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AND WELFARE

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MANUFACTURE AND USES OF CASEIN AND CO-PRECIPITATE

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INTRODUCTION

Casein is a long-established dairy product with many traditional industrial uses. In recent years, realization of the usefulness of casein and milk protein preparations as food ingredients has led to changing technology of manufacture. The general development of industrial technology and the new uses in foods have increased demand and led to rising production.

Casein production has generally increased since the 1930's. From an average of 74 000 tons in 1934-38, world production rose to 100 000 tons in 1953-57 and reached 156 000 tons in 1965. It has continued at about 150 000 tons since then (FAO, 1957, 1960); Anon., 1958; Commonwealth Economic Committee, 1965; Commonwealth Secretariat, 1969; Anon., 1970).

Since World War II, there have been notable changes in the production of casein by various countries. The USA was the leading producer until the early 1950's, when the introduction of price supports for dried milk caused virtual cessation of casein manufacture. Argentina then became the leading producer, manufacturing 36 000 tons in 1957. New Zealand produced 14 000 tons in that year and following a rapid rise in production became the leading manufacturer in 1961. By 1969, New Zealand production had increased to almost 70 000 tons—about 45% of the world total.

With declining production in Argentina since 1963 and, more recently, in France, Australia became the second largest manufacturer in 1968 with a peak of almost 32 000 tons in 1969-70. Phillips (1958) and McClelland (1965) described the changes in Australian production up to 1964.

Such changes in production patterns in many of the casein-producing countries have been due mainly to the effects of pricing policies which tended to favour alternative uses of skim-milk. In New Zealand and Australia, although relative prices are a factor, as shown by McClelland (1965), the rise in production has been strongly influenced by the change towards receiving whole milk rather than farm-separated cream at dairy factories.

The introduction of efficient methods of manufacture in plants designed to make an edible quality product also influenced production. These plants not only reduced manufacturing costs, improving the value of milk proteins relative to that of dried milk, but also elevated the status of casein for both industrial and food uses.

ACID CASEIN MANUFACTURE

General

Sutermeister & Browne (1939) gave a good account of the principles of casein manufacture and of the manufacturing methods in use at that time. Spellacy (1953) described manufacturing methods in detail. This review is therefore mainly concerned with developments since these books were published.

In manufacturing acid casein, the pH of skim-milk is reduced to ~4·6 so as to obtain a coagulum in a form suitable for further processing. This pH reduction is commonly achieved by the action of bacteria on the lactose in skim-milk or by the addition of an acid to the milk. Casein produced through the action of a bacterial starter is termed lactic casein. Casein precipitated by acid usually includes the name of the acid in its description, e.g., hydrochloric acid casein, lactic acid casein, but may simply be called acid casein. While there are differences in the manufacture of lactic casein and acid casein, many of the principles and steps in manufacture are common to both.

Lactic casein

The traditional method for forming the coagulum in lactic casein manufacture has been described by a number of authors (Spellacy, 1953; Luck, 1955; Patten, 1956; King, 1962, 1970). Skim-milk at ~26-27°C was filled into cheese vats and inoculated with 0·5 to 1·5% of a mixed lactic culture consisting usually of strains of *Streptococcus lactis* or *Str. cremoris*. After ~16 hours, coagulation had occurred and the temp. was raised by steam injection—'cooking'. Cheese knives were sometimes used to cut the curd before cooking. Cooking continued with gentle agitation for ~1 h until a temp. of ~60°C was reached, when the curd was allowed to settle and the whey drained off.

This process was time-consuming and laborious; effort was therefore devoted to improving its efficiency. Tarasov (1951) described a steam injector used to draw the coagulum from the vat and heat it from 30° to 58°C, at the same time breaking the curd into particles suitable for further processing. King (1962) described developments in New Zealand where it was found that the coagulum could be pumped without damage to granule formation during later cooking. Cooking was then performed by steam injection while the coagulum was being pumped. A holding pipe or vat was used to permit the curd to agglomerate and

shrink before separation from the whey. These advances made it possible to use large vats for the coagulation stage, usually vertical silo types of 4000 to 25 000 gal capacity, and to have a continuous process for cooking, washing and subsequent stages. One problem in using very large coagulation vats was that, in the time required to empty a vat by pumping, the pH of the coagulum could change and affect casein quality. Neff (1966, 1970) and Buchanan (1962) described a method of pH adjustment using addition of acid or alkali to the coagulum during pumping. This technique could also be used to overcome variations in lactic starter activity such as those ascribed to bacteriophage by Perry (1961). Dolby (1967b) advocated the use of skim-milk for pH adjustment.

Another approach to improvement of the lactic casein process was described by Olšanský & Hénik (1957). By using 1-4% of a thermophilic starter and 40-42°C as incubation temp., coagulation in 2-2½ h was achieved. The processes of coagulation, cutting, stirring, whey removal, washing, pressing and drying required a total of 5-6 h. Čeřovský & Basař (1963), Čeřovský & Kněz (1962) and Vindyš (1962) gave further details of the process. However, King (1962) considered that there was little economic advantage in rapid starter action as compared with the overnight 'slow set'—a conclusion supported by the adherence of New Zealand manufacturers to the latter procedure.

Acid casein

Even with the use of thermophilic starters, the time required for pH reduction in the manufacture of lactic casein is ample to achieve equilibrium conditions in the conversion of the micelles of calcium caseinate in milk to molecules or aggregates of casein virtually free from calcium. Where acid is added to skim-milk to make acid casein, conditions of time and temp. may not favour rapid attainment of such equilibrium conditions.

Earlier systems such as those described by Gordon (1961) involved the addition of acid to batches of milk at a temp., initially ~42°C and rising to ~49°C during mixing, so as to produce a curd suitable for further processing. Such processes generally required addition of a slight excess of acid and a substantial period of time for the curd to be in contact with the whey so as to remove any excess of calcium from the protein. Attempts were made to improve mixing of acid and skim-milk. Chappell (1935) and Clickner (1936) patented processes, which appear identical, for the mixing of acid and skim-milk in a baffled chamber before discharge into a chute with baffles designed to provide time for agglomeration of curd; mixing was carried out at the precipitation temp. Smart & Nelson (1957) devised a method using a mixing head before 20 ft of ⅜ in tubing. Dilute HCl was added at the head to milk at ~40°C. A rope-like mass discharged onto an endless screen belt or drum for whey removal.

The approach by Chappell & Clickner was very similar to that used in the 'Bellock Precipitator' as described by Buchanan (1957). Muller & Hayes (1962a) found that this method of mixing could lead either to high losses of casein as 'fines' or to a high ash content for the casein. They observed a slow increase in pH when the curd was held in the whey, indicating that equilibrium pH had not been achieved during mixing. If the pH of precipitation was low enough to give a low-ash product, losses of fines approached 9%. If the pH of precipitation was higher, say 4·4, a high-ash product resulted unless extra acid was added. It was found that at the normal precipitation temp. of 43-45°C a visible coagulum formed in <0·1 sec. This was considered much too short a time to achieve efficient mixing and completion of the reaction between acid and the casein micelles. Experiments showed that lowering the milk temp. to 30-35°C and pumping dilute acid through a spray to give a fine, cone-shaped discharge in the opposite direction to the flow of milk provided efficient mixing which occurred more rapidly than the coagulum formed. Steam injection after mixing was used to reach the precipitation temp of 44-46°C. Losses of fines were reduced to low levels and pH equilibrium was attained rapidly enough to permit the whey to be removed immediately from the curd. This technique proved commercially satisfactory and is now used in most Australian factories as well as in other countries. Strmiska (1968) described pilot-scale studies which confirmed the findings of Muller & Hayes (1962b). Miller (1966) also confirmed the effect of efficient mixing of acid and skim-milk on losses of fines and proposed a method for estimating such losses. For successful application of this mixing technique, uniform pumping rates for milk and constant-strength acid were desirable. Hatton (1962) described suitable equipment for diluting HCl to constant strength.

The principle of mixing acid with skim-milk at low temp., ~5°C, followed by an increase in temp. to effect coagulation was proposed by Ernstrom (1964) as a basis for the continuous manufacture of Cottage cheese. A further development of this principle has been described recently by Gwozdz & Hutin (1970a, b). By use of low milk temp., more dilute acid and a special mixing pump, a high degree of mixing efficiency was attained before injection of steam to reach the precipitation temp. Very low fines losses result.

Type of acid

The choice of method for reducing the pH of skim-milk to precipitate casein is largely governed by economics. In terms of cost of acid, a lactic fermentation process is attractive especially when, with large scale processing by modern methods, the tendency for higher capital and operational costs are minimized. These considerations have obviously determined the almost complete allegiance of New Zealand manufacturers to lactic casein manufacture.

King et al. (1962) pointed out that the early history of sulphuric acid casein manufacture led to unfavourable trade opinion on its quality. However, these authors showed that, with suitable conditions for precipitation, its quality can be identical with good quality lactic casein. This has been confirmed in Australia. One possible difficulty with the use of H_2SO_4 is the suitability of the whey for pig feeding. This factor was examined by McDowall et al. (1962).

In Australia and other countries where HCl is available in bulk in most areas at reasonable prices, the known ability of this acid to produce good quality casein for both industrial and edible purposes together with the relative simplicity of its use have favoured its acceptance for casein manufacture.

Lactic acid has been used as a precipitant. Oetiker (1960) described an interesting approach in which whey was fermented with a selected strain of *Lactobacillus bulgaricus*, the pH being held constant by continuous partial neutralization with lime. On completion of the fermentation, H_2SO_4 was added under conditions designed to give good crystallization of $CaSO_4$. The dilute lactic acid remaining as supernatant after settling of the precipitate of $CaSO_4$ was used to produce lactic acid casein of high quality.

An even more novel approach is described by Kuipers (1969a, b) in which the mother liquor from lactose manufacture was mixed with cheese whey, heated and filtered to remove precipitated protein. The filtrate was passed over a cation exchange resin to reduce the pH to ~1·8. This acid material was then used to precipitate casein, the whey being recycled through the lactose ion exchange process.

Other acids such as phosphoric or acetic have been considered. Muller & Hayes (1966) found phosphoric acid to be a suitable precipitant for casein. Because of its low dissociation constant excessive quantities of acetic acid are required to precipitate casein.

Washing

Čefovský et al. (1958), Vindyš (1962) and Čefovský & Kněz (1962) described a casein washer of the drum type in which the curd was moved by a screw through an inclined cylinder. Kajda (1967) patented a washing method involving use of acidified water at various temp. In studying the quality of casein then produced in Australia in relationship to the user's requirements, Muller (1959) concluded that the most positive quality improvement could be achieved through more efficient washing. Experiments showed that the impurities were removed from casein particles by a process of diffusion. He found that at least 3 washes for 15-20 min were necessary to achieve low lactose levels in the casein. Commercial-scale trials were initially based on rectangular, baffled wash vats of design similar to that of the 'Bellock Precipitator', and it was shown (Muller, 1960) that well washed casein could be obtained in such units. It was found that curd could be separated from whey or water with fair efficiency by running the mixture over screens of 90-mesh stainless steel gauze inclined at an angle of 60° from the vertical.

The rectangular design first chosen had some disadvantages (Muller & Hayes, 1960). The method of mixing skim-milk and acid in use with the Bellock Precipitator created large curd masses which had to be reduced to smaller particles for efficient washing. The use of 2 contra-rotating agitators in circular vats proved more effective than the agitation system in rectangular vats in reducing the curd particles to uniform size. The pattern of movement through both systems of particles marked with the fluorescent stain Thioflavine S (Hayes & Muller, 1960) proved to be little different. There was a slightly greater tendency for curd particles to by-pass through the circular vats but it was judged that this would be compensated for by the more uniform particle size. On the basis of these observations, and for convenience of plant layout, circular vats were adopted for use in a commercial 3-stage trial.

Before the development of the acid/skim-milk mixing system described by Muller & Hayes (1962b), it was necessary to use the 1st stage of these plants for 'acidulation'—agitation of the curd in the whey to firm the particles and obtain equilibrium in pH. Of the 3 stages in the trial commercial unit only 2 were therefore available for washing. With this unit it was possible to reduce the lactose content of casein to <0·15% with a milk flow of 1200-1300 gal/h, and 1000 gal water/h to each wash vat. In later trials, Muller & Hayes (1962a) established that milk flow rates of ~2200 gal/h gave satisfactory washing in 3-stage plants provided water flow rates were adjusted to maintain an average holding time of ~30 min/vat. For counter-current washing, ~1 gal water for each 2 gal skim-milk was optimum, whereas approx. equal volumes of water and milk were required for co-current washing. It was observed in these trials that contamination of water in the 1st wash tank by whey decreased efficiency. This factor is well recognized and a number of approaches have been taken to improve efficiency of curd-whey separation. Muller (1960), Buchanan (1962) and King (1970) described the McKenzie and Ridley system developed in New Zealand. In this unit, curd and whey are separated on a moving, inclined nylon endless screen, the curd being sprayed with water to remove surplus whey before the 1st wash. Buchanan (1962) described the use of vibratory screens for this purpose. Drew (1970) compared 2 types of vibrating screen with an inclined stationary screen and concluded the former were more efficient and their use for curd-whey separation should be considered. In a mathematical approach to assessment of casein washing efficiency, Zadow (1970, 1971) highlighted the importance of the whey separation stage.

This was one factor influencing the variation in lactose content of casein observed by Neff (1965). Other factors as outlined above include proper balancing of flow rates, especially in counter-current washing systems. Inclined screens have been found quite satisfactory in both Australia and New Zealand provided that the total washing capacity is adequate for the milk flow rate. Washing capacity of a particular plant can be increased by adding extra washing units or increasing whey separation efficiency. Zadow (1971) showed these alternatives clearly.

A further important consideration in the choice of separation process is to minimize loss of fine casein. Counter-current washing assists this by restricting the opportunity for fines to be lost to one outlet. Conditions of precipitation, pumping, agitation and temp. of washing must be chosen so as to maintain firm, friable casein particles and avoid creation of excessive fines. Even in a plant operating efficiently there will be a loss of 1-2% of the yield as fines in effluent wash water. Jebson (1969) described the use of hydrocyclones for fines recovery.

Wash water quality

Clark et al. (1920) emphasized the importance of adjusting the water used to wash casein to a pH of 4·6 to avoid the formation of a gelatinous layer over the curd particles in excessively acid water and the softening and redispersion of the curd in alkaline waters. A survey of casein wash waters from most Australian factories by Muller & Hayes (1961) led to recommendations for addition of acid to waters exceeding given levels of alkalinity. Rammell et al. (1964) showed that alkaline wash waters affected both the pH and Ca content of casein and that improvement resulted from acid addition to the water. As casein is much less soluble in H_2SO_4 than in HCl, the former is preferred for adjustment of the pH of wash waters so as to avoid softening of the curd should over-dosage occur. Wash waters should be clear, since any colour or turbidity is absorbed by the casein and discolours the product. Settling treatment of turbid waters with ferric aluminium sulphate can give a satisfactory wash water as the liberation of some H_2SO_4 during this reaction helps to reduce alkalinity. It has been observed, however, (Muller & Hayes, 1963) that residual Al salts, which may carry over from such flocculation treatments, can interact with the casein and increase its viscosity in solutions.

Pasteurization during washing

Most of the casein plants in New Zealand and Australia have been designed to produce an edible grade product (Newport, 1967; King, 1970). Achievement of the bacteriological standard required for this grade requires pasteurization of either or both the milk and curd. King et al. (1970) showed that, in lactic casein manufacture, pasteurization of the milk had little effect on the bacteriological quality of the product but did improve pH control and yield. Evans (1970) described the conditions necessary to ensure positive pasteurization of the curd during washing. Pasteurization of the curd is normally favoured in Australia for edible acid casein manufacture.

Pressing

The efficiency of the final dewatering stage in casein manufacture is of importance in minimizing the quantity of water to be evaporated during drying. The press normally used in Australia is a larger and modernized version of the Ireland-Chappel press as described by Spellacy (1953). This consists of 2 contra-rotating stainless steel rollers, the lower one being perforated to allow the water to escape. A moving backing plate helps to form a trough for the curd and by its movement feeds the curd between the rollers. The McKenzie and Ridley press as described briefly by Muller (1960) and King (1962) also uses opposed rollers but in this case the curd is fed through the rollers on a horizontal nylon belt. The Pillet press as described by Spellacy (1953) and Gordon (1961) is a screw press relying on the pressure applied by the screws to dewater the casein. A horizontal centrifuge is used in the plant described by Gwozdz & Hutin (1970a, b).

The final moisture of the curd from any press depends largely on the condition of the curd supplied to it. A firm, friable curd resulting from precipitation at the optimum pH and well washed in waters at the correct pH and at graded temp. to aid moisture expulsion from the particles during washing, will press well. The final moisture content is usually ~55-60%.

Drying

Spellacy (1953) gave a detailed account of the types of drying equipment in use for casein at that time. Of the types described, the 'Ultra' dryer manufactured by A. Pillet et Fils, France, was, until recently, the most common in Australia and New Zealand. In this equipment, the curd is moved by slowly travelling rakes over 2 trays of perforated stainless steel. The Proctor and Schwartz (USA) endless belt type of casein dryer is also used in Australia. In this, the curd is layered evenly on a moving perforated stainless steel belt to pass through 3 compartments at various air temp.

