a hypothesis it is difficult to account for the increase of the free form of the vitamin in the milk, when, owing to deprivation of carotene, the cow is forced to use its liver reserves. When thyroxine causes these liver reserves to be mobilized at an enhanced rate, the limited power of the mammary gland to esterify vitamin A is placed even more in evidence (Chanda & Owen, 1952). Nevertheless, it must be supposed that the gland does esterify the vitamin, since the present experiments show that the ester form is absent from the blood of cows which are deprived of carotene, while the bulk of the vitamin A in the milk is still in the ester form (Table 5).

The present experiments confirm the observation of Chanda (1953) that vitamin-A ester occurs in cattle blood, and show that it reflects the intake of β -carotene, as indeed the experiments of Thompson, Ganguly & Kon (1949) would lead one to expect. In the absence of evidence as to the relative rates at which the mammary epithelium can take up vitamin-A alcohol and vitamin-A ester, it is not possible to say in which form the major part of the vitamin A originating from dietary carotene is absorbed by the mammary gland. β -Carotene is absorbed from the gut into the intestinal lymphatics (Thompson *et al.* 1949; Glover, Goodwin & Morton, 1948; Eden & Sellers, 1950) chiefly as vitamin-A ester. From these lymphatics the vitamin-A ester will pass to the venous system, to the lungs and then to the general body circulation, so that the liver will have no better chance of intercepting it than will the mammary gland.

SUMMARY

1. Lactating cows were fed, in alternate periods, carotene-free diets and diets containing carotene; the intakes of carotene in the successive carotene periods increased as the experiment progressed.

2. In confirmation of the authors' previous results, carotene deprivation caused the amount of vitamin-A alcohol in the milk to increase.

3. The concentration of vitamin-A alcohol in the blood serum was not affected by the removal of carotene from the diet.

4. Carotene deprivation caused vitamin-A ester to disappear completely from the blood serum. The ester reappeared when carotene was again fed.

5. It is concluded from these results and from earlier results with thyroxine-treated animals that increases in vitamin-A alcohol in the milk of cows indicate a transfer of reserves of vitamin A from the cow's liver to the milk.

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The Excretion of Kynurenine and 3-Hydroxykynurenine by Man

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3-Hydroxykynurenine was first shown to be a tryptophan metabolite in animals in experiments with pyridoxine-deficient rats (Dalgliesh, Knox & Neuberger, 1951; Dalgliesh, 1952*b*). Later, Makino, Satoh, Fujiki & Kawaguchi (1952) claimed that 3-hydroxykynurenine is responsible for the diazo

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reaction and Weiss urochromogen reaction shown by the urine of patients suffering from acute tuberculosis. This suggested that in acute tuberculosis the function of pyridoxine-deficient enzymes might be deranged, and the purpose of this work was to investigate this possibility. It was found that the excretion of hydroxykynurenine by man is unrelated to tuberculosis and occurs in many cases

of fever of various origins. The excretory pattern of tryptophan metabolites found in fever is compared with that found when tryptophan is ingested by the normal human, and the significance of the results is discussed.

METHODS

Preparation of the urines. Overnight urine samples of approximately 200 ml. were obtained from patients having fever (taken as a body temperature exceeding 100° F). A portion was tested by the diazo reaction and the remainder, when appropriate, prepared for chromatography using methods described by Dalglish (1952*b*) and Charconnet-Harding, Dalglish & Neuberger (1953). Briefly, the urine was acidified with acetic acid, shaken with charcoal which had been deactivated with 4% stearic acid, the charcoal filtered off, washed with water, and the aromatic substances were eluted from the charcoal by 5 or 10% (w/v) aqueous phenol. Extracts from human, unlike rabbit and rat, urines deposited crystals if allowed to stand. These were isolated in some cases and identified as uric acid. Normally when precipitation occurred the precipitate was isolated and discarded. The phenol eluates, after filtration where necessary, were evaporated to dryness under N₂ at a low pressure and at about 50–60°. The residues were taken up in water for paper chromatography, any uric acid present remaining undissolved.

