nicotinamide as

Table 1. *Content of tryptophan and nicotinamide of salt solutions before and after incubation*

		Tryptophan content of salt solution (μg./ml.)		Nicotinamide content of salt solution and organisms (μg./ml.)	
Inoculum	Salt solution	Before incubation	After incubation	Before incubation	After incubation
E. coli 4*c*	Ammonium lactate	<20	<20	10	32
	Ammonium lactate + ornithine (2 mM) + DL-tryptophan (2·2 mM)	442·0	441·4	8	49
	Casein hydrolysate	87·6	87·7	25	40
Saline washings of broken-up *E. coli* 4*c*	Ammonium lactate + DL-tryptophan (1·9 mM)	382·8	383·1	0	27
Mixed cultures from rat caecum	Ammonium lactate	<20	<20	15	31
	Ammonium lactate + DL-ornithine (2 mM) + DL-tryptophan (2·2 mM)	440·9	385·4	12	33
	Casein hydrolysate	87·7	71·2	10	25

compared with the control (without tryptophan) is greater in washed than in untreated cells. This effect is presumably due to the decreased nicotinamide synthesizing activity of the cells, caused by the washing.

Concentrations of DL-tryptophan up to about 0·1 mM had no stimulating effect on the nicotinamide synthesis by washed *E. coli* in ammonium lactate solution. At a concentration of 0·3 mM a marked increase was observed and the stimulating effect reached its maximum at concentrations of 1–2 mM. Higher concentrations did not produce any further increase in nicotinamide synthesis.

Table 2. *The effect of repeated washings of* E. coli 4 c *on the nicotinamide synthesis in ammonium lactate solution and on its increase by tryptophan*

(In all tubes the concentration of the cells was about 40×10^6 organisms/ml.)

	Nicotinamide content of salt solutions and organisms (μg./ml.)			
No. of washings ...	0	3	6	9
Salt solution				
Ammonium lactate	28	15	7·5	5·0
Ammonium lactate + 2 mM-DL-tryptophan	28	28	29	28

Table 3. *The effect of various concentrations of* DL-*tryptophan on the nicotinamide synthesis in ammonium lactate solution by washed* E. coli 4c

Tryptophan concentration (mM)	Nicotinamide content of salt solution and organisms (mμg./ml.)
0	10
0·1	11
0·3	15
1·0	25
2·0	25
5·0	24
10·0	24

Table 4. *Nicotinamide synthesis by washed and by broken* E. coli 4c *in ammonium lactate solution in the absence or presence of ornithine, tryptophan or both*

Compound added	Washed *E. coli*; nicotinamide content of salt solution and organisms (mμg./ml.)	Broken *E. coli*; nicotinamide content of salt solution (mμg./ml.)
None	6·3	7·5
DL-Ornithine (2 mM)	50·0	50·0
DL-Tryptophan (1 mM)	32·5	32·5
DL-Tryptophan (2 mM)	38·5	35·5
DL-Ornithine (2 mM) + DL-Tryptophan (1 mM)	49·0	48·0
DL-Ornithine (2 mM) + DL-Tryptophan (2 mM)	49·0	50·6

Synthesis of nicotinamide was also observed on incubation of washed *E. coli*, broken cells (Table 4), and undialysed or dialysed washings (Table 5) of disintegrated cells, in ammonium lactate solution alone or with either tryptophan, ornithine or both tryptophan and ornithine. Ornithine increased the synthesis considerably as did tryptophan, but the synthesis in the presence of ornithine was not markedly affected by simultaneous addition of tryptophan. When the same experiment was carried out in saline phosphate solution no nicotinamide was formed in the absence or presence of tryptophan alone, but nicotinamide was produced in the presence of ornithine by undialysed and dialysed cell washings. No nicotinamide was formed in saline phosphate solution containing tryptophan by washed cells even in the presence of glucose, or by saline washings of broken cells. No nicotinamide was formed by washed cells, broken cells or cell washings in 1% saline solution in the presence of ornithine or tryptophan or both of them. No nicotinamide was formed by the exhaustively washed cell fragments in either solution with or without added tryptophan or ornithine.