More recently, many factories in Australia and New Zealand have installed a vibratory type of dryer as described by Practical Dairy Developments Ltd (N.Z.) (1964b) and Neff (1966). In this unit, commonly referred to as the Bates dryer, the curd passes through a mill to reduce it to an even particle size and then travels by means of a vibratory action over an upper tray of perforated stainless steel before transfer by a star wheel to a lower tray. The dryer is in 2 sections—a pre-dryer with 2 trays reducing moisture content to 30-35% and a final dryer with 4 trays. Transfer from the pre-dryer to the final dryer is by means of an air stream with separation by a cyclone. Advantages apparent with this form of dryer are the completely positive movement of curd through the system which eliminates overdrying of portions of the casein, the higher efficiency of heat utilization and the flexibility to cope with varying types of curd at differing flow rates. Max. capacity is equivalent to ~4000 gal milk/h.

Patchett (1968, 1969) compared the steam requirements for Pillet and Bates dryers. Scott (1954) gave details of the steam requirement for various phases of the traditional lactic casein process. Vickers (1967) reported a proposal to use an indirect, oil-fired air heater instead of steam-heated coils for casein dryers. The use of natural gas for direct heating of a Bates dryer has recently been introduced in Victoria.

Other developments in casein dryers have been reported. Spellacy (1953) described the Sheffield continuous air suspension dryer in which the curd is carried in air streams through 2 drying chambers constructed similarly to cyclone air separators. A somewhat similar dryer was described by D'yachenko (1958) who estimated a heat requirement of 1200 kcal/kg moisture evaporated. Cesul et al. (1963) described a continuous dryer of the fluidized bed type consisting of a vertical cylinder with a top overflow of product. The heat requirement in this case was found to be [illegible] kcal/kg water evaporated. Compared with the table dryers then used in the Polish dairy industry this represented ~50% reduction of steam consumption. A similar dryer was described by Filonenko et al. (1963).

The Berk ring dryer (F. W. Berk & Co. Ltd., London) has been adapted for casein drying in one Australian factory. In this dryer, the curd is fed continuously into large stainless steel ducts in the form of a ring. High velocity air, heated, in this instance, by direct burning of propane gas, circulates the curd through the system. A manifold with adjustable baffles separates the dried particles from the heavier moist particles by centrifugal force. The dried particles are separated from the outgoing air by cyclones. Kajda (1970) described mechanical and heating procedures in casein drying.

Treatment after drying

Neff (1966) gave details of the steps in casein manufacture subsequent to drying. He stressed the necessity for adequate tempering to provide for efficient cooling and blending of the casein. This step is important to achieve a uniform moisture content for efficient grinding. Grinding is usually required to provide a uniform mesh size for ease of dissolving casein. The grinding operation is followed by sieving into various mesh sizes and bagging. King (1970) described these processes as used in New Zealand with particular reference to the bin transfer method of blending as studied by Jebson (1963, 1965) and King & Jebson (1970).

Fig. 1, reproduced from King (1970) with kind permission of the author and the New Zealand Journal of Dairy Science & Technology, illustrates the complete process of lactic casein manufacture normally used in New Zealand. Fig. 2, by courtesy of the manufacturers, Bell Bryant Limited, illustrates a typical layout of equipment as used in Australia for HCl casein manufacture.

Economics

McDowall (1961) detailed the theoretical considerations determining the yield of lactic casein and Zeromski & Chucklova (1959a, b) proposed a formula for calculating yield. Imbs & Frąckowiak (1951) and Neitzke (1967) give manufacturing costs in Polish and German factories. King (1962) estimated that the savings possible by modernizing lactic casein manufacture at that time were 30% of the operational costs.

SPECIALIZED CASEINS

Rennet casein

The manufacture of rennet casein was outlined by Sutermeister & Browne (1933) and Neff (1966) and treated in detail by Pedersen (1949) and Spellacy (1953). Possibly because this type of casein is mainly used for plastics manufacture for which world demand is small relative to that for acid casein, there are not many references in the literature to new developments in its manufacture. The continuous process of separation of curd from whey, washing, pressing and drying has been adopted for rennet casein (Neff, 1966). Muller et al. (1962) found that by addition of $CaCl_2$ to the milk to increase the rate of rennet action it was practical to form the coagulum in a suitable

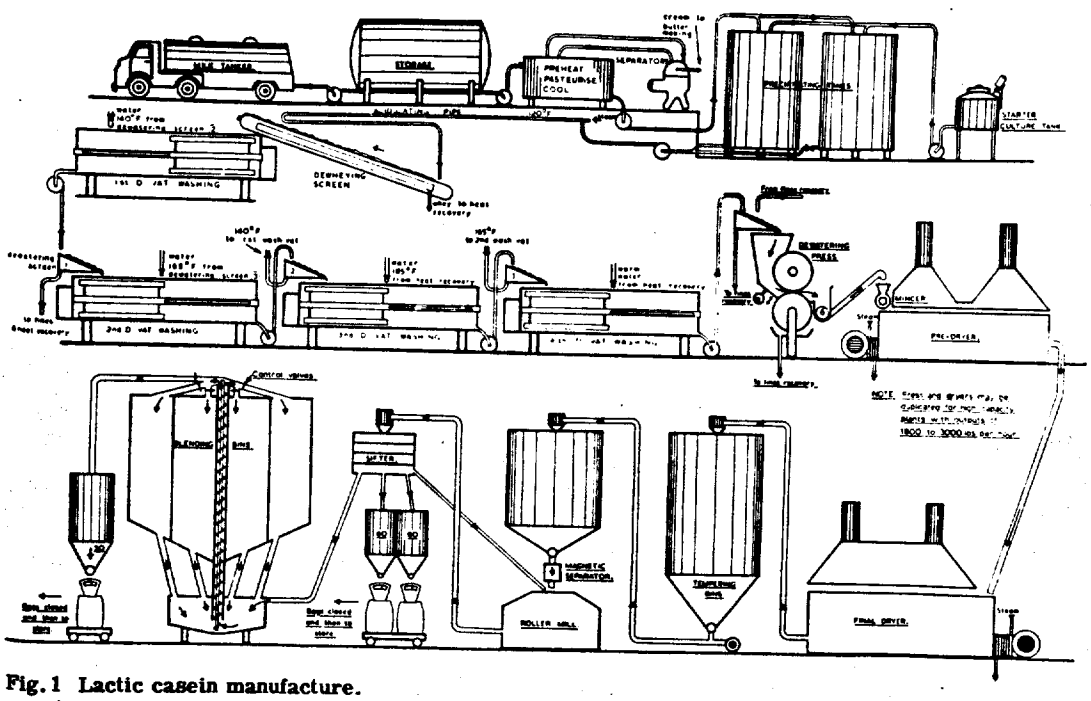

Fig. 1 Lactic casein manufacture.
From King (1970).

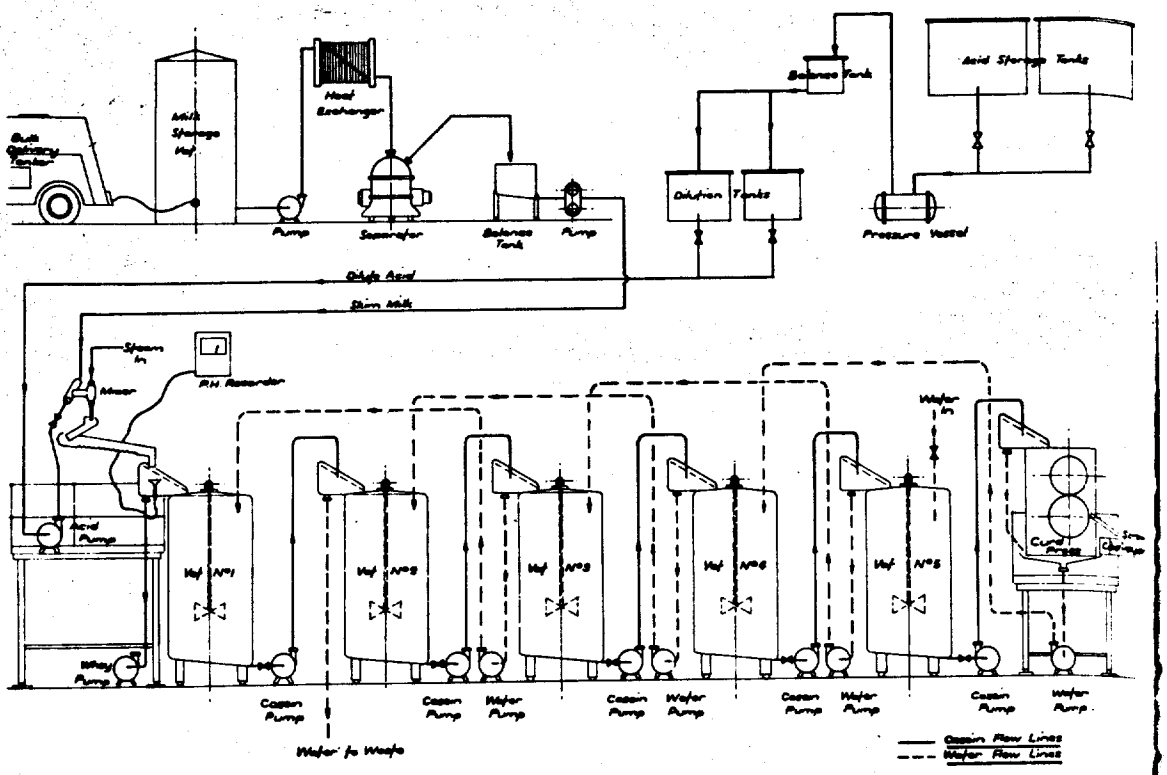

Fig. 2 Hydrochloric acid casein manufacture.

holding vessel and pump it to a steam injector for precipitation before continuous washing. Weal (1970) outlined trials in which steam injection with passage through a holding tube was used to give continuous cooking. Berridge (1964) patented the process of treating the milk with rennet at low temp. and then obtaining rapid coagulation by feeding the milk on to a heated metal surface. While the main emphasis was on cheese, this process was claimed to be suitable for rennet casein manufacture. Practical Dairy Developments (1964a) patented a continuous process using air injection to give a floating curd. It is understood that this process is no longer used, possibly because of the effect of occluded air on the quality of resultant plastics. Dolby (1967a) discussed techniques in conventional rennet casein manufacture of avoiding defects due to occluded air.

Frømyr & Brandsaeter (1952) studied methods of washing rennet casein. Schulz & Hetzel (1959b) described the manufacture, by addition of synthetic plastics to skim-milk before renneting, of a rennet-casein plastic which had a long setting time and could be moulded after wetting.

Low-viscosity casein

After investigation of the uses of acid casein in the paper coating industry, Muller (1961) concluded that the trend towards higher solids in coating dispersions was favouring adhesives other than casein. The increase in the % of casein in a high solids coating reflected the log. increase with concn. of the viscosity of casein solutions (Salzberg & Georgevits, 1956). Following study of possible chemical and enzyme treatments in manufacture of a low-viscosity casein, Muller & Hayes (1963) chose a pepsin treatment for commercial development. Their continuous process involved treating part of the skim-milk with pepsin and blending the treated mixture with the remaining milk before precipitation, washing and drying as usual. Muller et al. (1966) gave details of the gross and amino acid compositions of pepsin- and 'Pangestin' (trade name of an alkaline protease) treated caseins with the suggestion that they might be useful as raw materials for caseinate manufacture.

Salzberg & Simonds (1967) patented a process involving treating milk with an alkaline protease until viscosity of the casein was reduced by approx. ½, precipitating the casein and mixing it with pigment for paper coating.

Other processes

A number of other approaches to casein manufacture have been reported. Sharp (1950) and Sharp & Shields (1951) patented processes for mixing air and the precipitant simultaneously with skim-milk to obtain a foam of coagulated casein. Deb (1969) reported that casein of good quality could be made from

soured whole milk by treating it with warm water and churning to separate the fat before precipitating the casein. Trexler (1952) patented a process for adjusting skim-milk at 2-10°C to pH 4·6 with acid and then heating to 70°C to give a fine granular precipitate which could be spray-dried as a suspension. In British and German patents, Jensen (1954, 1959) detailed a process of adding glycerol and a base, or glycerol, an acid and H_2O_2 to milk before casein manufacture. The additions were claimed to lower the moisture and lactose contents.

In a US patent, Waugh (1956) described an interesting process for making a water-soluble casein. The Ca concn. of milk at temp. ranging from 15 to 60°C was adjusted to 0·06-0·08% to form aggregated casein. The temp. chosen determined whether centrifuging was necessary to separate the casein from the whey. This casein was dispersed in water and treated with a sequestering agent to reduce its Ca content to <0·2%. Ion exchange was used to remove other salts.

General accounts of acid casein manufacture were given by Albonico (1967), Anon. (1968) and Park (1968). Buchanan (1967) outlined proceedings at the IDF Seminar on Casein and Caseinates in 1967. Further details of this seminar are available (International Dairy Federation, 1968). Useful papers on casein manufacture were presented at this seminar by Buchanan et al. (1967) and Newport (1967). These were summarized by Fox (1968). The chapter on casein and whey products in Webb & Whittier (1970) gives a good summary of developments.

STANDARDS AND METHODS OF ANALYSIS

Standards

The increasing emphasis on quality for acid and rennet casein, especially when food uses are involved, has been highlighted by the recent issue of quality standards and the interest in analytical techniques. Standards for casein were published by the Indian Standards Institution (1966), New Zealand Dairy Produce Board (1967), US Federal Register (1968) and the International Dairy Federation (1969). The Australian Exports (Dairy Produce) (Commonwealth of Australia, 1955) Regulations have been amended twice in recent years to provide for high quality edible and industrial casein.

Methods of analysis

Frieberg (1959) discussed methods then used in New Zealand for casein analysis. The New Zealand standards cited above contain methods of analysis, as do the US standards and the Indian standards. The British Standards Institution (1968) published methods for the analysis of acid casein and for rennet casein. A recent comprehensive set of standard methods was published by the Standards Association of Australia (1970). The IDF has appointed groups of experts to prepare standard methods of chemical and bacteriological analysis.

For the determination of the very small amount of lactose in high quality casein, Richards (1959) proposed a method using an anthrone reagent. There is a tendency now to use for this purpose the phenol-sulphuric acid method described by Lawrence (1968) as this is somewhat simpler. At low lactose levels, the browning reaction between protein and lactose on heating, usually at 105°C for 5 h, can be used as a guide to lactose content. Rammell & Croft (1969, 1970) found measurement of absorbance at 460 nm more accurate than a subjective grading of colour. Hayes & Dunkerley (1970) found reflectance measurements of colour useful as a control measure.

For determination of free acidity, Southward & Dolby (1967) found that the extraction at bp as given in the British Standard method (1963) was difficult to handle; they considered that an extraction at 60°C for 30 min with manual agitation every 10 min was better. Rammell (1968) reported total acidity figures from a large number of samples; he modified the British Standard method to improve reproducibility. Southward & Dolby (1966) showed the pH of a casein suspension to be influenced by the pH of coagulation.

As a quick method of estimating TS in a casein dispersion, the New Zealand Dairy Research Institute (1966) proposed the use of refractive index (RI) measurements. The formula was TS = 490·67R − 651·67 (where R = RI at 40°C). Dunkerley (1970), for a co-precipitate dispersion in NaOH, found that RI = 0·0016TS + 1·345. He also proposed a rapid control method for gravimetric determination of TS.

Mummery (1955) modified the English Papermakers' Association test for insolubility so as to make it quantitative. Joerin (1969) referred to difficulties in using new sieves for particle size analysis. Brickey (1970) proposed using 20% tetrasodium EDTA to dissolve casein sufficiently to use rapid wet sieving as a means of extracting extraneous matter.

Microbiology

Vodičková-Rašková (1962) advocated drying casein immediately after making the curd as a means of avoiding proteolytic activity and reducing the microflora. She found that the bacterial count decreased during storage of dried casein. Freke (1969) observed an exponential decrease in coliform count in dried casein at room temp. Freke (1968) modified the pre-enrichment broth for estimation of salmonellae by adding $NaHCO_3$ to dissolve the casein.

Viscosity and Ca content

The viscosity characteristics of casein have a major influence on its usefulness in various applications. Dolby (1961a) evaluated 3 methods for determining a viscosity index and selected a technique using a 15% solution in borax as giving a better spread of results and being less subject to variations due to pH. Using this method, Dolby (1961b) found the viscosity to depend on the pH at which the lactic casein was coagulated and showed that washing at >82°C or drying at 88°C for 4 h increased viscosity. Hayes & Muller (1961) studied the viscosity-pH curves for solutions in various alkalies, confirmed that the concn.-viscosity relationship for normal casein was logarithmic and showed a linear relationship between the log. of viscosity and the reciprocal of absolute temp. They

found that the variation in viscosity with pH of precipitation was associated with changing Ca levels. Schober et al. (1961) also studied the influence of Ca on viscosity of more dilute casein solutions. As the estimation of Ca was very important in these studies, Sawyer & Hayes (1961) developed a titration method using EDTA. This was simplified by Dunkerley & Hayes (1967) for use as a routine control test in co-precipitate manufacture.

In more detailed studies on the viscosity of caseinates in dispersions of high concn., Hayes et al. (1968) showed that with ~1·0% Ca or Sr in the pH range 5·2-6·0, a casein dispersion could form a gel as temp. increased. The gel reliquified on cooling. No gel formed with Ba, Al or Mg but, on cooling, Mg preparations separated into 2 phases, the supernatant being enriched in κ-casein and β-casein. κ-Casein and a mixture of κ- and β-caseins gave reversible gels at similar levels of Ca and pH. Towler & Dolby (1970) showed that the level of Ca in acid casein could be affected by the temp. of precipitation as well as the pH. The removal of Ca from the curd was affected by the pH and Ca content of the wash water.