Paper chromatography (cf. Dalglish, 1952*b*). Samples of the urine concentrates were run overnight on paper chromatograms (descending technique) using the organic layer from a freshly prepared butanol:acetic acid:water mixture (4:1:5, v/v). Hydroxykynurenine was detected in particular by its characteristic yellow-green fluorescence under ultra-violet illumination, by the almost immediate formation of a brown-black spot on treatment with ammoniacal silver nitrate and by the pink colour given with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde in dilute HCl). Kynurenine was detected in particular by its fluorescence and behaviour with Ehrlich's reagent. The *R_F* values of kynurenine and hydroxykynurenine were shown to agree with those of authentic specimens. The relative amounts were judged visually from the size and intensity of the spots. When appreciable amounts of hydroxykynurenine were present it could be clearly seen as a yellow spot on the untreated chromatogram. The method of use of the various reagents has already been described (Dalglish, 1952*b*). In addition, hydroxykynurenine reacted extremely rapidly with the ferric chloride ferricyanide reagent for phenols devised by Barton, Evans & Gardner (1952). Attempts to develop a reagent using tetrazolium salts were unsuccessful.

In view of the widespread use of Dent's (1948) 'map of the spots', the positions of kynurenine and hydroxykynurenine were determined on two-dimensional chromatograms run by the ascending technique in phenol and then collidine, using metal frames (Datta, Dent & Harris, 1950). Kynurenine and hydroxykynurenine corresponded closely in positions with phenylalanine and tryptophan, respectively. The two former could be distinguished by their fluorescence, but Ehrlich's reagent was inapplicable, owing to excessive background, unless collidine was removed by successive washing of the paper with ethanol and ether.

Weiss reaction and diazo reaction. The Weiss urochromogen reaction (production of a golden-yellow colour on treatment of the urine with dilute permanganate) was carried out

according to the Merck Index (1940*a*). It is an unspecific reaction which might be given by many reducing substances, and is therefore not easy to interpret. For routine examination we used the diazo reaction, which calls for comment. The procedure used, based on procedures described in the Merck Index (1940*b*) was as follows: equal volumes (about 5 ml.) of urine and 0.5% (w/v) sulphanic acid in approx. 0.5*N*-HCl were mixed, and 3–5 drops of a 0.5% (w/v) NaNO₂ solution added, followed by 6–8 drops of NH₃ solution (sp.gr. 0.880). The tube was then vigorously shaken. A positive reaction was one in which, after shaking, a pink or red colour appeared in the foam. Many substances of possible occurrence in urine, such as phenols, glyoxaline derivatives and pyrroles (cf. van den Bergh reaction) give in solution a red or orange colour with the diazo reagent, and in fact some degree of red coloration in the solution was found with all urines examined. Most of these on shaking gave a yellow foam quite distinct from the pink foam found when hydroxykynurenine was present. Some experiments were carried out with authentic hydroxykynurenine and with 3-hydroxyanthranilic acid. The latter contains the same aromatic system as hydroxykynurenine, and has been isolated from the urine of a tuberculous patient (Musajo, Spada & Coppini, 1952). It was thought likely that it would behave similarly to hydroxykynurenine in the diazo reaction. On submitting either hydroxykynurenine or hydroxyanthranilic acid to the diazo reaction, a red colour was produced in solution, but no foam was formed on shaking. On adding protein (a drop of serum) or detergent and again shaking, the hydroxykynurenine gave the typical pink foam, whereas the foam from the hydroxyanthranilic acid was yellow. The 'foam' method of carrying out the diazo reaction seems satisfactory for human urines. That this is fortuitous, however, is shown by the fact that almost any rabbit urine will give a positive result by this technique, even though no hydroxykynurenine can be detected chromatographically.

Feeding experiments. Two normal males took by mouth 10 g. DL-tryptophan as a slurry in water. Further water (about 1 l. altogether) was taken and urines were collected for the periods 0–8 and 8–24 hr. The urines were worked up and examined chromatographically as described above. In a further experiment a normal male was injected intravenously with 0.5 ml. typhoid vaccine. After a short while the body temperature started to rise, and 10 g. DL-tryptophan was taken by mouth as before. The body temperature remained at 101.4° F for about 12 hr., and urine excreted during this period was collected and examined.