The enzyme systems involved in the nicotinamide synthesis were contained in the saline washings of the broken cells and retained their nicotinamide synthesizing activity after dialysis. *E. coli* 3*c* behaved similarly to *E. coli* 4*c*.

Experiments with rats

When rats (Table 6) were given rising doses of nicotinamide by mouth the urinary elimination of nicotinamide methochloride increased proportionately to the dose administered. The increase varied individually in the different rats, but in the same rat it rose in proportion to the dose given for doses between 2·5 and 50 mg. The response to intraperitoneally administered nicotinamide was in all six rats lower than when nicotinamide was given by mouth. When tryptophan was given by mouth in doses of 50 mg. and more, there was an increase in nicotinamide methochloride output proportional to the dose administered up to about 200 mg. Above this level no further increase of nicotinamide methochloride elimination occurred in any of the six rats. When tryptophan was injected intraperitoneally no increased nicotinamide methochloride output was observed in either rat after doses up to 100 mg. and only a very slight increase in two rats after doses of 200 mg.

DISCUSSION

Ellinger & Abdel Kader (1949*a*) showed that tryptophan increased the nicotinamide synthesis by mixed cultures from the rat faeces or caecum content, but not that by pure cultures of *E. coli*. This action had been attributed to an ability of some organisms of the mixed cultures, but missing from *E. coli*, to split the tryptophan molecule in such a way that the

Table 5. *Nicotinamide synthesis by undialysed saline washings of broken cells of* E. coli 3c *and* 4c *and dialysed washings of* E. coli 4c *in ammonium lactate and saline phosphate solutions in the absence and presence of* DL-*ornithine and* DL-*tryptophan*

Salt solutions	Compound added	Nicotinamide content of washings: Undialysed *E. coli* 3c (mμg./ml.)	Undialysed *B. coli* 4c (mμg./ml.)	Dialysed *E. coli* 4c (mμg./ml)
Ammonium lactate	None	6·3	10·5	12·5
	DL-Ornithine (2 mM)	35·0	40·0	33·5
	DL-Tryptophan (2 mM)	25·0	37·0	40·0
	DL-Ornithine (2mM) + DL-tryptophan (2mM)	35·0	40·0	35·0
Saline phosphate	None	0	0	0
	DL-Ornithine (2 mM)	15·0	25·0	12·5
	DL-Tryptophan (2 mM)	0	0	0
	DL-Ornithine (2mM) + DL-tryptophan (2 mM)	15·0	26·0	12·5

Table 6. *The urinary elimination by rats of nicotinamide methochloride following oral or parenteral administration of nicotinamide or* DL-*tryptophan*

Compound tested	Dose (mg.)	Route of administration	Increase in nicotinamide methochloride output (mg.) Rat 1	Rat 2	Rat 3	Rat 4
Nicotinamide	2·5	Oral	1·704	1·099	1·068	2·062
	5·0	Oral	5·128	2·423	2·524	5·146
	10·0	Oral	8·138	5·871	6·249	9·402
	20·0	Oral	15·133	10·210	11·947	16·670
	50·0	Oral	28·389	18·280	28·885	40·117
	10·0	Intraperitoneal	2·931	4·136	5·424	5·688
DL-Tryptophan	20	Oral	0	0	0	0
	50	Oral	1·246	0·631	0·300	1·530
	100	Oral	3·132	1·395	0·450	2·400
	200	Oral	5·909	2·565	1·149	4·076
	400	Oral	6·493	2·550	1·125	3·039
	50	Intraperitoneal	0	0	0	0
	100	Intraperitoneal	0	0	0	0
	200	Intraperitoneal	0·840	0·085	0	0

Experiments with two more rats revealed essentially similar results.