Apart from the influence of manufacturing techniques, the viscosity of casein can vary naturally. Dolby (1964) reported a seasonal variation in the viscosity index. Southward & Dolby (1968), in a study of changes during a year in the viscosity index and adhesive strength of casein from 6 individual cows, found a progressive decrease in viscosity and increase in adhesive strength. They considered the effect to be lactational rather than seasonal. The casein from 1 cow had a consistently low viscosity as did that from its identical twin. This was ascribed to the presence of the α_{s1}-casein A.

CASEINATE MANUFACTURE

Sodium caseinate

Sodium caseinate is the most commonly used water-soluble form of casein. Its manufacture normally involves the preparation of an aqueous colloidal solution of acid casein by addition of an alkali, usually NaOH, to achieve a final pH of ~6·7. The solution is then spray- or roller-dried, spray-drying being more usual. The normal manufacturing technique was described by Burston et al. (1967), Fox (1968) and Webb & Whittier (1970). The basic problems of the process arise from the logarithmic increase in the viscosity of caseinate solutions as concn. increases.

Spray dryers vary in design and in the flexibility of their atomizing systems to cope with varying viscosity in the solution fed to them. For each spray dryer there is a max. viscosity at which the feed solutions can be successfully dried. With sodium caseinate solutions preheated to 90-95°C this max. usually corresponds to TS content of ~20%. Under suitable conditions, a solution with a max. of 25% TS may possibly be dried successfully.

The limitation imposed by such low solids in feed solutions means that the hourly output of a spray dryer used for sodium caseinate is only ~ ¼ of the output of the same dryer when used for milk concentrated to 45-50% TS. The cost of spray drying sodium caseinate is therefore relatively high.

Possible alternative approaches to spray- or roller-drying a solution have been proposed. Menke (1951) mixed wet curd with dry $NaHCO_3$ and dried the mixture in thin layers at temp. increasing from 45° to 80°C. The dried product was then ground to a powder. After studies of the drying characteristics of sodium caseinate, Keey (1964) concluded that spray-drying to 10% moisture should be followed by a batch-operated rotary dryer. King (1966) proposed a method of mixing wet curd with alkali and forming it into particles suitable for drying in, e.g., a pneumatic dryer. Vorob'ev (1959) used this technique with a shelf-drying plant. Sair (1969) patented a technique of reacting dry ground casein with minimum water and alkali followed by compaction under pressure into an almost glassy mass. The mass was dried and then ground. Suzuki (1968) patented a method involving stirring a caseinate solution into a foam which was dried and ground. Krause (1953) stirred wet curd at 15°C in ammonia in an enclosed vessel at a pressure of 6 atm. After 30 min, the temp. was raised to 68°C and the product dried under a partial vacuum of 100 mm Hg.

Steinsholt (1969) discussed the production of sodium caseinate from rennet casein.

Other caseinates

Sutermeister & Browne (1939) outlined the manufacturing techniques and uses for a number of specialized compounds of casein with different metallic ions. Many of these compounds have limited application in the pharmaceutical field. Calcium caseinate is the most common and finds use in both pharmaceutical preparations and as a food ingredient. The specifications for this product vary with its end use but they frequently include a limitation of the Ca content to within the range of 1·0-1·5%.

The most common technique for preparing a calcium caseinate involves the reaction of a good quality acid casein with $Ca(OH)_2$. It is possible to achieve a Ca content of 1·0-1·5% at a pH of ~6·5 by use of this reagent alone. At this pH value and Ca level, care is necessary in the application of heat during the dispersion process. As shown by Hayes et al. (1968), there is increasing aggregation of protein as temp. increases. It is important to ensure that the casein has equilibrated in Ca content and pH before heating to temp. > 45°C. With care in techniques, a dispersion with TS as high as 28% and relatively low viscosity can be prepared by this method.

Where the specifications for a calcium caseinate call for higher pH values with a limit for Ca, an alkali such as NH_4OH is used to increase pH to the value required.

Other methods for preparing a calcium caseinate have been reported. Srinivasan et al. (1963) added $Ca(OH)_2$ in a sucrose solution to give 1% Ca in a solution of casein in NH_4OH. The product was roller dried. It was suggested by van Kreveld (1963) that the sugars glucose, galactose, lactose and lactulose

may be used as well as sucrose for this purpose. A $Ca(OH)_2$ solution in fructose gave a copious white precipitate on mixing with sodium caseinate.

D'yachenko & Zhdanova (1961) reported obtaining a water-soluble edible protein by partially hydrolysing casein or co-precipitate with pancreatin. Limited hydrolysis resulted in no bitter taste. Nitsche (1957) dissolved casein at <45°C in an aqueous solution of calcium lactate and magnesium sulphate. When evaporated at <45°C and ground to a powder, a water-soluble product resulted. Paterson (1955) patented a process for preparing aluminium caseinate.

USES OF CASEIN

Industrial uses

Sutermeister & Browne (1933) provided a very useful account of the industrial uses of casein. As pointed out by Poarch (1967) the development of other adhesives has resulted in reduced casein usage for plywood, glues, paper coating and plastics. However, these and similar uses still represent important outlets for casein and have received attention in recent literature. Génin (1958) described the use of casein as a stabilizer in latex paints. Eldon (1956) used an unstable ammonium salt mixed with casein as a base for paints or cement. Génin (1961) discussed the use of casein in preparation of reinforcing agents for rubber. Blažek & Hvězda (1964) proposed the use of a solution of casein in triethanolamine as a cement for finishes to leather.

To obtain a casein more suitable for use in glues, Yaffi (1957) passed hot wet casein curd through 2 cooled, contra-rotating rollers. Dean (1957) used heated rollers on dry casein mixed with alkali metal polyphosphate and sufficient water to dampen. Salzberg & Kinney (1958) used differential rollers at 2 temp. on moist casein.

Muller (1961) outlined problems in the use of casein in paper coating. The question of manufacturing low viscosity casein for this purpose has been dealt with earlier in this review. National Dairy Products Corporation (1966) sought patents on the treatment of casein to reduce viscosity for paper coating by using EDTA to sequester Ca and urea, thiourea, urethane or ammonium thiocyanate as a fluidizing agent. Mozingo & Salzberg (1956) used oxychlorination with a mixture of an inorganic chloride and H_2O_2 to make a casein stable for several months in a water solution. Daniloff (1953) claimed that a stable casein solution of low viscosity could be obtained by heating a dispersion in borax and boric acid and adding preservatives. Weakley et al. (1961) found that, in borax dispersions, a complex, which was useful in paper coating, formed between dialdehyde starch and casein. Jayme & Rohmann (1965) used reflectance in the IR region to determine casein-clay ratios on coated paper.

Schulz & Hetzel (1959a) found that casein from formalin-treated milk could be acid-precipitated and, by polymerization with synthetic resins, could be used to produce plastics with new properties.

The use of casein as a clarifier of wine has been described by O'Neal et al. (1951) and Cruess (1963) and, for beer, by Vancraenenbroeck & Loutie (1964) and by Vancraenenbroeck et al. (1967).

Food uses

Poarch (1967) outlined the growing importance of casein as a food ingredient. From early use in improving the quality of sausages, applications in the USA had been extended to such products as breakfast cereals, baked goods, simulated whipping cream, powdered toppings, desserts, coffee whiteners, imitation milks and instant breakfasts. At that time (1967), there were at least 26 food products containing casein or sodium caseinate on the US market and annual usage was estimated at 8000-10 000 tons. Borst (1970) estimated that the annual usage of casein for food purposes in the USA had grown to ~30 000 tons of the total imports of 45-50 000 tons. He included animal foods in the estimate. Calf milk replacers are being made from casein and whey solids.

Other accounts of the use of casein and milk proteins in food were given by Kerkkonen et al. (1963); Nasakin & Kundin (1968); Buchanan (1968, 1969); van Kreveld (1969); Muller (1969); Hynd (1970) and Muller (1971).

As pointed out by Borst (1970), the main reason for the growth in food uses of casein lies in its functional properties. The ability of the protein to bind water and emulsify fat is of particular significance. Pearson et al. (1965) reported, however, that in meat in the pH range 5·3-5·6 skim-milk had greater emulsifying capacity than soya sodium proteinate or potassium caseinate.

The usefulness of sodium caseinate in meat products is well known. The main deterrent to greater use of caseinate in such products is, apparently, a legal one. Many countries will permit such additives only if they are quantitatively detectable in foods. The question of detecting milk protein in the presence of meat protein has been under active examination in Europe. A number of methods have been evolved by: Kutscher et al. (1961); Stadelmann (1962); Wyler & Siegrist (1965); Baal & Leget (1965); Braun (1966); Fleischmann (1964); Kotter et al. (1966); Thalacker (1966); Tsvetkova (1968); Thorson et al. (1969); and Olsman (1970). These efforts are likely to be connected with the recent W. German amendments to permitted additives in sausages (Strobel & Hocherl, 1969).

Making use of the functional properties of casein was well exemplified in the manufacture of textiles from spun fibres of casein as described by Sutermeister & Browne (1933), Spellacy (1953), Peterson et al. (1948) and Malik & Agarwal (1968). This basic technique has been applied to the manufacture of foods such as artificial meats from casein or soya protein. The literature in this field is mainly in the form of patents, e.g., Boyer (1956), Anson & Pader (1957), Kjelson & Page (1965), Mykleby (1966), Dechaine & Callaghan (1966, 1967) and Page & Bauer (1968).

Lenderink (1950, 1959) produced a foaming agent from casein by partial hydrolysis with $Ca(OH)_2$ at pH 10-12. Carnation Co. (1951) produced an egg white substitute by treating casein with a protease while Patterson (1955) proposed a mixture of a caseinate and hexametaphosphate for this purpose. Freund & Danes (1960) produced strong gels from caseinate, pyrophosphate and a calcium compound. These were claimed to have applications in meat products, cosmetics and textiles.

Casein is soluble in some acids at pH 2-3. Kennedy & Bernhart (1953) proposed the use of a dried casein lactate for invalid feeding. Hayes et al. (1970) reported making stable dispersions of up to 15% protein in several acids at pH 2-3. These could be mixed with starch as a basis for gluten-free cakes. When they were heated with sugars, gels formed.

Bernhart et al. (1955) evolved a method for making an incompletely (50-65%) soluble casein capable of forming a stable suspension in water. For this, he acid-washed rennet casein before neutralizing and spray-drying a slurry.

Cuthbertson & Childs (1960), in preparing a human dietary preparation, made use of the fact that a roller-dried calcium caseinate can absorb more than its own wt. of fat. Mathur & Shanbhag (1968) homogenized a paste of wet acid casein curd with skim-milk, added sugar and evaporated the water to obtain a protein-rich sweetened condensed milk. Kende & Ketting (1959) mixed acid casein with cereals and meat flavouring, adjusted the pH to 4·9-5·2 and the water content to 55-60% before heating at 90°C for 20-70 min to obtain, on cooling, a product like cheese. Gray & Cohan (1969) prepared a biscuit (cookie)-like product by mixing casein, sugar and flour in hot water with ~20% shortening. $NaHCO_3$ in the mixture produced sodium caseinate and CO_2 which aerated the mass. Todd & van Abbe (1958) sprayed glyceryl mono-oleate in alcohol onto a food product consisting of casein and sodium glycerophosphate. This treatment improved dispersibility. Wagner (1964) discussed the nature of a brownish-red discoloration of processed cheese in which casein was used for adjustment of composition.

The above references tend to support the observation by Borst (1970) that few food applications for casein are based only on its contribution to nutrition. Functional considerations are generally more important to the food processor.

CO-PRECIPITATE

Manufacture

In the 1950's it was observed that the proteins precipitated by acid or $CaCl_2$ from heated milk comprised both casein and whey proteins, i.e., a co-precipitate.

The development of these observations into commercial manufacture of co-precipitate was motivated by the following 3 considerations:-

(i) The desire to improve recovery of protein from milk—recovery is ~80% when casein is manufactured.

(ii) The concept that the range of functional properties in milk proteins for foods could be increased.

(iii) The hope that the slightly improved nutritive value of the casein-whey protein combination could increase the rôle of milk proteins nutritionally.

The work on co-precipitate has been outlined by Génin (1966), Fox (1968), Nielsen (1969) and Webb & Whittier (1970). The early development of this product was mainly in the USA and USSR. In the USA, Scott (1952) added alkali to skim-milk to reduce the acidity to ≤0·12% so as to inhibit precipitation during heating to ~90°C. Acid was slowly added to precipitate the protein, which was then washed and dried. Howard et al. (1954) used a similar batch method without acidity adjustment of the milk. These authors referred to dispersing the product with alkali at pH 6·6-7·2 and drying it either in that form or mixed with whey proteins and skim-milk to obtain nutritionally superior products. Scott (1958) described techniques of dispersing the co-precipitate from his earlier process so as to achieve max. solubility and hydration of the protein. The technique involved adding a suitable alkali to achieve pH 6·6-6·7, then adding NH_4OH to pH 8·4 and heating. During spray drying, excess NH_3 was evaporated and a final pH of 6·6-7·3 obtained. If less solubility and hydration was desired, the pH of the dispersion was adjusted to ~5·8.

Lowenstein (1961a) produced a soluble form of protein from Scott's co-precipitate by mixing it with a diglyceride and lecithin and homogenizing the mixture in skim-milk. He claimed improved wettability, dispersibility and solubility. Another product by Lowenstein (1961b) was made by adding carrageenan or an alginate to a skim-milk concentrate and then mixing this with a hot dispersion of co-precipitate at pH 7·5-9·5 before spray drying. The product was claimed to have improved water binding capacity. To obtain a spray-dried co-precipitate of low water binding capacity for use in baked goods, Lowenstein (1965) added 1% of lime to a slurry of co-precipitate in water to reach pH 7·0-7·4 and then added an equal amount of a Ca salt such as $CaCO_3$.

Engel & Singleton (1968, 1969) produced a co-precipitate by heating skim-milk for 10 min at 82-99°C, concentrating to about 30% solids, applying a heat treatment at 138°C for 15 sec, cooling to 101°C and acidifying to obtain a curd which was then washed, ground and spray dried.

The type of co-precipitate made by the US processes could be expected to have a Ca content in the range 0·7 to 2·0%. The work in the USSR was on the production of a co-precipitate of high Ca content— ~3%. D'yachenko et al. (1953) developed a method involving adding $CaCl_2$ to milk heated to 85-95°C to obtain ~[illegible] recovery of the protein. The continuous precipitation process and plant was described by D'yachenko (1961). Arbatskaya et al. (1962) described a version of the process in which skim-milk was preheated. $CaCl_2$ injected and then steam injected to increase the temp. to 95-97°C. The proteins were separated

and pressed in a horizontal centrifuge before drying on a belt type dryer. Rostrosa et al. (1968) showed that, when milk was heated to 80-95°C after addition of 1·25 g $CaCl_2$/l., ~40% of added Ca was bound with the coagulum in low acid milk and ~20% in high acid milk.

Co-precipitation has been taken further to include co-precipitates of milk proteins with other proteins. Pokrovskiĭ & Levyant (1968, 1970), by using heat and calcium lactate, produced a co-precipitate of milk and blood proteins. The product was considered suitable for a wide range of culinary purposes. Sato & Ito (1970) showed that, if the Ca content was adjusted to 110 mg/100 ml, casein and egg white protein would co-precipitate after heating at 80°C for 5 min. In a German patent, Friedel & Friedel (1953) described precipitation of milk protein by rapidly heating the milk to 91°C and adding citric or tartaric acid. During or after the coagulation, silicate was added to facilitate separation of the protein particles.

In Australia, Buchanan et al. (1965) modified the $CaCl_2$ precipitation process of D'yachenko et al. (1953) to produce a curd which would withstand a thorough washing process. The technique used in Australian casein plants for mixing acid and skim-milk was adopted for spraying $CaCl_2$ into milk heated to 90-95°C. A holding tube after the spray provided suitable conditions for aggregation of the curd before separation from the whey prior to washing. These authors showed that the product could be dispersed prior to spray drying by using sodium tripolyphosphate (STPP) the % of which was varied to make products of different water-binding capacities.

In view of its major effect on functional properties, Muller et al. (1967) sought to control the Ca content of co-precipitate. These authors found that, by varying the time of heating at ~90°C of milk with changing additions of $CaCl_2$, and by precipitation at controlled pH, a co-precipitate could be produced within a narrow range of Ca content. Commercial development of the process was based on the type of casein plant illustrated in Fig. 2. Fig. 3 shows such a plant adapted to make a co-precipitate. The process was patented (CSIRO, 1969a, b, 1970a, b; Muller et al., 1970).

Properties

Smith & Snow (1968) studied the effects of pH, Ca and fat content on the solubility of co-precipitate and on the whiteness of dispersions. They showed the importance of applying adequate mechanical energy, with either a colloid mill or an homogenizer, during the dispersion process. Hayes et al. (1969a) developed a method for determining a solubility index related to commercial practice in using ground co-precipitate. This index was used in studies of the relationships between dispersion techniques, Ca content, pH, solubility and whiteness of dispersions.