RESULTS

The conclusion of Makino *et al.* (1952) that hydroxykynurenine is responsible for the diazo reaction is confirmed. Wherever a positive reaction was encountered the urine was found by paper chromatography to contain hydroxykynurenine, and no hydroxykynurenine was found in those urines showing a negative reaction. Altogether some hundred urines were examined, including normal controls. The results are summarized in Tables 1–3. Table 1 summarizes cases in which hydroxykynurenine excretion was observed, and shows that the excretion is not dependent on sex or drug

treatment. Table 2 summarizes all fever patients whose urine was examined chromatographically, whilst Table 3 similarly summarizes non-febrile cases. The only apparent common factor in the positive cases is the existence of fever. In Table 3 it will be observed that hydroxykynurenine excretion was not observed in acute thyrotoxicosis, in which the metabolic rate is raised without accompanying fever, or in various other non-febrile states associated with weight loss. Kotake & Tani (1952) have recently reported the excretion of hydroxykynurenine in insulin-treated diabetes. We have examined a large number of cases of insulin-treated diabetes (Table 3), and in no case was hydroxykynurenine excretion observed. In view of the marked tendency for diabetes to co-exist with other disorders it seems probable to us that the excretion observed by Kotake & Tani may be ascribed to fever due to complications, and not to diabetes as such.

When DL-tryptophan was taken by mouth by the normal human, considerable amounts of kynurenine were excreted in the urine, but no hydroxykynurenine. It is of interest that D-kynurenine was excreted as well as the L-isomer (the two are distinguishable chromatographically; Dalgliesh, 1952*a, b*), though the L-isomer predominated. Similar conversion of D-tryptophan into D-kynurenine has been observed *in vivo* in the rabbit (Kotake & Ito, 1937), rat (Dalgliesh, 1952*b*) and man (Langner & Berg, 1953), although the conversion does not take place with isolated systems (Knox & Mehler, 1950). Kynurenine excretion was also observed in some of the febrile cases excreting

hydroxykynurenine. With patients excreting large amounts of hydroxykynurenine, a much smaller amount of kynurenine excretion occurred, whereas when hydroxykynurenine excretion was low, no kynurenine could be detected.

Ehrlich's *p*-dimethylaminobenzaldehyde reagent revealed many indolic compounds which were not investigated in detail. In all cases some tryptophan was excreted. Tryptophan is known to be normally excreted by the human (e.g. Albanese & Frankston, 1945). The four principal Ehrlich-reacting compounds otherwise encountered are briefly summarized in Table 4 (*A-D*). In almost all urines, two fast-running indolic compounds, *A* and *B*, appeared. One or both of these may correspond to the ether-soluble tryptophan metabolite of Sarett & Goldsmith (1949) or to the 'aberrant metabolite' of Albanese (1944) and Albanese & Frankston (1944).

Table 2. *Summary of fever cases in which the urine was examined chromatographically*

Diagnosis	No. of cases	
	Excreting hydroxykynurenine	Not excreting hydroxykynurenine
Tuberculosis (excluding mild cases)	4	2
Bronchitis	2	2
Syphilis and bronchitis	1	—
Influenza and bronchitis	—	1
Pneumonia	1	2
Pneumonia and pernicious anaemia	—	1
Pleural effusion	—	1
Influenza	1	—
Bronchopneumonia	—	1
Lupus erythematosus	1	1
Cor pulmonale	1	1
Epilepsy and paroxia	1	—
Jaundice	—	1
Leukaemia	1	—
Tonsillitis	—	1
Thrombophlebitis	—	1
Peptic ulcer and pyrexia due to malignancy	—	1
Coronary thrombosis	—	2

Table 1. *Excretion of hydroxykynurenine in fever, showing independence of sex and drug treatment*