latter could be utilized by *E. coli* for the synthesis of nicotinamide. When washed coli, instead of untreated coli, were examined for their ability to synthesize nicotinamide and the action of tryptophan on this synthesis, it was found that tryptophan increased nicotinamide production by the washed cells as compared with the control in ammonium lactate solution in a similar way as was found with mixed cultures. The systematic examination of the effect of repeated washings on the nicotinamide production by *E. coli* and on the tryptophan action on the synthesis (Table 2) showed that washing gradually diminished the synthesizing power of the organism and that this was restored by added tryptophan. The apparent relative increase in nicotinamide synthesis by added tryptophan as compared with that in ammonium lactate alone was, therefore, due to the restoration of tryptophan removed by washing. This fact indicated that the stimulating effect of tryptophan on the nicotinamide synthesis by mixed cultures might possibly be due to a reduced tryptophan concentration of the medium. That this was so, was shown by investigation of the tryptophan content after incubation of mixed cultures (Table 1). This was considerably reduced during incubation and nicotinamide synthesis. Whether the unchanged tryptophan content of the salt solutions of pure *E. coli* cultures during growth and nicotinamide synthesis was due to tryptophan formation equal to tryptophan destruction or to the fact that changes were too small to be detected by the method of assay could not be decided.

The experiments show, at least, that with pure cultures of *E. coli* nicotinamide synthesis is not accompanied by a marked consumption of tryptophan as in the case of mixed cultures. The concentration of tryptophan optimal for maximum nicotinamide formation was found to be 1–2 mM (Table 3) and the concentration of about 2 mM was, therefore, used in all experiments.

The facts discussed so far, namely, that tryptophan stimulates nicotinamide synthesis by *E. coli* in ammonium lactate solution only when its concentration in the cells is suboptimal, that its concentration

in mixed cultures is suboptimal and consequently the nicotinamide synthesis by these mixed cultures is stimulated by added tryptophan and that rising concentrations of added tryptophan increase nicotinamide synthesis by washed coli cells proportionally to its concentration only until the optimal level is reached, can best be explained by a catalytic action of tryptophan in the nicotinamide synthesis by *E. coli*. Moreover, the fact that no nicotinamide is formed by undialysed or dialysed washings of broken cells when tryptophan, but no other source of nitrogen, is present in the salt solution (Table 5) supports this hypothesis.

As had been shown (Ellinger & Abdel Kader, 1949*a*) nicotinamide synthesis by *E. coli* is independent of the intactness of the cell structure. This is confirmed by the results shown in Table 5. Moreover, the fact that the enzyme system is contained in the washings of the broken cells and can be dialysed without loss of activity, made it possible to remove all soluble and dialysable substrates from the enzymes.

It had been suggested that ornithine might be an intermediate in the biosynthesis of nicotinamide (Klein & Linser, 1932; Guggenheim, 1940; Ellinger & Abdel Kader, 1949*a*). In the presence of ornithine, tryptophan had very little or no effect on nicotinamide synthesis by either of the coli preparations. If ornithine is formed as an intermediate the findings would mean that tryptophan is mainly involved in the early stages of the nicotinamide synthesis, i.e. from ammonia and lactate to ornithine.

The experiments on rats (Table 6) confirmed the findings of Schweigert & Pearson (1947) and of Ellinger & Abdel Kader (1949*a*) that tryptophan increases the nicotinamide methochloride output to a far higher degree when given orally than parenterally. The differences were, however, much greater than described before; of six rats only two showed a slight increase after an intraperitoneal administration of 200 mg. DL-tryptophan. These results indicate clearly the importance of the intestinal flora for the nicotinamide-saving action of tryptophan. If the tissues are concerned with this action at all, this can only be of secondary importance.

The fact that rising doses of tryptophan increase the nicotinamide formation, as indicated by the urinary elimination of its methyl derivative, up to a maximum which could not be raised by larger doses of tryptophan though it could be by nicotinamide application, indicated that a kind of tryptophan saturation has to be reached to obtain optimum nicotinamide formation. The maximum was reached in each of six rats of about 300 g. when 200 mg. of DL-tryptophan were orally administered. If one were to assume an even distribution of the ingested tryptophan over the whole body, this would mean that saturation was reached at a concentration of 3·3 mM. This assumption of even distribution is certainly incorrect and a comparison between rat and *E. coli* is not admissible. But it may be noted that in both rat and *E. coli* maximum nicotinamide formation was observed with tryptophan concentrations of the same order. The results with the rat also support the conception of a catalytic action of tryptophan in nicotinamide biosynthesis. However, there are some apparent objections to this conception: a catalytic action would mean that tryptophan would play the role of a coenzyme in the nicotinamide synthesis and the methyltryptophans substituted in the indole nucleus would, therefore, be metabolite antagonists to a coenzyme. No such case has been described so far. The concentration of tryptophan producing optimal nicotinamide synthesis compared with the amount of nicotinamide synthesized is high and this is unusual for a catalyst. But catalytic reactions are known which require a fairly high concentration of the catalyst, e.g. nickel in catalytic hydrogenation or aluminium chloride in Friedel and Craft's reaction.