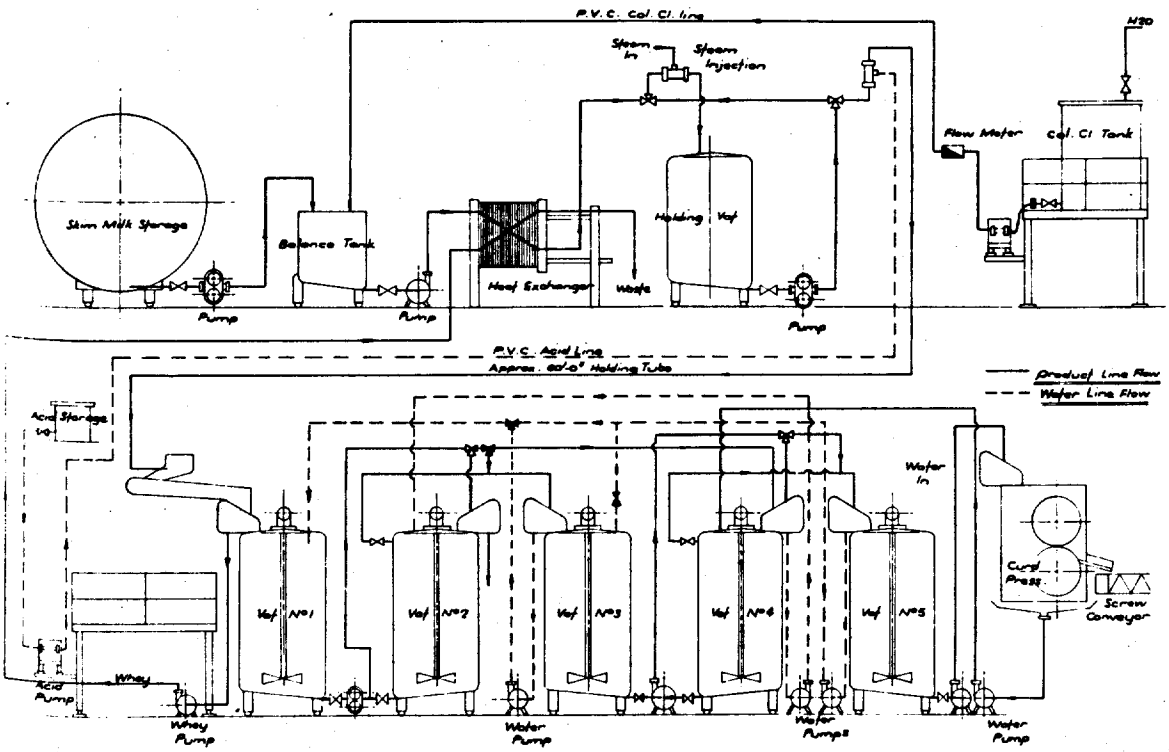

Fig. 3. Co-precipitate manufacture.

Hayes et al. (1969b) reported on the viscosity-pH-temp. relationships of co-precipitate with various Ca contents. They found that the response of a low-Ca co-precipitate to Ca addition differed from that of casein and attributed this to the influence of the protein-protein linkages formed on heating milk. Sawyer (1969) reviewed the formation of a complex between β-lactoglobulin and κ-casein in milk on heating.

As shown by Muller et al. (1966), the amino acid content of co-precipitate differs slightly from that of casein because of the inclusion of whey proteins. Hill & Leary (1968) proposed a method for estimating the whey protein content of co-precipitate. This was based on amperometric determination of the cystine content. A further method proposed by Koning & van Rooijen (1970) used a specific ninhydrin reagent to determine cysteine.

Uses

The nutritive value of milk proteins was reviewed by Henry (1957). The nutritional value of co-precipitate was a factor in its choice as the form of milk protein for the Australian milk biscuit as described by Buchanan & Townsend (1969), the biscuit pre-mix of Henderson & Buchanan (1970) and the infant food for lactose intolerant patients produced by Henderson et al. (1970).

Functional properties are also important. Muller et al. (1970) showed that small changes in pH, Ca or STPP level affected the quality of a spray-dried co-precipitate for baked goods. Bready (1966) produced a pastry glaze from a fully hydrated high-Ca co-precipitate. Kinsella (1970), in studies on the functional chemistry of milk products in candy and chocolate, saw possibilities for further developments using co-precipitate. Thomas (1969, 1970) found advantages in using co-precipitate in processed cheese. Wong & Parks (1970) produced a deep fried co-precipitate curd and Schaap & Torenvliet (1969) used a high-Ca co-precipitate as the base for a spread.

FLAVOUR OF MILK PROTEINS

Freedom from flavour defects is very important in many of the applications of milk proteins as food ingredients. On storage, milk proteins tend to develop unpleasant flavours variously described as gluey, stale, burnt feathers or musty. Good progress has been made in research into the origins of the flavours. Ramshaw & Dunstone (1969a, b) found a large range of volatile components in the steam distillate of gluey casein. Their findings suggested that the flavour resulted from a mixture of compounds with some synergistic effect from *o*-aminoacetophenone, a compound of low volatility possibly arising from breakdown of tryptophan. The type of compounds which seemed significant in the flavour spectrum and their experiments on manufacture of casein and co-precipitates indicated that non-enzymic browning reactions were involved in the flavour. It appeared that reducing substances produced by this reaction subsequently degraded to flavour components. Ramshaw & Dunstone (1970a) reported trials in which dispersions of milk proteins were heated to encourage this degradation so that the volatile flavour components could be removed during spray drying. By using browning inhibitors (1970b), they also obtained improved flavour stability of low-Ca co-precipitate, where the longer heating time for the milk can initiate browning reactions.

In commercial manufacture of casein and co-precipitate, application of the principles of avoiding browning reactions through reducing lactose to low levels and avoiding heating as far as possible at all stages, certainly gives products of improved flavour stability.

That this may not be the complete answer was suggested by Walker (1970). On the basis of comparing ferricyanide reducing values with flavour of low-lactose casein he concluded that the browning reaction did not contribute significantly to development of musty off-flavour.

Sharma & Hansen (1970) linked development of gluey flavour on heating casein with breaking of ester phosphate bonds. Ramshaw & Leary (1970) found that UV treatment of casein gave unpleasant odours as well as gluey flavour. The treatment appeared to accelerate degradation of tryptophan but not the browning reaction.

Industry has sought to obtain the best flavoured product by such techniques as reducing the lactose content by thorough washing and avoiding excessive heating at any stage of manufacture so as to minimize browning reactions. The manufacture of caseinate from fresh wet curd and minimizing its storage time before use also helps in obtaining the best flavour. Apart from these precautions, other treatments have been applied to the solution before spray drying, especially when caseinate was being made from dry casein. Morinaga Milk Industry Co. (1970) patented a treatment with activated charcoal. Wingerd et al. (1962) patented a method of stabilizing the flavour of casein by treating the skim-milk with trypsin, oxidizing with H_2O_2, precipitating the casein with HCl, washing and drying.

CONCLUSIONS

It is evident from this review that the trends in research and commercial development are creating for casein a much wider rôle than that of a traditional raw material for adhesives and plastics. The emphasis in casein manufacture has been on modern, hygienic, continuous processes capable of giving a high quality product suitable for food applications. In studies on the uses of casein, its functional properties have been emphasized and good progress has been made on reducing off-flavour development during storage. The introduction of co-precipitate has provided a wider choice of functional and nutritive properties to the food processor.

More study is needed on the ways in which the various forms of milk protein can best fulfil their obvious rôle in food processing. However, the substantial developments over the last 20 years suggest that the food uses of casein and co-precipitate will continue to expand with benefit to both the dairying industry and the consumers.

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WEANING AGE AND DIETARY LIVER NECROSIS IN THE RAT

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THE purpose of the experiments here described was to find a method of adjusting the sensitivity of groups of rats to the development of acute dietary liver necrosis. Direct comparisons between groups of rats more and less sensitive to the development of liver necrosis could thus be made and so provide another approach to the study of the disease.

In a preliminary experiment it was found that in a group of young male black-and-white rats weaned on their 17th day of age the median survival time was 6 days after the low-protein diet was first offered. This result was so striking in comparison with the survival time (14-43 days) in previous experiments with young male albino rats weaned on their 24th day (Naftalin, 1951, 1952) that the effect of age at weaning was further investigated. Since other factors in these experiments may have been different, direct comparison on litter-mates was made.

METHODS

Animals. Male rats of the hooded Lister strain of the Rowett Research Institute, caged individually, were used in all three experiments. Environmental air temperature (dry bulb) was 72-76° F. throughout the experiments.

Diets (table I). The Rowett Research Institute stock rat-cube diet (Sengupta and Howie, 1948-1949) supplemented with cow's liquid milk was given during pregnancy and for the first 17-19 days of lactation. This diet was followed by a semi-synthetic high-protein diet containing casein 16 ; brewers' yeast * 3 ; lard 7 ; sucrose 68 ; McCollum's salt mixture 185 (B.D.H., to which 0·022 g. potassium iodide had been added per kg. salts) 4 ; cod liver oil 2 per cent. Choline chloride (200 mg./100 g. diet) was added to every diet, and the following B vitamins (mg. per 100 g. diet) to the diets containing casein :—aneurin hydrochloride 0·3, riboflavin 0·3, pyridoxin 0·3, Ca pantothenate 2·0, inositol 0·5, nicotinic acid 4·0.

On the 38th day of age the young rats were offered a semi-synthetic low-protein diet containing as source of protein either casein 8 and brewers' yeast 3 (sucrose 78), or brewers' yeast 18 per cent. In expt. 1 the "vitamin-free" casein of Glaxo Laboratories Ltd.,

* Dried yeast powder (Pulv. saccharomycetis cerevisiæ exsicc.), Pharmaco-Chemical Products Ltd., London and Burton-on-Trent.

Greenford, Middlesex, was used in both high- and low-protein diets. As the stock of this casein was all used in this experiment and it is not now obtainable commercially, the casein of " low-vitamin " content of Genatosan Ltd., Loughborough, was used in the high-protein diet of expt. 2. In a pilot experiment in which Genatosan casein " low-vitamin " content had been used in the high- and low-protein diets,

TABLE I

Management of experimental animals, including procedure for rearing rats before low-protein diets were given

	Expt. 1 July-Sept. 1951	Expt. 2 Oct. 1951-Jan. 1952	Expt. 3 Feb.-May 1953
Mothers' diet	Rowett Institute stock rat cubes + cow's milk	Rowett Institute stock rat cubes + cow's milk	Rowett Institute stock rat cubes + cow's milk
Litter	1st	1st	1st
High-protein diet	Contained 16 per cent. Glaxo " vitamin-free " casein	Contained 16 per cent. Genatosan casein " low-vitamin " content	Contained 16 per cent. Glaxo casein " C " unextracted
Age of young when high-protein diet first offered (days)	19	17	17
Age when weaned (days)	17 (group A) 19 (,, B) 25 (,, C)	17 (groups D, F, H) 25 (,, E, G, I)	17 (group J) 20 (,, K) 25 (,, L)
Age when low-protein diet first offered (days)	38	38	38
Duration of experiment (days after feeding on low-protein diet)	72	73	72

no liver necrosis occurred in the experimental animals. In expt. 2, therefore, in addition to groups D and E, fed on the diet containing 8 per cent. of Genatosan " low-vitamin " content casein, 4 groups were fed on diets containing yeast but no casein. Groups F and G received a diet containing yeast 18 ; lard 7 ; sucrose 69 ; McCollum's salt 185 (+KI) 4 ; cod liver oil 2 per cent., and choline chloride 200 mg./100 g. diet. Groups H and I received a similar diet except that maize-starch 30, and sucrose 39, were the added carbohydrates ; this diet was known from previous unpublished work to lead to a high incidence of necrosis. The diet containing sucrose as the only carbohydrate was used to find out whether a change of carbohydrate influenced the development of acute liver necrosis under the conditions of the experiment. In expt. 3, Glaxo casein " C ", unextracted, was used in both high-protein and low-protein diets.

Feeding. All rats were allowed to eat to appetite and had free access to water.

Procedure. The methods of rearing the rats are shown in table I. Batches of rats were weaned on the 17th, 19-20th or 25th day of age. In expt. 1, three rats from each of 12 litters were studied; those in group A were weaned on the 17th day, in group B on the 19th, in group C on the 25th day. Two or three rats remained with their mother till the 25th day of lactation but only one of these was used. In expt. 2 there were 13 litters, of which the first six litters supplied litter-mates to groups D (weaned 17th day) and E (25th day) (Genatosan

TABLE II

Age at weaning and incidence of acute liver necrosis in expts. 1-3

Expt.	Group	Age when weaned (days)	Low-protein diet (L.P.D.)	No. of rats tested	No. dead with acute liver necrosis	Median survival time (days after eating L.P.D.)
1	A	17	Glaxo	12	11	10
	B	19	" vitamin-free "	12	10	13
	C	25	casein	12	9	28
2	D	17	Genatosan casein	10	0	...
	E	25	" low-vitamin " content	10	0	...
	F	17	Yeast-sucrose diet	10	8	16
	G	25		10	3	... *
	H	17	Yeast-maize-starch-	9	9	18
	I	25	sucrose diet	8	2	... †
3	J	17	Glaxo casein " C "	8	8	6
	K	20	unextracted	8	8	6
	L	25		8	4	... ‡

* One rat died on each of the following days after eating the low-protein diet; 19, 35, 46.
† One rat died on each of the following days after eating the low-protein diet; 20, 51.
‡ One rat died on each of the following days after eating the low-protein diet; 2, 12, 20, 26.

casein diet); groups F (17th day) and G (25th day) (yeast-maize-starch-sucrose diet); and groups H (17th day) and I (25th day) (yeast-sucrose diet). Litters 7-10 supplied rats to groups D and E and groups H and I; litters 11-13 were allocated to groups H and I. Three rats remained with their mother till the 25th day. In the third experiment, three male rats from each of 8 litters were allocated to 3 groups, J, K and L, and were weaned on their 17th, 20th or 25th day of age. In each litter the number of the rats was made up with male and female siblings so that each mother suckled 10 rats till the 17th day of lactation, 4 rats till the 20th and 2 rats till the 25th day.

RESULTS AND DISCUSSION

The incidence of acute liver necrosis and the median survival time are shown in table II. In expt. 1, the difference in survival time between the rats weaned on the 17th day and those weaned on the 19th day is very small and does not approach significance. However,

the difference in survival time between these two groups and the rats weaned on their 25th day is highly significant ($P < 0{\cdot}001$), although differences in incidence of liver necrosis are not significant.

The interactions between survival time, incidence of acute liver necrosis and age at weaning were clearly demonstrated in expts. 2 and 3. In expt. 2, 17 out of 19 rats weaned on their 17th day died of acute liver necrosis (median survival time 17 days) in contrast to 5 deaths among 18 litter-mates weaned on the 25th day (groups F and H *v.* G and I). Feeding with either a mixture of sucrose and maize-starch or sucrose only gave the same results. In expt. 3, 4 out of 8 rats weaned on their 25th day survived the experimental period of 10 weeks after their 38th day ; every rat in both the groups weaned earlier died.

The increased survival time and lower incidence of acute liver necrosis in the groups of rats weaned late may perhaps be explained by a general non-specific improvement in their nutrition or by the presence in their mother's milk of some specific inhibitor of necrosis. If a specific inhibitor is implicated, vitamin E and factor 3 of Schwarz (1951, 1952) must be among the substances considered. The possibility that the rats weaned later derive from their mothers' milk a sufficient quantity of vitamin E to delay or prevent necrosis does not seem likely, because the mothers were fed on a vitamin-E-free diet from the 17th or 19th day *post partum*. Factor 3 of Schwarz has been found in a wide variety of natural substances. It is present in many American-grown yeasts, but not as a rule in the European-grown ; the differences are probably due to the methods of cultivation and not to the yeasts themselves. Little is known about the concentration of factor 3 in animal tissues or animal secretions. However, it can be said that by investigating differences between rats weaned early and late, a new approach is opened to the study of dietary liver necrosis.

Liver-necrogenic quality of casein

In expt. 1 Glaxo "vitamin-free" casein was used in both high-protein and low-protein diet. This led to a high incidence of liver necrosis (groups A, B and C). In expt. 2, Genatosan "low-vitamin" casein was used in the high-protein diet given to all the rats. When this brand of casein was also used in the low-protein diet liver necrosis did not result (groups D and E). In expt. 3, Glaxo casein C, unextracted, was used : this also induced a high incidence of liver necrosis in rats weaned early. It is therefore apparent that the make of casein has a definite effect in determining the incidence of liver necrosis. Thus the description of a casein by the manufacturers as "vitamin-low" or "unextracted" has no bearing on its capacity to induce liver necrosis. Experiments directly comparing Glaxo casein C with Genatosan "low-vitamin" casein have been conducted and are reported elsewhere (Naftalin, 1953).

Summary

Acute dietary liver necrosis was studied in young male hooded Lister rats fed on diets low in casein or high in yeast, without added vitamin E. The rats were housed in an environmental temperature of 72-76° F. and were allowed to eat to appetite.

In three experiments, groups of rats weaned on their 17th day of age had a shorter median survival time than their litter-mates weaned on their 25th day, and in two of these experiments the incidence of acute liver necrosis was significantly greater in the rats weaned early.

The investigation has revealed marked differences in the capacity of different caseins to induce liver necrosis in rats.