Sex	Diagnosis	Drug treatment
M.	Tuberculosis	Streptomycin and <i>p</i> -aminosalicylic acid
M.	Tuberculosis	Streptomycin and <i>p</i> -aminosalicylic acid
F.	Tuberculosis	<i>iso</i> Nicotinic acid hydrazide and <i>p</i> -aminosalicylic acid
M.	Tuberculosis	<i>iso</i> Nicotinic acid hydrazide and streptomycin
F.	Bronchitis	Penicillin and ephedrine
M.	Bronchitis	Penicillin and aminophylline
M.	Syphilis and bronchitis	Penicillin
F.	Pneumonia	Penicillin and codeine
F.	Influenza	None
F.	Lupus erythematosus	Cortisone
M.	Cor pulmonale and pleurisy	Procaine penicillin
M.	Epilepsy and paroxia	None
M.	Leukaemia	None

Table 3. *Non-febrile cases in which no hydroxykynurenine excretion was observed*

Diagnosis	No. of cases
Acute thyrotoxicosis	6
Sarcoidosis	1
Prolapsus intervertebralis	2
Myasthenia gravis	1
Polyneuritis	1
Psychotic depression	1
Muscular dystrophy	1
Rheumatoid arthritis	1
Bronchogenic carcinoma	1
Coronary infarct	2
Diabetes and muscular atrophy	1
Diabetes	43

Table 4. *Behaviour of some unknown tryptophan metabolites, and of some known compounds*

(R_F values from descending chromatograms on Whatman no. 1 paper. Solvent mixture; butanol:acetic acid:water; 4:1:5, v/v.)

Substance	Colour with Ehrlich's reagent	Mean R_F value
<i>A</i>	Blue	0.88
<i>B</i>	Blue	0.75
<i>C</i> (? indican)	Orange-red, developing slowly	0.44
<i>D</i>	Blue	0.33
Indoleacetic acid	Blue	0.88
<i>N</i> ^α -Acetyltryptophan	Blue	0.86

The slower-running of the pair, substance *B*, is present in the greater amount. Substance *A* showed some resemblance in chromatographic behaviour to both indoleacetic acid and *N*^α-acetyltryptophan (Table 4), though slight differences appeared on paper electrophoresis. As indoleacetic acid is known to occur in human urine (e.g. Pacheco, 1953), *A* might represent this substance. Substance *D*, which only appeared occasionally, runs more slowly than tryptophan and probably contains a fairly hydrophilic system. Substance *C* appeared often, but not always, and from its chromatographic behaviour we consider that it almost certainly represents indican. It slowly gives an orange-red or brown colour with Ehrlich's reagent; indican is well-known to give such a colour with *p*-dimethylaminobenzaldehyde. If the chromatogram is sprayed with dilute acid and heated, substance *C* gives a blue or green-blue spot, whereas similar treatment with alkali has no effect. Indican is readily hydrolysed by acid (the resultant indoxyl being oxidized to the blue indigo) but is stable to alkalis. Substance *C* on chromatograms gives a yellow-brown colour on treatment with nitrous fumes, couples slowly with diazotized sulphanilic acid to give a red colour, but does not give a ninhydrin reaction nor reduce ammoniacal silver nitrate. All these are the expected properties for indican, but unfortunately we had no authentic material for direct comparison.

DISCUSSION

Hydroxykynurenine is not excreted in all cases of fever. It was observed in almost half of the cases examined, but the patients available to us were those in a general hospital. They did not include any with infectious diseases (e.g. measles, typhoid fever, scarlet fever, etc.), which have long been recognized to give rise to diazo-positive urines. Moreover, with drug treatments now available, patients are not often febrile for long after admission to hospital, and the fever may in some cases have wholly or partly dropped before collection of urines for examination. Thus it is likely that if

all types of fever patients were examined during the course of an untreated fever, an appreciably higher proportion would be found to be excreting hydroxykynurenine.