SUMMARY

1. The effect of tryptophan has been studied on the biosynthesis of nicotinamide by washed and broken *E. coli*, by undialysed and dialysed washings and washed fragments of the broken cells, in various salt solutions.

2. The effect of rising doses of tryptophan on the nicotinamide methochloride elimination by rats has been examined.

3. Nicotinamide was formed by washed and broken *E. coli* and by their undialysed and dialysed washings, but not by exhaustively washed cell fragments, in salt solutions containing sources of nitrogen other than tryptophan, such as ammonium ions or ornithine; the formation was increased by the addition of tryptophan.

4. No nicotinamide was formed by these cell preparations in the presence of tryptophan when no other source of nitrogen was present.

5. The effect of rising doses of tryptophan on the nicotinamide synthesis by washed *E. coli* reached a maximum at concentrations of 1–2 mM.; a further increase of the concentration did not increase the synthesis of nicotinamide.

6. In rats comparative experiments on the effect of tryptophan administered either orally or intraperitoneally confirmed the importance of the intestinal flora for the action of tryptophan on the nicotinamide metabolism. By feeding rising doses of tryptophan it was shown that as in *E. coli* a saturation point could be reached.

7. The bearings of these findings on the possible role of tryptophan in the biosynthesis of nicotin-

amide are discussed. It could best be explained by a kind of catalytic action of tryptophan on the nicotinamide synthesis

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Since this paper was submitted, Heidelberger, Abraham & Lepkowsky (1948) have reported feeding DL-tryptophan labelled in the β-position of the side chain with ^{14}C to rats; nicotinamide methochloride eliminated after ingestion contained a labelled C atom in the carboxylic acid group. This does not, however, prove, as is claimed by these authors, that 'the conversion proceeds in the same fashion in the rat as in Neurospora'.

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Some Observations on the Amino-acid Distribution of Collagen, Elastin and Reticular Tissue from Different Sources

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By histological methods three types of connective tissue can be shown to be present in skins; collagen, elastic fibres and reticular tissue. Collagen, in the form of white bundles of interweaving fibres, makes up the greater part of the skin; the elastic fibres are pale yellow in colour and occur mainly in the grain layer; reticular tissue occurs around fat deposits and possibly as sheaths round the collagen fibre bundles (Dempsey, 1946). It is extremely difficult to isolate these three different tissues from skins. Tissues which stain similarly can be isolated fairly readily from other parts of the animal body: elastic fibres from the ligamentum nuchae (Vandegrift & Gies, 1901) and reticular tissue from lymph nodes (Bate-Smith, 1947) and from the large fat deposits (Maximow & Bloom, 1935; Dempsey, 1946), and some studies have been made on them. Before these tissues can be assumed to be identical with those occurring in skin, however, more conclusive evidence than staining reactions is required.

The aim of the present investigation was to determine what major differences in composition existed between the three main types of connective tissue, and to obtain information as to whether similar tissue preparations from different sources had the same composition.

EXPERIMENTAL

Analytical methods

Total N, amide N, amino N and titration curves were determined as described by Bowes & Kenten (1948*a*).

Preparation of samples

Ox-hide and alkali-treated sheepskin collagen were prepared as previously described (Bowes & Kenten, 1948*a*, *b*). Reticular tissue was prepared from lymph nodes and from the adipose tissue of ox. The wet lymph nodes were sliced, extracted with 5% (w/v) NaCl, washed and macerated with many changes of 30% ethanol in water. The fibrous mass was