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EFFECT OF DIFFERENT MAKES OF CASEIN ON THE PRODUCTION OF ACUTE DIETARY LIVER NECROSIS IN THE RAT

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From 1949 till the summer of 1951, the casein used in diets to induce acute liver necrosis in rats was the "vitamin-free" product of Glaxo Laboratories, Ltd., Middlesex, England. When this casein became unavailable commercially, "low-vitamin" content casein of Genatosan, Ltd., Loughborough, England, was substituted. The next two experiments were conducted in the autumn of 1951 under those conditions which had been found to be most favorable for the production of the disease; *i.e.*, when the rats were housed in an environmental temperature of about 75°F. and allowed to eat to appetite. In these latter experiments,[1] no rat suffered from liver necrosis. This paper reports in more detail the results obtained by a direct comparison, using littermates, of two batches of casein, Genatosan casein "low-vitamin" content, batch no. 078689, supplied in 1951, and Glaxo lactic casein "C" (unextracted), batch no. UA529, supplied in 1947. It was not possible to make a direct comparison with the sample of Glaxo "vitamin-free" casein used in previous experiments.

Methods

Two experiments lasting from February to April, 1952 and from April to July, 1952 are described. The environmental temperature of the animal house was 74°–78°F. Young male rats of the hooded Lister strain, Rowett Institute stock, were weaned on their 17th day, instead of their 24th day, as is customary in this Institute, as it had been found that groups with a higher degree of sensitivity to liver necrosis were thus obtained.[1, 2]

The *diets* contained casein 16 (Glaxo lactic casein "C" unextracted or Genatosan casein "low-vitamin" content), brewers' yeast 3 (Pharmaco-Chemical Products Ltd., London), sucrose 68, commercial lard 7, McCollum's salts 4, cod liver oil (B.P.) 2 per cent, choline chloride 200 mgm./100 gm. diet, and B vitamins but without added vitamin E. These diets were offered from the 17th to 37th day of age and were followed on the 38th day of age by diets in which the casein was reduced to 8 per cent and the sucrose increased to 76 per cent. The rats were always allowed to eat to appetite.

Groups. In experiment 1, six male rats from each of five litters were allocated at random to 3 groups, A, B, and C, on their 17th day of age. Groups A and B were given diets containing Genatosan casein "low-vitamin" content. The rats in group B were given in addition 0.5 μg. vitamin B_{12} orally three times a week from their 38th day of age. Group C was given diets containing Glaxo lactic casein "C." In experiment 2, six male rats from each of twelve litters were allocated at random to 2 groups, D and E. Three rats from each litter were put into each group, and fed on diets containing either Genatosan casein or Glaxo lactic casein "C."

TABLE 1

INCIDENCE OF ACUTE LIVER NECROSIS IN EXPERIMENTS 1 AND 2

Expt. no.	Group	Make of casein use in diets	No. of rats tested			No. dead with acute liver necrosis	Median survival time days‡	Remarks
1	A	Genatosan* casein "low-vitamin" content	2	from each of 5 litters	=10	4	—	One rat died on each of the following days after feeding experimental diet; 11, 12, 24, 28.
	B	Genatosan casein "low-vitamin" content + vit. B_{12}**	2		=10	1	—	One rat died 8 days after feeding experimental diet
	C	Glaxo† lactic casein "C" unextracted	2		=10	9	7	Two rats died on each of the following days after feeding experimental diet; 4, 5, 7; and three on the 13th day.
2	D	Genatosan casein "low-vitamin" content	3	from each of 12 litters	=36	16	—	See graph
	E	Glaxo lactic casein "C" unextracted	3		=36	33	9	See graph

* Genatosan Ltd., Loughborough, Leicestershire, England.
** Vitamin B_{12}: Cytamen (Glaxo) 0.5 μg. in 0.1 ml. water given by mouth 3 times a week from 38th day.
† Glaxo Laboratories, Ltd., Greenford, Middlesex, England.
‡ Days after feeding experimental diet; *i.e.*, days after 38th day of age. Survivors killed on 47th day in experiment 1 and on 72nd day in experiment 2.

Results and Discussion

The accompanying *table* and graph (TABLE 1) show the incidence of acute liver necrosis and the median survival time of the rats. It is obvious that batch UA529 of Glaxo lactic casein "C" induced the higher incidence of liver necrosis.

Since Genatosan casein "low-vitamin" content might be low in vitamin B_{12}, this vitamin was given to one group of ten rats (group B - TABLE 1). The addition of this vitamin may have delayed necrosis, but the numbers studied were too small for the result to be statistically significant.

In experiment 2, among the rats fed on the diet containing Glaxo lactic casein "C" (group E), 2 died from acute liver necrosis on their 36th day of age, another on its 37th, and a fourth on its 38th day; *i.e.*, before the percentage of casein in the diet was reduced from 16 to 8. György and Goldblatt,[3] in their original report on "Hepatic injury on a nutritional basis," used a diet containing 18 per cent casein (brand unspecified). Schwarz,[4] using a diet containing 15–30 per cent of his casein VI, obtained a high incidence of liver necrosis. In an earlier experiment,[5] in which a diet containing 19 per cent Glaxo "vitamin-free" casein was used, one rat among five died from acute liver necrosis. The results of experiment 2 are further evidence that a high level of protein *per se* in the diet does not necessarily prevent dietary liver necrosis.

Glaxo lactic casein "C" batch UA529 was a crude product of New Zealand

it from "Kasein Hammarsten Merck 2242" by repeated acid precipitation from dilute hot alkaline solution and thus purified it still further. Even when casein VI was incorporated at a level of 15 or 30 per cent of the diet, a high incidence of liver necrosis resulted. The unpurified casein Merck 2242 did not induce liver necrosis. As a result of their studies with a water-washed casein and an oxidized casein, Hove, Copeland, and Salmon[8] came to the conclusion that acute liver necrosis or massive lung hemorrhage in rats depended on a simultaneous deficiency of Vitamin E and a casein co-factor. More recently Schwarz[9, 10] has reported that a substance ("Factor 3") which prevents liver necrosis is present in a variety of natural materials including American crude casein and "vitamin free" casein prepared by repeated alcohol extraction. It should be emphasized that Glaxo lactic casein "C" (unextracted) is a crude product and, therefore, it is not possible to state without further investigation whether the difference between its liver necrogenic properties and those of Genatosan casein "low-vitamin" content is due to a difference in content of "Factor 3" or whether there is some other reason.

Summary

(1) There was a higher incidence of acute liver necrosis amongst rats housed in an environmental temperature of 74–78°F. and allowed to eat to appetite, when the casein in the diet was the lactic casein "C" (unextracted) of Glaxo Laboratories, Ltd., Middlesex, England, than when the "low vitamin" content casein of Genatosan Ltd., Loughborough England, was used.

(2) Glaxo lactic casein "C" (unextracted) is a crude product of New Zealand origin. Genatosan casein "low-vitamin" content is prepared from British liquid milk with or without the addition of reconstituted dried skim milk of New Zealand origin.

Acknowledgments

I am indebted to Dr. W. F. J. Cuthbertson of Glaxo Laboratories, Ltd., and to Mr. F. A. Todd of Genatosan Ltd., for information about the preparation of the caseins used in the experiments described in this paper.

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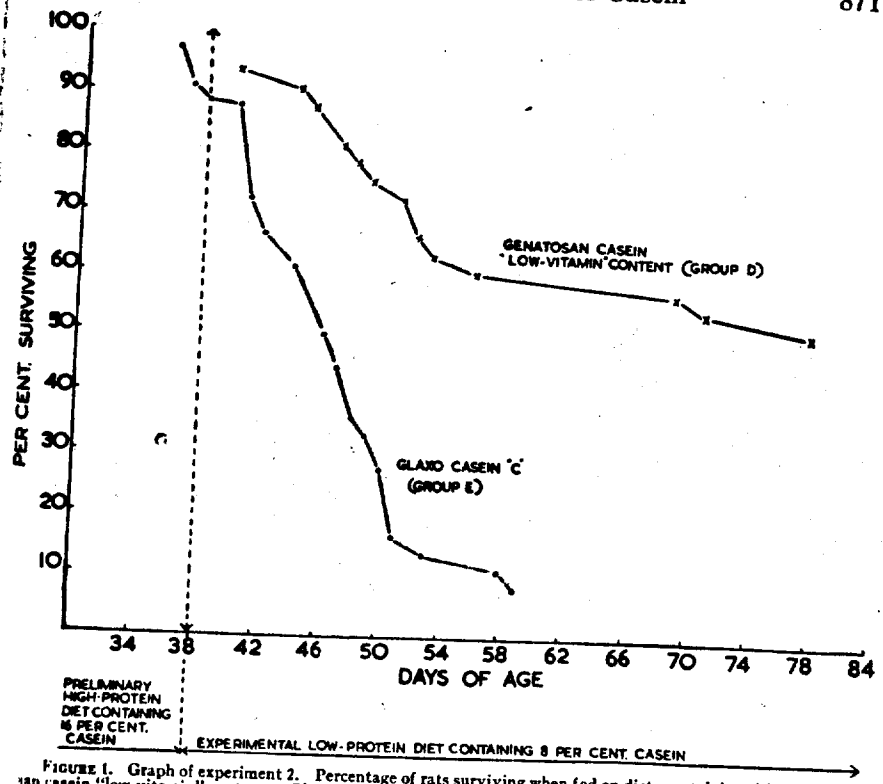

FIGURE 1. Graph of experiment 2. Percentage of rats surviving when fed on diets containing either Genatosan casein "low-vitamin" content or Glaxo casein "C." In group D (Genatosan casein) one rat died on 53rd day of age from lung hemorrhage; one rat died on 60th day of age from severe diarrhea and one rat was killed on 60th day of age. None of these rats had liver necrosis and are omitted from the calculation of percentage surviving. In group E (Glaxo casein "C") four rats died from liver necrosis before the low-protein diet was fed.

origin.[6] Genatosan casein "low-vitamin" content prepared by acid precipitation, followed by extraction with methylated spirits and ether, is usually made from British liquid milk, although reconstituted dried skim milk of New Zealand origin is sometimes added if a sufficient quantity of liquid milk is not available.[7] In two earlier experiments[2] when no liver necrosis resulted, an unnumbered batch of Genatosan casein "low-vitamin" content was used. In the experiments 1 and 2 of the present paper, batch No. 078689 of Genatosan casein "low-vitamin" content was made from a mixture of milks of British and New Zealand origin. Unfortunately, no information is now available about the unnumbered batch of the earlier experiments. The differences between caseins prepared from British or New Zealand milk may be due to age or to differences arising in preparation or to differences in the milks themselves, which may arise from differences in the feeding systems.

That caseins differ in their ability to lead to liver necrosis was first reported by Schwarz in 1944.[4] Despite this, many workers have omitted to mention the brand of casein they employ. Schwarz's casein VI which induced liver necrosis may have been regarded as specially treated casein because he prepared

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Noncasein Protein to Casein Ratio of Feeding Formulas

Effect on Blood Component Levels in Normal Infants

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In an earlier study[1] it was observed that the levels of certain blood constituents changed from day to day in the early postpartum period in accordance with a definite pattern. It was further noted that when breast milk was compared to cow's-milk formula, a significant difference was observed in plasma urea nitrogen levels, the levels on breast milk being much lower. At that time it was hypothesized that this difference was due to the nature of the protein distribution in the two milks. The ratio of noncasein protein to casein is roughly 7 : 3 in breast milk and 1 : 3 in cow's milk.[2] By noncasein protein is meant albumin, globulins, and proteoses.[3]

It was the purpose of this study to evaluate the effect of the ratio of noncasein protein to casein of the feeding formula on the levels of certain blood constituents and the changing levels of these constituents during the first month of life. For this purpose, two feeding formulas were prepared, similar except that one contained a high ratio of noncasein protein to casein approximating that in breast milk, and the other a low ratio of noncasein protein to casein. It was decided to use undenatured noncasein protein because of its greater solubility and palatability and because heat-denatured whey protein is predominately albumin and not truly representative of noncasein protein.

Three groups (15 in each group) of healthy newborn infants were selected, matched according to weight and sex. One group was fed the formula low in noncasein protein ("casein formula"); the second group was fed the formula high in noncasein protein. For simplicity this is referred to as the "albumin formula." The third group was fed at the breast.

The experimental period was 30 days after the second day of life. Blood samples were analyzed, at intervals indicated in the charts, for hematocrit value, plasma sodium, potassium, chloride, carbon dioxide content, sugar, urea, protein, calcium, phosphorus, and blood pH. The breast milk was analyzed for fat, protein, lactose, sodium, potassium, chloride, calcium, and phosphorus, on the 7th and 30th days. Records were kept of weight gains and the volume of formula consumed.

PREPARATION OF THE FORMULAS

Undenatured noncasein protein was prepared from cow's-milk whey by precipitation in acid solution as the polyphosphate at pH 3.[4] The precipitate was separated by decantation and filtration, and was then washed. The noncasein proteins were then suspended, and salts were removed by ion exchange resins. Whey contains only approximately 0.5% protein, and 1200 gal. had to be processed for this study. Casein was prepared in the usual commercial manner by precipitation at pH 4.7 with lactic acid. The precipitated casein was filtered and washed several times.

To pasteurized cow's milk was added cream, refined peanut oil, sucrose, vitamin C, and vitamin D. This mixture was divided into two batches. To one batch (casein formula) was added the casein and calcium lactate. To a second batch (albumin formula) was added the noncasein protein and calcium lactate. Both formulas were homogenized, analyzed and adjusted for electro-

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lyte content, concentrated under vacuum, and then spray-dried. The powdered products were packaged in Cellophane bags, which were stored in cans under nitrogen. Each Cellophane bag contained enough powder so that when diluted to 1 qt. the solution would contain 12.8% solids. This quart would also contain 400 I. U. of vitamin D and 100 mg. of vitamin C. The vitamin C was added not only for its nutritional value, but because it increased the stability of the preparation after dissolving. Table 1 lists the analytical data obtained on the two formulas. The formulas were prepared for us by the Dean Milk Company, of Rockford, Ill.

To prepare the day's formula, the contents of one Cellophane bag were made up to a quart by mixing well with lukewarm water, and the mixture was terminally steam-sterilized at 208 F (97.8 C) for 30 minutes after being put in the nursing bottles. For the first six days 0.3 ml. of a solution of B-complex vitamins and vitamin C was distributed throughout the 24-hour formula just before feeding. This preparation (prepared by Endo Products, Inc.) contained the vitamins per milliliter as follows: ascorbic acid, 100 mg.; thiamine, 3 mg.; riboflavin, 0.7 mg.; nicotinamide, 10 mg.; pyridoxine 0.5 mg., and pantothenyl alcohol (Panthenol), 2 mg. The B-complex vitamins and ascorbic acid were added to eliminate the possibility that these had been destroyed during the processing or on standing. From the seventh day, 0.3 ml. of a water-soluble polyvitamin preparation including vitamins A and D and mixed tocopherols (Vifort, Endo Products, Inc.) was distributed throughout the formula just before use. This same material was administered to the breast-fed infants.

TABLE 1.—*Comparison of Breast-Milk Composition on the Seventh and Thirtieth Days After Parturition with That of the Synthetic Formulas Used*

Feeding Formula		Protein, per Cent	Carbohydrate, per Cent	Fat, per Cent	Na, mEq./L.	K, mEq./L.	Cl, mEq./L.	Ca, Mg./100 Ml.	Phosphorus, Mg./100 Ml.
Breast milk 7 days post partum Formula B	Mean	1.42	6.96	2.92	14.7	18.1	18.9	26.3	18.0
	Std. dev. ±	0.31	0.78	0.54	3.10	1.93	2.91	2.12	2.44
	Ext. range	1.1-1.9	5.4-8.2	2.4-3.1	9.2-19.5	14.8-20.3	14.6-24.5	24.0-32.0	14.5-23.3
Breast milk 30 days post partum Formula B	Mean	1.03	6.37	2.98	9.94	14.5	13.6	31.0	16.7
	Std. dev. ±	0.11	0.43	0.41	3.84	0.83	4.5	3.54	1.28
	Ext. range	0.78-1.31	5.2-7.3	1.4-4.5	5.9-20.0	13.2-16.5	8.5-26.0	25.3-37.6	14.7-18.3
Formula A: 60% noncasein protein, 40% casein	.....	2.1	6.8*	3.3†	9.0	13.5	7.9	55.0	48.0
Formula C: 11% noncasein protein, 89% casein	.....	2.1	6.8*	3.3†	8.0	13.8	8.2	50.0	43.0

* Sucrose, 4.8%; lactose, 2%.
† 80% cow's-milk fat, 20% peanut oil.

ANALYTICAL METHODS

Blood was collected from the finger tip, and the hematocrit value was determined by a method previously described.* Plasma and milk sodium and potassium were determined by the flame photometer.† Chloride was determined by mercurimetric titration for both milk and plasma.‡ Urea nitrogen was determined by the diacetyl monoxime method.§ Calcium in plasma was determined colorimetrically as the calcium alizarin complex.[9] Milk calcium was determined by the same method after ashing, redissolving, and oxalate precipitation. Phosphorus in plasma was determined by the molybdenum blue color.[10] Milk was first wet-ashed with sulfuric acid and 30% hydrogen peroxide at 120 C before analysis for phosphorus by the molybdenum blue color.[11] CO_2 content was determined by a method described previously.[5] Plasma glucose was analyzed by a modification of the method of Folin and Wu.[1] pH was determined at bedside and compared to a healthy adult control as described before.[12] Lactose was estimated in milk by the method of Folin and Wu for glucose as compared to a lactose standard. Protein in both milk and plasma was analyzed by the biuret method after precipitation of the proteins and fat extraction with Bloor's reagent.[1] The plasma proteins were compared to a pooled serum, standardized by Kjeldahl distillation. In the Kjeldahl method the factor 6.38[13] was used to convert nitrogen values to protein content. It is of interest to note that

* References 1 and 5.
† References 1 and 6.
‡ References 1 and 7.
§ References 1 and 8.

with the biuret method, breast-milk proteins yield about 90% of the color observed with plasma proteins for the same total nitrogen content. The breast milk standard for protein was a pooled sample of breast milk assayed by the Kjeldahl method.[13] The alcohol-ether mixture (Bloor's reagent) not only extracts fats but also removes lactose which tends to interfere in the biuret method. It is necessary to dilute the milk 1:3 before adding the Bloor's reagent to remove the lactose and N. P. N. The synthetic formulas were analyzed by Kjeldahl distillation of precipitated protein with trichloracetic acid.[13] Total fat content of milk was first analyzed by solvent extraction and weighing and also by the Babcock centrifugal method.[3] Since the results agreed within the limits of experimental error of both methods, most of the data on the fat content of breast milk was by the Babcock method only.