The amount of hydroxykynurenine excreted in some cases seemed much too large to have been derived from dietary tryptophan, and we consider it likely that the excretion is due to a high rate of breakdown of body-proteins 'flooding' the enzyme systems responsible for tryptophan breakdown. Albanese & Frankston (1945) presented evidence that the small amount of tryptophan normally found in human urine is derived from protein breakdown and not from the diet. We have noticed in the present work that hydroxykynurenine excretion is accompanied by a considerably increased excretion of uric acid as is to be expected during general tissue breakdown. Increased uric acid excretion was also observed in thyrotoxicosis, though here it was not accompanied by hydroxykynurenine excretion. Owing to individual variations in the rate of protein breakdown and in the size and activity of the available enzyme pool, it is to be expected that the threshold for excretion of a substance such as hydroxykynurenine would not be exceeded in all cases.

The fact that hydroxykynurenine, but little or no kynurenine, is excreted in fever, whilst the normal human taking an excess of tryptophan by mouth excretes kynurenine and not hydroxykynurenine, is very striking. The place of kynurenine and hydroxykynurenine in tryptophan metabolism is summarized in Fig. 1 (cf. Dalglish, 1951, 1952*b*). (It is possible that kynurenine and hydroxykynurenine themselves are not the normal intermediates but that derivatives such as phosphates (cf. Charconnet-Harding *et al.* 1953) are involved. These, if formed, might well be excreted as kynurenine or hydroxykynurenine. In this discussion the terms kynurenine and hydroxykynurenine are considered to cover possible intermediates at the kynurenine and hydroxykynurenine levels of oxidation, respectively.)

Both kynurenine and hydroxykynurenine are split by the same enzyme, kynureninase. It has been shown by Knox (1953) that in the rat the potential rate of formation of kynurenine exceeds the potential rate of breakdown by kynureninase. The first step in tryptophan oxidation is moreover adaptive, increasing this difference between the potential rates of kynurenine formation and breakdown. A similar state of affairs may well exist in man. Besides being attacked by kynureninase, any kynurenine formed can be removed in three other ways: (a) by oxidative deamination or transamination to give, after ring closure, kynurenic acid, (b) by conjugation involving one or more of the so-called 'detoxication' mechanisms, and (c) by ring

oxidation to give hydroxykynurenine. The absence of significant amounts of kynurenic acid in human urine suggests that reaction (a) is quantitatively of slight importance. The absence of appreciable amounts of conjugated derivatives of kynurenine (or hydroxykynurenine) from our chromatograms suggests that (b) is also of slight importance. The absence of any appreciable amount of anthranilic acid or derivatives from human urine suggests that kynurenine is not normally present in sufficient amount (or possibly not at the correct site) for the action of kynureninase to be quantitatively significant. The greater part of normal kynurenine would therefore appear to go by route (c) to hydroxykynurenine. The results obtained on feeding tryptophan to the normal man are consistent with this view. The large excretion of kynurenine would indicate its formation at such a rate that the enzymes normally further metabolizing it are flooded, so that kynurenine is excreted as such. The absence of hydroxykynurenine excretion would indicate that hydroxykynurenine is further metabolized as fast as it is formed, which would also suggest that the enzyme converting kynurenine into hydroxykynurenine is not adaptive.

More difficult to explain is the fact that when hydroxykynurenine is excreted in fever the amount is always greatly in excess of any kynurenine that might be present. This indicates that if the apparent block in fever is simply due to one enzyme being present in limiting amount, this block occurs after the hydroxykynurenine stage. It is of interest that not more than possible traces of conjugated derivatives (sulphate, etc.; cf. Dalgliesh, 1952*b*) of hydroxykynurenine were observed. This suggests that either the human cannot deal with an excess of hydroxykynurenine by using 'detoxicating' mechanisms available to the rat, or that the hydroxykynurenine is excreted before it can be attacked by the appropriate enzymes. As sulphate and glucuronide excretion normally occur in man, the second explanation seems the more likely.