COMMENT

Excepting protein distribution, attempt was made to make the high noncasein-protein formula (Formula A) as close in composition as possible to the high-casein formula (Formula C). This was only approximately achieved, as seen in Table 1. The level of protein was chosen at 2.1%, since this is approximately the protein level usually found in infant feeding formulas, such as with homogenized cow's milk diluted 2:1. It should be noted that this level is considerably higher than that found in breast milk. The fat levels in Formulas A and C are higher than that observed in breast milk. Purified peanut oil was chosen as the fat supplement because of its lack of allergenicity[14] and to supply adequate amounts of the essential unsaturated fatty acids.[15]

The caloric content of the synthetic formulas, calculated on the basis of 4 Cal/gram for protein and carbohydrate and 9 Cal/gram of fat, is 65.3 Cal/100 ml. The mean value of 7th-day breast milk is calculated to be 59.7 Cal/100 ml., while the mean values of 30th-day breast milk is calculated to be 56.4 Cal/100 ml. These figures may be compared

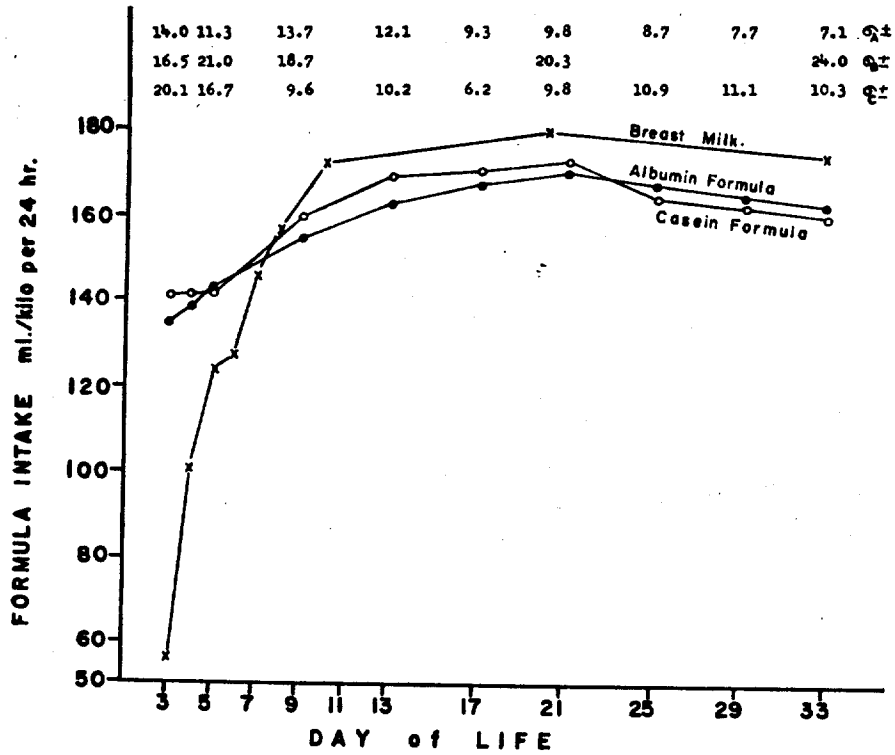

Fig. 1.—Formula intake of the groups on albumin formula, casein formula, and breast milk for the experimental period. In addition to the amounts indicated, water was given ad lib. Standard deviations, σ_A, σ_B, σ_C, are indicated for the groups on the albumin formula, breast milk, and casein formula, respectively, on the days indicated. Fifteen infants in each group.

to those of Hytten,[16] who found a mean value of 59.4 Cal/100 ml. in 121 cases on the seventh day of life, but in uniparae.||

Thus the infants given the synthetic formulas were fed a formula of higher caloric content than those on breast milk. This was only partly compensated for by an increased volume of intake of breast milk. The mean value on the 15th day for breast milk (Fig. 1) was 180 ml/kilogram, while that for Formulas A and C was 173 ml/kilogram. This is only a 4% increase in intake, while the caloric difference ranges, on breast milk, from 9.1% on the 7th day to 14% on the 30th day less than on the synthetic formulas.

The differences in caloric intake were not reflected in the rate of gain in weight, as can be seen from Figure 2. The slow gain in weight for the first six days on breast milk correlates with the low breast-milk output for this period (Fig. 1). The intake of Formulas A and C was the demand of the infant, since he was offered more than he consumed at each feeding. The values of Figure 2 do not include the total fluid intake, in that they do not include the water intake of the infant.

|| Hytten used the figure of 57.8 Cal/100 ml., calculated on the basis of 3.75 Cal/gram of lactose. This figure of Hytten has been adjusted to 59.4 Cal/100 ml., using the figure of 4.0 Cal/gram for lactose to compare with our figures. The figures of 3.74 Cal/gram for glucose and 3.96 Cal/gram for sucrose are usually given. Since these figures are from the bomb calorimeter and are only approximations in human nutrition, we have used the rounded figure of 4.0 Cal/gram. for both sucrose and lactose.

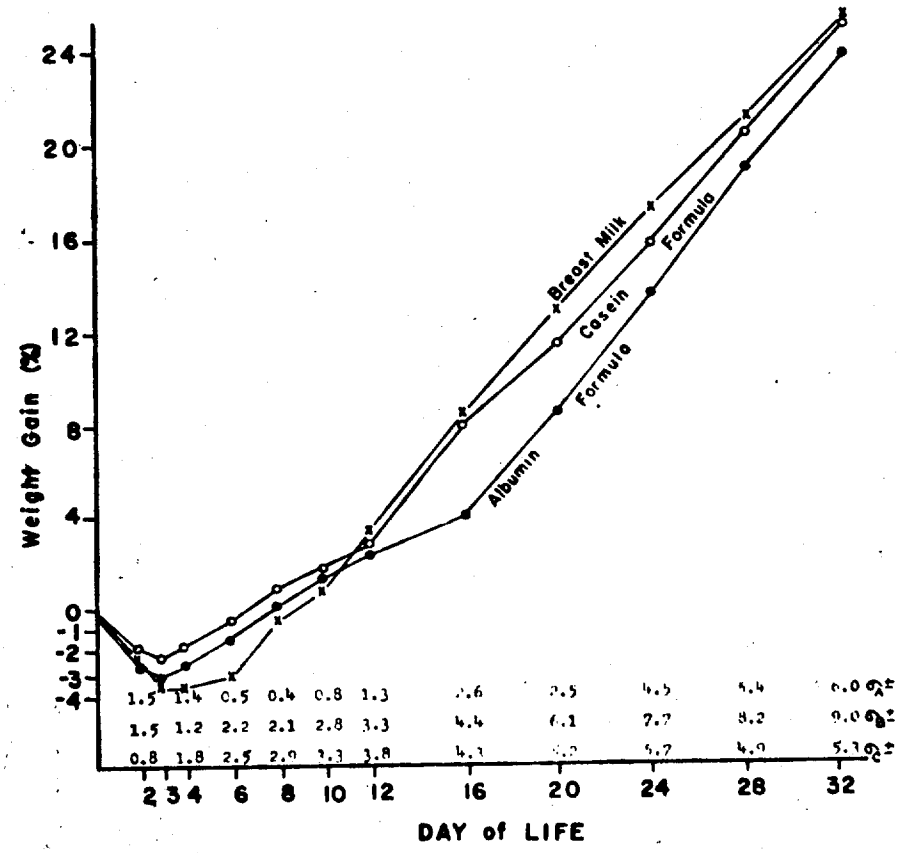

Fig. 2.—Comparison of weight gain of healthy infants on the albumin formula, casein formula, and breast milk during the experimental period. Note the slow gain on breast milk for the first six days of life which correlates with the lesser intake (Fig. 1) during that period. Standard deviations, σ_A, σ_B, σ_C, are indicated for the groups on the albumin, breast milk, and casein formulas, respectively. Fifteen infants in each group.

Figure 2 is not intended as an evaluation of the relative merits of the different formulas, since it has been shown before that where weight gain alone is the criterion, large numbers of infants must be employed.[17] Figure 2 does indicate that in this series, weight gain in all groups was in the normal range as compared with the usual expected weight gain.[18]

Breast-milk composition varied widely, as can be seen from the standard deviations and extreme values listed in Table 1. Thus there were individual infants who gained above the mean value for the group for a 30-day period and who were consuming less than 180 ml/kilogram by the 15th day of a formula whose caloric content was as low as 51 Cal/100 ml. Thus it may be concluded that the caloric intake for the synthetic Formulas A and C was in excess of requirement. Comparing 7th- with 30th-day breast milk, one notes certain obvious changes in the composition of the milk. The changes in protein and fat parallel the changes observed by Hytten.[16] This author noted a rise in carbohydrate level. Our observations indicate a drop in this level after 30 days. A drop in electrolyte levels other than calcium is noted. There is a progressive decline of serum calcium levels as pregnancy progresses.[19] Since two-thirds of the total calcium deposition in the fetus takes place in the last month of pregnancy, the lower calcium levels in early breast milk as compared with later breast milk probably reflect exhausted stores which are subsequently replenished.

Figure 3 tends to confirm the previous observation that urea nitrogen levels are lower for breast milk than on cow's-milk formula. That this is statistically significant is apparent when one considers that from the 7th to the 32d day of life the mean values for plasma urea N for the infant given breast milk did not exceed 9 mg/100 ml., with a standard deviation of ± 2 mg/100 ml. One factor is undoubtedly the fact that the infants given the synthetic formulas had a higher protein intake. N. P. N. and urea levels are higher on high protein intake.[20] Since the protein nitrogen was in excess of requirement, the higher urea levels then indicate a greater requirement for excretion of excess nitrogen. The differences between Formula A and Formula C cannot be explained on this basis, since they contained the same amounts of protein nitrogen. It should also be noted from Figure 3 that the values for plasma urea nitrogen are

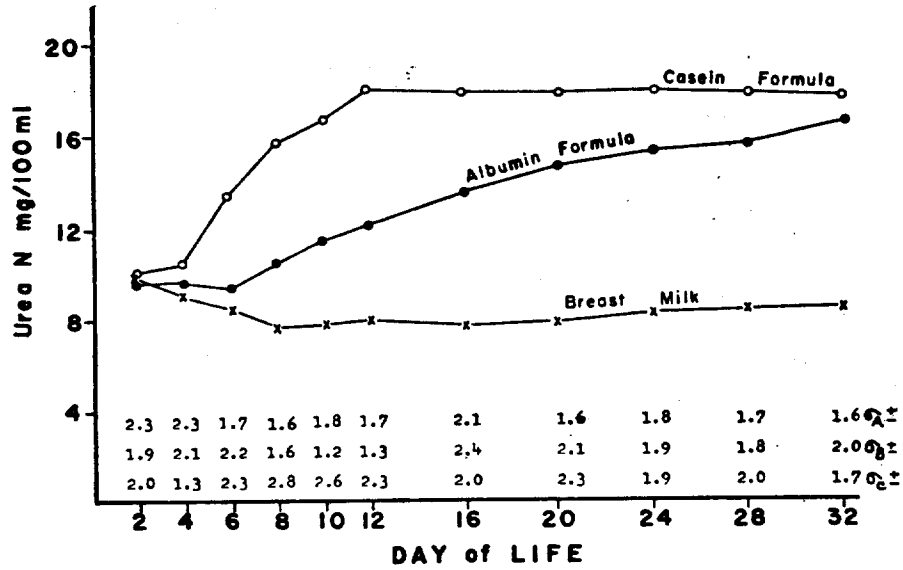

Fig. 3.—Plasma urea nitrogen levels for the infants on the three formulas during the experimental period. Standard deviations, σ_A, σ_B, σ_C, are indicated for the groups on the albumin formula, breast milk, and casein formula, respectively. Fifteen infants in each group.

converging for the albumin and casein formulas. It is suggested that the increasing urea N levels on the albumin formula represent a lessened ability of the infant to utilize the excess protein nitrogen consumed. This explanation finds some support in the decreasing protein content of breast milk on the 30th day as compared with the 7th day and the indicated lessened protein requirement per kilogram of body weight.

Applying the *t*-test [21] for significant difference of the means between nonprotein nitrogen levels observed on Formulas A and C, these differences are significant from the 6th to the 32d day at the 1% level. Further, nine cases of the 15 on the casein formula exceeded the value of 20 mg/100 ml. at some time during the month. None did so on the albumin formula. An explanation which is offered for these phenomena is that the amino acid composition of lactalbumin and lactoglobulin more closely resembles the plasma proteins in their composition than casein does. Casein is not a complete protein and needs to be fortified with certain amino acids.[22] Thus the albumin protein nitrogen may be more efficiently utilized. If this is true it should reflect itself in increased plasma protein levels on the albumin formula. That this is the case is noted in Figure 4.

In spite of the large protein intake for the casein formula (more than twice that on breast milk), the mean plasma protein level is lower on this formula than on breast milk. Applying the *t*-test for significance between means, the difference between the plasma protein level for both breast milk and casein formula is significantly lower than that on the albumin formula from the 20th to the 32d day. The albumin formula contained approxi-

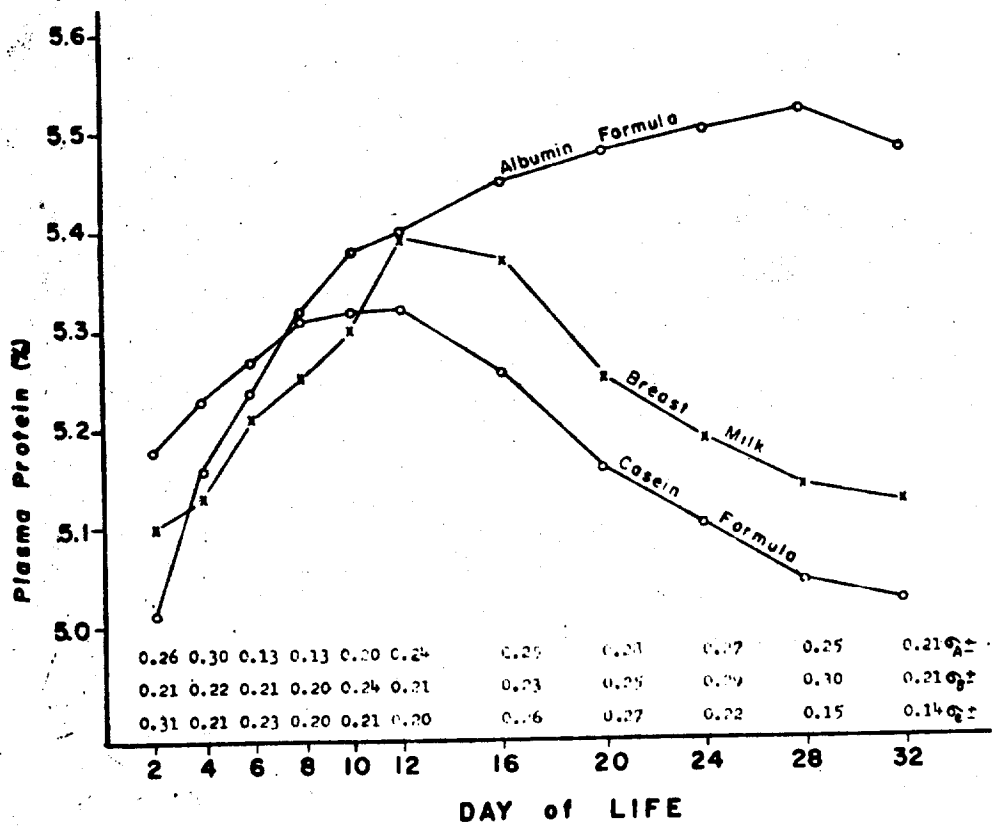

Fig. 4.—Plasma protein levels for the infants on the three formulas during the experimental period. Standard deviations, σ_A, σ_B, σ_C, for the groups on the albumin formula, breast milk, and casein formula, respectively, are given for the days indicated. Fifteen infants in each group.

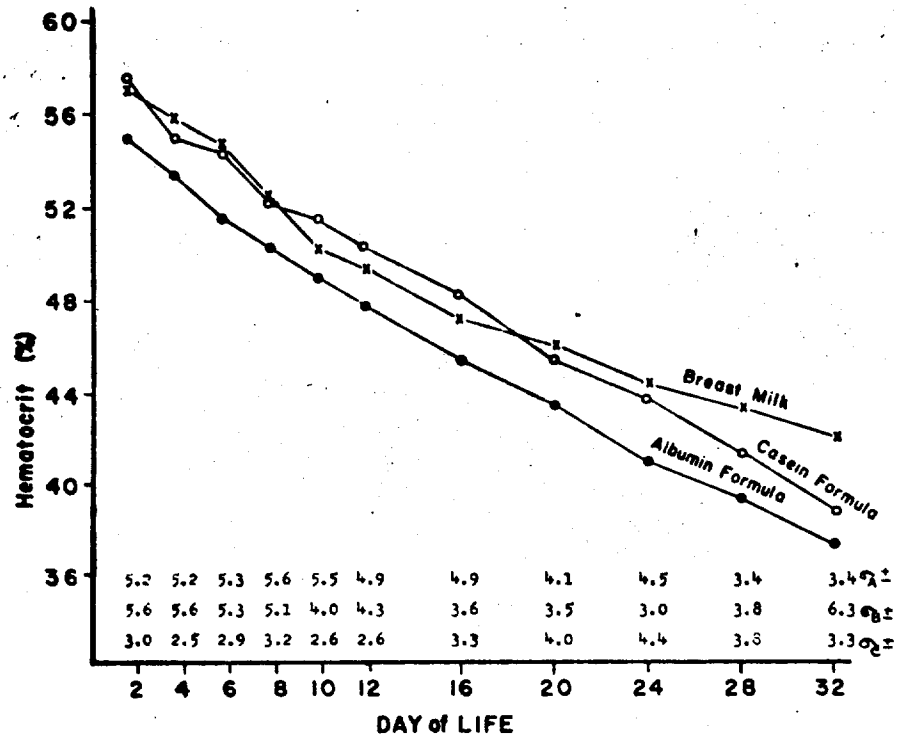

Fig. 5.—Percentage of packed red cells (hematocrit value) of the blood of infants on the three formulas for the experimental period. Standard deviations, σ_A, σ_B, σ_C, for the groups on albumin formula, breast milk, and casein formulas, respectively, are listed for the days indicated. Fifteen infants in each group.

mately twice the total noncasein protein found in breast milk, and this may serve as an explanation for this observation. The casein formula contained approximately 75% of the noncasein protein found in breast milk. Thus the plasma protein patterns seemed to follow the noncasein-protein content of the formula in this study.

The observations above correlate with the studies of Weech [23] in dogs. This author showed by two different techniques that lactalbumin was almost twice as effective as casein in raising the serum albumin and serum protein levels in protein-depleted dogs. Colostrum which is only one-third casein, and thus resembles breast milk in protein distribution, was closer to lactalbumin with respect to its ability to regenerate plasma protein, while mature cow's milk with a relatively low ratio of noncasein protein to casein more nearly resembled casein in this respect. In the study of Weech, the ratio of efficiency in raising plasma protein levels was 1.82 : 1.43 : 1.14 : 1.00 for lactalbumin, cow's colostrum, cow's milk, and casein, respectively.

The observations on plasma protein levels for the different groups of infants suggest explanation on the basis of different degrees of hydration. This does not find support from the hematocrit values as observed in Figure 5. In Figure 5 it is noted that the curves for the casein-formula group and albumin-formula group are roughly parallel. If these two curves are plotted as percentage drop from initial value, they are almost identical. The mean hematocrit level for the breast-milk group on the 32d day is highest for that day. This difference is significant at the 1% level when the t-test is applied. From the shape of the curve one may speculate that this difference may have become greater if extended for a longer period of time.

Examination of Table 1 indicates that breast-milk sodium levels were substantially

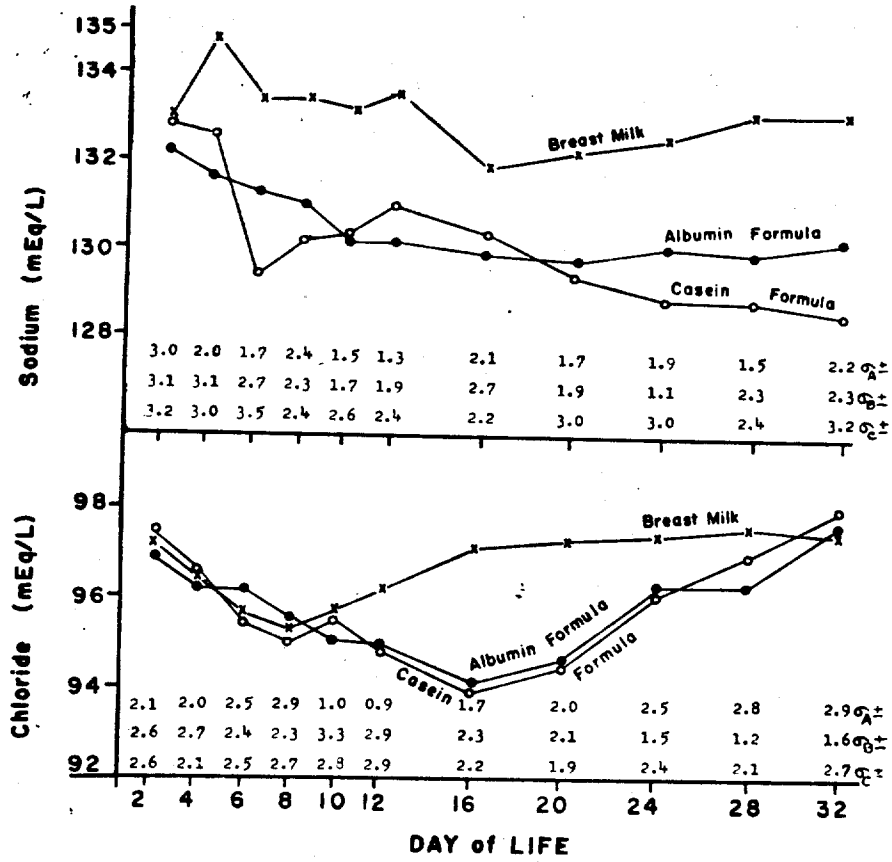

Fig. 6.—Plasma sodium and chloride levels for the groups on the three formulas for the experimental period. Standard deviations, σ_A, σ_B, σ_C, for the groups on albumin formula, breast milk, and casein formula are given. Fifteen infants in each group.

TABLE 2.—*Comparison of Mean Values Obtained for Various Finger-Tip Blood-Plasma Constituents on the Second Day of Life with That After Thirty Days on the Three Diets*

Day of Life	Formula	Urea N, Mg./100 Ml.	Protein, per Cent	Na, mEq./L.	K, mEq./L.	Ca, Mg./100 Ml.	P, Mg./100 Ml.	Hct., per Cent	Cl, mEq./L.	Blood pH	Glucose
2	Mean (all infants).....	9.8	5.1	132.8	5.3	10.0	7.6	56.5	97.2	7.34	44.4
	Std. dev. ±.....	2.1	0.28	3.1	0.33	0.49	0.47	5.0	2.4	0.051	4.1
32	Albumin (mean)	10.3	5.6	130.4	5.1	10.3	6.9	37.2			
	Std. dev. ±.....	1.6	0.21	2.2	0.31	0.30	0.48	3.4			
	Breast (mean)	8.3	5.1	133.3	5.4	10.3	6.9	41.9	97.9* ±2.5	7.36 ±0.048	81.7* ±11.2
	Std. dev. ±.....	2.0	0.21	2.3	0.30	0.29	0.59	6.3			
	Casein (mean)	17.5	5.0	128.7	5.0	10.1	8.1	38.7			
	Std. dev. ±.....	1.7	0.14	3.2	0.25	0.29	0.77	3.3			

* No significant difference between the means for all infants on the three formulas and therefore the mean for all 6 cases.

TABLE 3.—*CO_2 Content of the Plasma from Finger-Tip Blood of Healthy Infants Maintained on Breast Milk and the Two Formulas*

Day of Life	Albumin		Breast		Casein	
	Mean CO_2, mM./L.	Std. Dev. ±	Mean CO_2, mM./L.	Std. Dev. ±	Mean CO_2, mM./L.	Std. Dev. ±
20.....	20.8	2.8	20.7	2.0	20.5	1.2
32.....	19.5	2.7	21.2	1.9	19.1	1.9

higher on both the 7th and the 30th day than the sodium levels of the synthetic formulas. This circumstance is reflected to some extent in the plasma sodium values obtained on breast milk (Fig. 6). Referring to Table 2, the plasma sodium levels on the 32d day of life for the three groups are in the following order: breast milk > albumin formula > casein formula. These differences are statistically significant at the 1% level when the *t*-test is applied. It is suggested that the difference is partly due to the requirement to excrete larger amounts of phosphate on the synthetic diets (Table 1). Further, since the protein content of these synthetic diets is higher than requirement, some of the sodium may have been utilized in excreting organic acids derived from the deaminated amino acids.

The lower plasma chloride levels for the synthetic formulas at the midpoint of the study (Fig. 6) would correlate with the lower chloride contents of the formulas as compared with breast milk (Table 1). However, by the 32d day no significant difference was observed. Since on the 32d day the chloride levels are essentially the same for the three formulas but sodium levels are different, one might expect a more acid blood pH in patients on the synthetic formulas than in patients on breast milk. The initial carbon dioxide plasma level for the 45 cases was 20.5 mM/liter, std. dev. ±3.0. No significant difference in plasma carbon dioxide levels was observed on the 20th day (Table 3), but by the 30th day carbon dioxide levels for infants on breast milk were significantly higher than for those on synthetic formulas. This would correlate with changing sodium to chloride ratios, as observed in Figure 6. Direct blood pH measurements yielded mean values of 7.38 for the breast milk group, 7.35 for the albumin group, and 7.33 for the casein group on the 30th day. However, the differ-

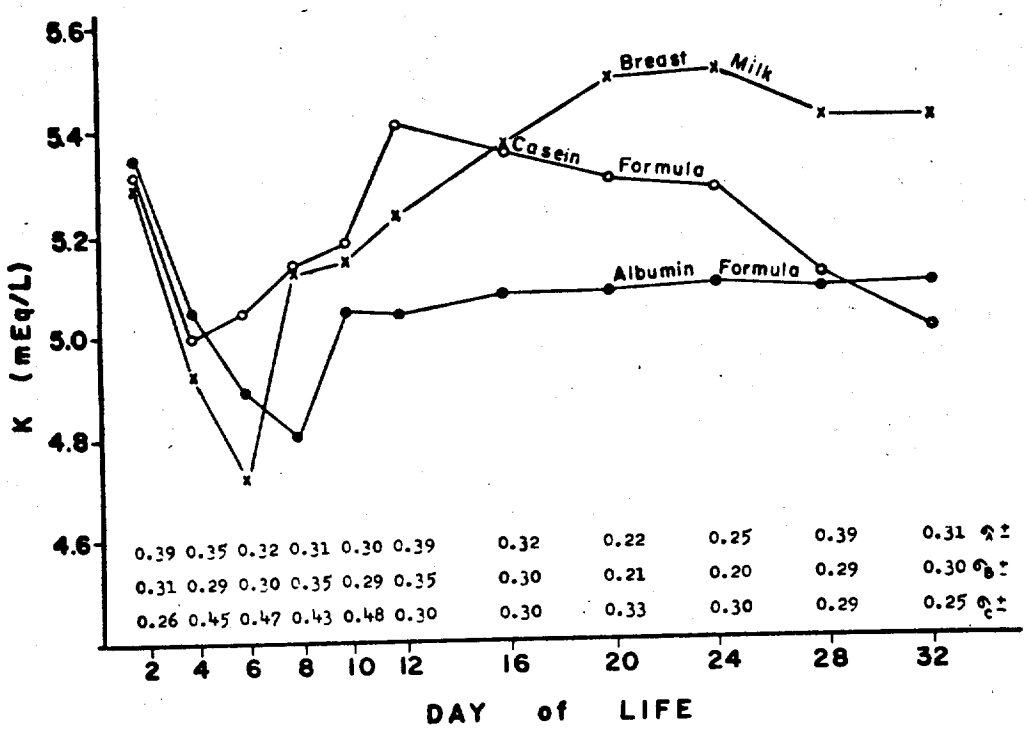

Fig. 7.—Plasma potassium levels for the groups on the three formulas during the experimental period. Standard deviations, σ_A, σ_B, σ_C, for the groups on the albumin formula, breast milk, and casein formula, respectively, are given. Fifteen infants in each group.

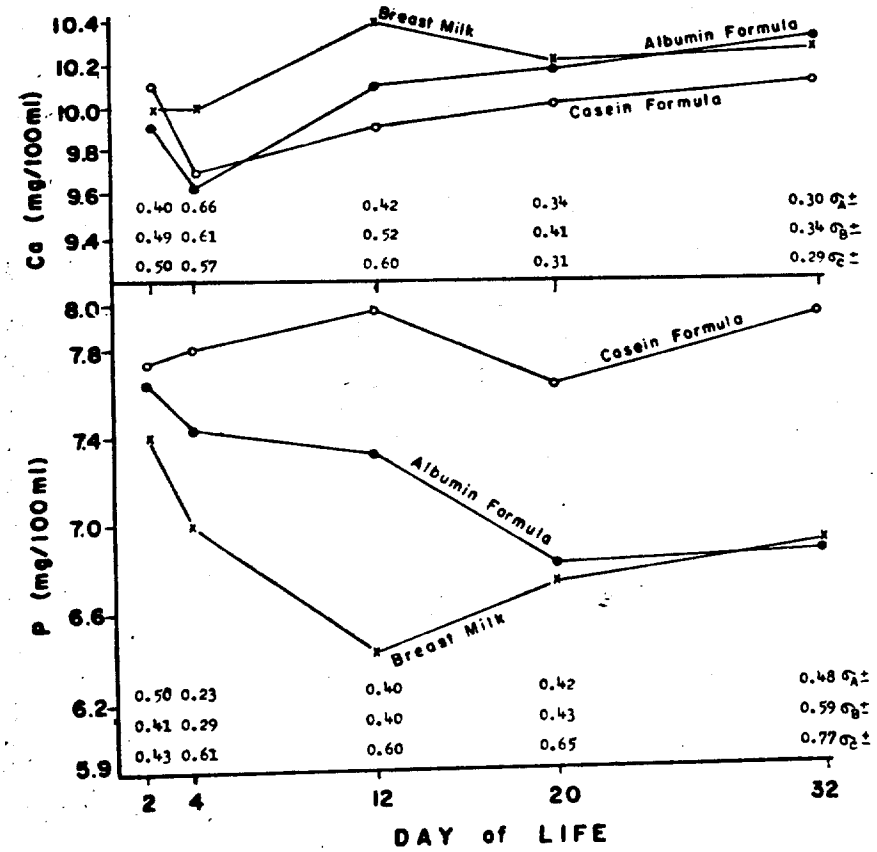

Fig. 8.—Plasma calcium and phosphorus levels for the infants on the three formulas during the experimental period. Standard deviations, σ_A, σ_B, σ_C, for the groups on albumin formula, breast milk, and casein formulas, respectively, are given. Fifteen infants in each group.

ences were not statistically significant when the *t*-test was applied and the figures are lumped in Table 2.

Plasma potassium levels (Fig. 7) dropped during the first four to eight days post partum. This fall corresponded roughly to the gradual disappearance of neonatal jaundice. Subsequently, the values rose to a level which stabilized except for those from the casein formula group, which continued to fall from a high level, so that at 32 days there was no significant difference between the plasma potassium levels in this group and those given the albumin formula. The plasma potassium levels for those given breast milk were significantly higher at the end of the study. Potassium intake on breast milk was highest, since breast-milk potassium levels on the 7th and 30th days were higher than potassium levels of the synthetic formulas. It is worthy of note that the highest point for plasma potassium levels for the casein-formula group (Fig. 7) comes on the 12th day, which correlates with the highest point of plasma urea nitrogen levels on this formula (Fig. 3).

The studies on plasma calcium and phosphorus levels are listed in Figure 8. The observation of Gittleman and Pincus ¶ that calcium levels drop in the first week post partum on a cow's-milk formula was also observed with the synthetic formulas. This is not true for the breast milk, as was also observed in the reference cited. In Figure 8 it is apparent that the values subsequently converge so that the albumin-formula group and the breast-milk group show similar values at the end of the 32d day. The plasma calcium level for the

¶ Reference 24 and cited in Reference 25.