In a preliminary communication (Dalgliesh & Tekman, 1953) we advanced as possible explana-

tions (a) that the enzymes involved were markedly temperature-dependent, so that, for example, the relative rates of two successive reactions might have been altered by change of temperature, and (b) that pathways for metabolism of endogenous and exogenous tryptophan differed. We also suggested that an alternative pathway from tryptophan to hydroxykynurenine might have been involved, possibly via 7-hydroxytryptophan. As 7-hydroxytryptophan has now been synthesized (Ek & Witkop, 1953) and shown not to be attacked by the enzyme system converting tryptophan into kynurenine (Udenfriend, Clark & Titus, 1953) such an alternative pathway seems unlikely. It is more probable that any difference in the metabolism of endogenous and exogenous tryptophan is due to the liberation of intermediates in amounts, and/or at sites, of varying favourability for further metabolism. In order to investigate the possible temperature-dependence (or the possible adaptive nature) of steps in tryptophan degradation, it would be necessary to do enzymic experiments. No record has yet appeared of a successful attempt to isolate the enzyme system bringing about the hydroxylation of the aromatic ring of kynurenine.

The explanation of the results obtained in the present work is therefore still in doubt. When tryptophan was given by mouth to a normal human in whom an artificial fever had been engendered, no hydroxykynurenine was excreted, suggesting that our results are due to a difference between exogenous and endogenous tryptophan metabolism. On the other hand, the fever was of short duration, and in a few patients with sustained fever who were followed for some days, it was found that there was sometimes a delay after onset of fever before the appearance of hydroxykynurenine in the urine. Hydroxykynurenine excretion always ceased with loss of fever. Although our results are inconclusive, they are reported at this stage as it is felt that further investigation must await a method for the quantitative determination of hydroxykynurenine. Unfortunately no method at present exists for the specific estimation of this substance in the type of complex mixture obtaining in urine.

Fig. 1. Summary of tryptophan metabolism.

SUMMARY

1. It has been found that in a large proportion of cases the febrile human excretes hydroxykynurenine. This excretion is, in particular, unrelated to tuberculosis or diabetes. It is attributed to a high rate of breakdown of body protein.

2. In states associated with weight loss but not accompanied by fever, hydroxykynurenine excretion could not be detected.

3. The normal human taking an excess of tryptophan by mouth excretes much kynurenine, but no, or negligible, hydroxykynurenine.

4. Possible reasons for this difference in excretory patterns are discussed.

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The Metabolism of Progesterone by Animal Tissues *in vitro*

1. FACTORS INFLUENCING THE METABOLISM OF PROGESTERONE BY RAT LIVER AND THE INVESTIGATION OF THE PRODUCTS OF METABOLISM

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Table 1 lists the steroids closely related to progesterone which have been isolated from human pregnancy urine (see Samuels & West, 1952, for references) and which are therefore assumed to be metabolites of the hormone. By *in vivo* studies, only three of these steroids have been shown conclusively to be derived from progesterone, namely, pregnane-3 α :20 α -diol (pregnanediol) (Venning & Browne, 1937), pregnan-3 α -ol-20-one (pregnanolone) (Dorfman, Ross & Shipley, 1948) and 5 α -pregnane-3 α :20 α -diol (Kyle & Marrian, 1951). In spite of indications from *in vivo* studies that the liver is involved in progesterone metabolism (see Samuels & West, 1952, for references), Zondek (1941) and Engel (1944) were unable to demonstrate inactivation of

the hormone by this tissue *in vitro*. However, using more sensitive chemical methods, Samuels (1950) showed that the $\alpha\beta$ -unsaturated ketonic group of progesterone underwent some change when incubated with rat-liver mince.

Table 1. *Human urinary steroids related to progesterone*

Pregnane-3:20-dione
 5 α -Pregnane-3:20-dione
 Pregnan-3 α -ol-20-one
 5 α -Pregnan-3 α -ol-20-one
 5 α -Pregnan-3 β -ol-20-one
 Pregnane-3 α :20 α -diol
 Pregnane-3 β :20 α -diol
 5 α -Pregnane-3 α :20 α -diol
 5 α -Pregnane-3 β :20 α -diol
 Pregnan-3 α -ol

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