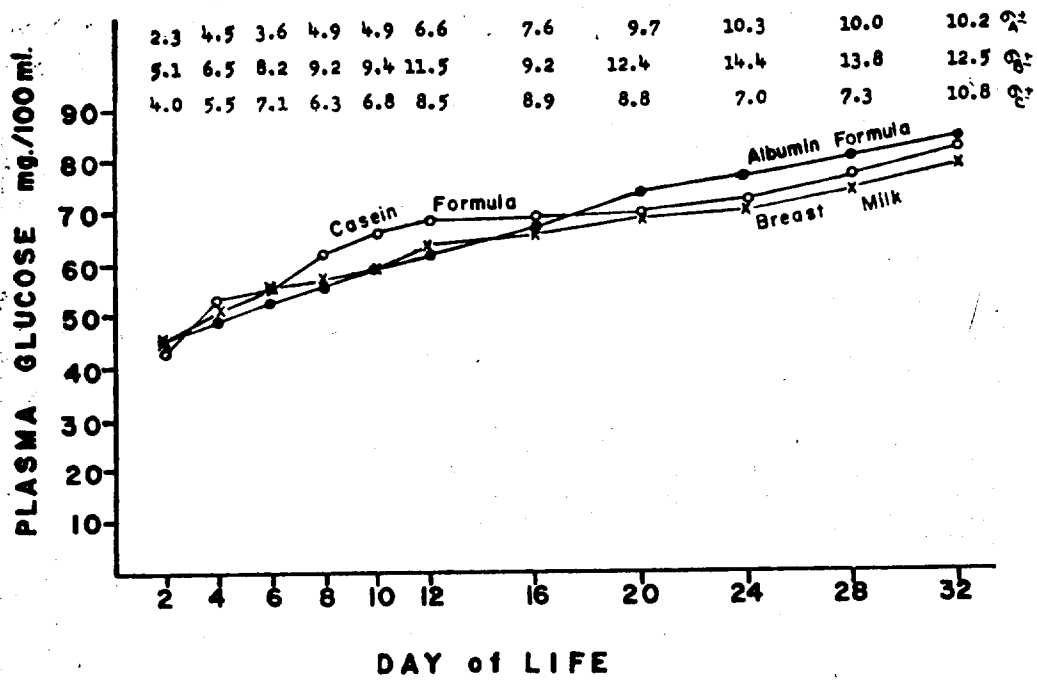

Fig. 9.—Plasma glucose levels for the infants on the three formulas during the experimental period. Standard deviations, σ_A, σ_B, σ_C, are given for the groups on albumin formula, breast milk, and casein formula, respectively. Fifteen infants in each group.

group on the casein formula is significantly lower. An explanation for this phenomenon is suggested from the plasma phosphorus values (Fig. 8).

Almost from the outset, the plasma inorganic phosphorus levels for the casein-formula group were significantly higher than the plasma phosphorus levels for the groups given the albumin and breast-milk formulas. This difference may be explained by the fact that organic phosphate is more readily absorbed than inorganic phosphate.[26] Bovine casein analyzes for 0.82% casein-bound phosphorus. Thus, from Table 1, the albumin formula (Formula A) would calculate to 6.9 mg/100 ml. and the casein formula (Formula C) to 15.3 mg/100 ml. of casein-bound phosphorus. Assuming that breast-milk protein is roughly one-third casein,[2] one may calculate the casein-bound phosphorus of breast milk as approximately 3.4-3.8 mg/100 ml. depending on the lactation date after parturition. Thus the intake of casein-bound phosphorus is substantially higher on the casein formula. If one accepts the thesis that casein-bound phosphorus is more readily absorbed than inorganic phosphate, then it follows that plasma phosphorus levels would be highest on the casein formula. This, in turn, would serve to suppress the plasma calcium levels for the infants on this formula. Gittleman and Pincus also observed higher plasma phosphorus levels on cow's milk than on breast milk.[24]

No significant difference was found in plasma glucose levels between the three formulas (Fig. 9). A gradual rise in plasma glucose levels is found, so that after 30 days the plasma glucose level has been almost doubled (Table 2).

Table 2 summarizes data for the various plasma constituents on the 2d and 32d days of life. The figures for the means are rounded off to significant figures. For the second day of life, data are given for 45 infants before the study was initiated and are the expected values to be found with the analytical methods employed. For the 32d day, where no significant differences were found, the data are statistically pooled. The pooling included glucose and chloride levels. For the other plasma

constituents, the levels are given for the groups on each formula. It is apparent from Table 2 and Figures 3-9 that in listing "normal values" for infants in the first month of life, one must take into account the age of the infant and the particular formula which is being fed to the infant.

SUMMARY

Two feeding formulas were prepared, similar, except that one contained a ratio of noncasein protein to casein of approximately 3 : 2 (A) and the other a ratio of approximately 1:8 (C). Three groups of infants (15 in each group) were fed A, C, and at the breast (B) for 30 days after the second day of life. Blood samples were analyzed at intervals for hematocrit value and plasma Na, K, Cl, CO_2, pH, sugar, urea, protein, calcium, and phosphorus. Total intake was essentially the same on Formulas A and C but somewhat higher for breast milk after the seventh day of life. Breast milk was analyzed for fat, protein, carbohydrates, Na, K, Cl, Ca, and phosphorus on the 7th and 30th days. Caloric content of breast milk was 59.7 Cal/100 ml. on the 7th day and 56.4 Cal/100 ml. on the 30th day. A and C were calculated to 65.3 Cal/100 ml. No significant difference in weight gain was observed between the three groups.

Differences found in plasma urea levels were in the order C>A>B. Plasma protein levels with A were higher than those on B or C. Potassium levels were highest with B, but B contained higher amounts of potassium than A and C. CO_2, Na, and Cl levels suggested that blood pH was in the order B> A> C at the end of the experimental period. Direct blood pH measurements suggested differences in the same order, but these were not significantly different when analyzed statistically. For the group receiving C, phosphate levels were highest and calcium levels were lowest at 30 days. C contained by far the highest amount of casein-bound phosphate. Hematocrit levels declined steadily, with no significant difference being noted until the 30th day, when the level for the group on breast milk became significantly higher. Glucose levels rose steadily during the 30-day period for all groups and were not significantly different.

From this study one may conclude that "normal" blood levels of certain constituents studied in this report on healthy infants depend upon the age of the infant and are influenced by the protein level and the ratio of noncasein protein to casein in the formula. Further, noncasein cow's-milk protein is more efficiently utilized by the infant in building plasma proteins than casein.

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Changes in Dietary Casein in Mouse

Effects

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Introduction

Kwashiorkor is regarded as predominantly a manifestation of protein deficiency in man, and the associated liver damage has been considered a major factor in the development of liver cancer during adult life.[1] Attempts to develop a satisfactory experimental model in rodents reproducing the full pathological picture of this disease have not proved satisfactory without force feeding.

While much information is available on the nutritional requirements of mice,[2,3] relatively few reports have been published regarding the pathology of lesions in the liver and other organs produced by various diets in this animal in contrast to the numerous studies in the rat.[4-12] While it is generally accepted that lesions are similar to those found in the rat, there is evidence of considerable strain differences in the lesions produced.[13,14] During studies on hepatic carcinogenesis, several groups of mice were maintained on synthetic diets differing predominantly as to casein content. Although not designed systematically from a nutritional viewpoint, these studies provide some additional information regarding the effects on the liver and other organs of varying protein levels in this species. They also emphasize that only some of the features of the kwashiorkor syndrome can be produced by either a restricted- or low-casein diet in this species.

Material and Methods

Mice of 3 different lines fed on diets containing various levels of casein were studied for a period of 100 days (Table 1). Animals in Groups 6 and 7 were maintained for 324 days. They were kept in groups of 6 to 10 animals per cage. The following diets were utilized.

1. *Low-Protein Diet.*—Ingredient percentages were: vitamin-free casein, 4; sucrose 65; alphacel, 15; Mazola oil 10, salt mixture U.S.P. XIV, 4; vitamin mixture 2 (Nutritional Biochemical Co.). Due to the high alphacel content, this diet had a considerable bulk, thus corresponding somewhat to certain high-residue maize diets used in parts of Africa.

2. *High-Protein Diet.*—Percentages were vitamin-free casein, 20; sucrose 59, alphacel, 5; Mazola oil, 10; salt mixture U.S.P. XIV, 4; vitamin mixture, 2.

3. *Intermediate-Protein Diet.*—Percentages were vitamin-free casein, 12; sucrose, 77, Mazola oil, 5; salt mixture U.S.P. XIV, 4; vitamin mixture, 2.

4. *Low-Protein Diet with Cystine Supplement.*—Ingredient percentages were as in Low-Protein Diet except for the addition of 0.5% L-cystine at the expense of sucrose.

Control mice were fed on Purina Laboratory Chow. All animals were weighed weekly with the exception of Groups 3 and 5 which were weighed daily in order to maintain similar growth curves. Additional Hauschka mice fed on 20% diets for periods up to 400 days were also available for study.

At the end of the experiment, the animals were killed and the organs fixed in 10% buffered

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TABLE 1.—*Experimental Mouse Groups According to Strain and Diet*

Group	Strain	Sex	Survivors 100 Days	Diet	Period of Diet, Days	Approx. Age	Serum Protein, Gm/100 Ml	How Killed
1	C3H	M	20	Purina Laboratory Mouse Chow	100	8 weeks	--	Killed
2	C3H	M	12	High-protein (20% casein)	100	idem.	6.45	idem.
3	C3H	M	20	Low-protein (4% casein)	100	idem.	4.45	idem.
4	C3H	M	10	Low-protein+0.5% cystine	100	idem.	6.45	idem.
5	C3H	M	8	High-protein restricted *	100	idem.	6.92	idem.
6	C3H	M+F	24	Intermediate-protein (12% casein)	Up to 324 days	16 weeks	--	idem.
7	C3H	M+F	103	Intermediate-protein + Carcinogen (12% casein)†	Up to 324 days	idem.	--	idem.
8	CFW	M	7	High-protein (20% casein)	100	8 weeks	--	idem
9	CFW	M	19	Low-protein (4% casein)	100	idem.	5.60	idem.
10	Hauschka	M	13	Low-protein (4% casein)	100	idem.	6.18	idem.

Groups 1-7: C3H mice from Jackson Memorial Laboratories, Bar Harbor, Maine; Groups 8-9: CFW, local random-bred mice of CFW line; Group 10: Hauschka, albino mice, random-bred from line developed by Hauschka at Institute for Cancer Research, Philadelphia.

* Group 5: Restricted amounts of the high-protein diet (20% casein) were fed to maintain mean weight identical to that of animals in Group 2.

† Group 7: The carcinogens were *p*-dimethyl-aminoazobenzene (DBA), carbon tetrachloride, and thioacetamide, singly or in combination. Animals were killed at varying intervals up to 324 days.

formalin. Paraffin sections were stained routinely with hematoxylin and eosin. Special stains were carried out as necessary and included Congo Red, Perl's stain, periodic acid-Schiff, and Van Kossa's stain. Fat was demonstrated by oil red O on frozen section.

Results

Discussion will be predominantly limited to the more significant lesions in the C3H mice, Groups 1-5, in which changes were consistent. Lesions in the liver resulting from the administration of carcinogens in Group 7 will not be described here, but in general they were similar to those described by others in mice. Where pertinent, reference will be made to the CFW and Hauschka lines which were less consistent in their histological reactions, and in which focal lymphocytes and mononuclear cell infiltration in the liver, kidneys, and lungs were common incidental findings.

Weights.—The C3H mice in Groups 1, 2, and 6 grew well. The mean weight of animals on 20% casein was approximately 10% higher than animals on Purina Laboratory Chow. The mean weight of those on 4% casein (Group 3) fell from 21 gm. to 17 gm., at which weight they remained until the end of the experiment. They appeared sick and unthrifty, in contrast to Group 5 which appeared well and active. Group 4 fell to 17 gm. in 28 days, after which their weight rose slowly to the initial weight. The weight trends in the other strains were similar.

In Group 7, the weight curve of mice given carbon tetrachloride was similar to the controls; the remaining mice on *p*-dimethyl-aminoazobenzene (DBA) or thioacetamide gained weight poorly and showed a terminal loss.

Blood Chemistry.—At autopsy, the calcium and protein levels were estimated on pooled sera for Groups 2-5 of C3H mice, and the results are given in Tables 1 and 2. The serum proteins in the C3H mice on a low-casein diet were markedly diminished. No obvious marked fall in serum protein was seen in the restricted-casein and cystine-supplemented groups.

Histological Findings

Liver.—C3H, Groups 1, 2, and 6; Purina Chow, High-Protein and Intermediate-Protein Diets: The livers were essentially normal in the C3H groups. Three of the CFW mice (Group 8) on 20% casein showed amyloidosis (Fig. 1).

C3H, Group 3; Low-Protein Diet: The livers showed scanty mild fatty change, the

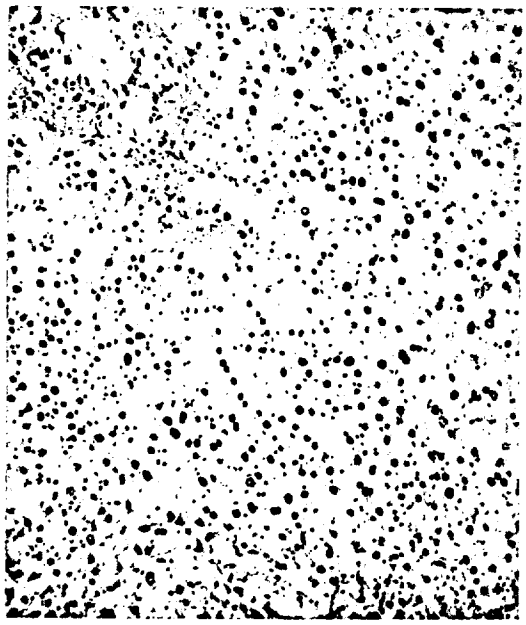
TABLE 2.—*Calcification in Animals Receiving High-Protein and Low-Protein Diets (100 Days)* *

Group	Diet	Heart	Aorta and Blood Vessels	Kidney	Lung	Total in Group	Serum Calcium, mEq/L
1	Purina Chow, C3H	0	0	0	0	20	--
2	High-Protein, C3H	7(±)	0	0	0	12	5.6
3	Low-Protein, C3H	19(+±)	5(++)	10±	6(+)	20	5.0
4	Low-Protein + Cystine, C3H	8(+)	0	0	0	10	4.8
5	Restricted High-Protein, C3H	5(+)	0	0	0	8	5.1
8	High-Protein, CFW	0	0	0	0	7	--
9	Low-Protein †, CFW	4(+)	2(+++)	1(+)	0	12	5.4
10	Low-Protein, Hauschka	4(+)	4(+++±)	6(+)	0	13	5.2

* Figures in parenthesis indicate mean degree of calcification for animals with calcification.
† Five of 10 animals who died before 100 days also showed calcification, in 2 of which there was 3+++ calcification in the aorta.

fat being diffusely scattered in fine droplets throughout the cytoplasm, especially in the midzonal and central regions (Fig. 2). The periportal cells also showed a fine vacuolar fenestration due to increased glycogen deposition. In the majority of livers, increased polyploidy and frequent focal eosinophilic degeneration of individual cells was also observed, so that hyperplastic and atrophic cells were in close apposition. In some livers, mitoses were prominent, suggesting increased regeneration. Scanty ceroid deposition was also noted in the Kupffer cells.

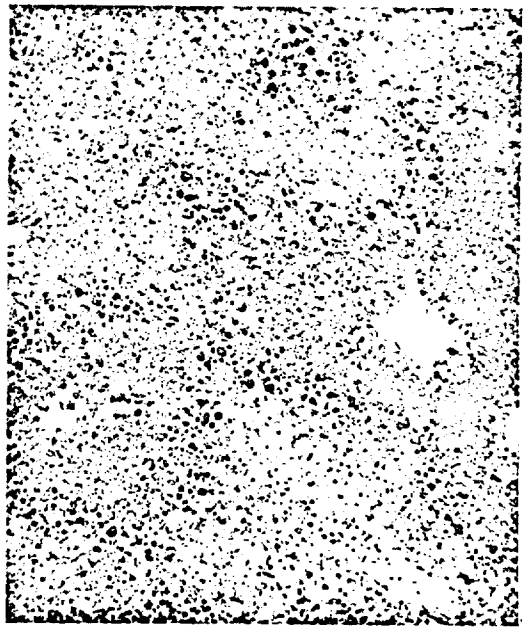
Fig. 1.—Liver from CFW mice fed 20% casein for 100 days showing moderate amyloidosis. Hematoxylin and eosin; × 110.



There was no evidence of fibrosis, and no amyloid was observed. In the Hauschka and CFW mice, the degree of fatty change was less than in the C3H mice and more diffuse.

C3H, Group 4; Low Protein Diet + Cystine: In these mice, there was greater fatty change than in Group 3. In contrast to the low-protein group, the fat was definitely periportal, and large globules and fatty cysts were frequent (Fig. 3).

Fig. 2.—Liver in animals on C3H mice fed low-protein diet for 100 days: slight fine fatty change involving predominantly midzonal and central lobule. Hematoxylin and eosin; × 100.



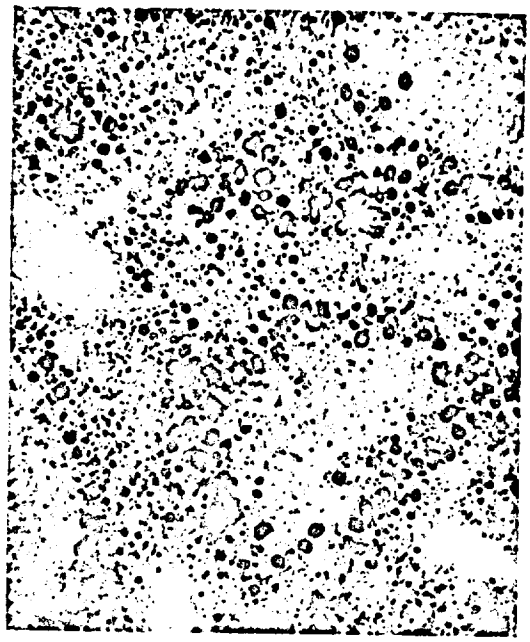


Fig. 3.—Liver in C3H mice fed low-protein diet supplemented with cystine for 100 days. Moderate fatty change is present in the periportal region. The fat is deposited in large droplets and in microcysts in contrast to the liver in Figure 2. Hematoxylin and eosin; × 100.

C3H, Group 5; High-Protein Diet Restricted: These livers (C3H) showed slight fatty change, which was more diffuse than in Group 3 and without definite lobular distribution.

Comment: The above findings suggest that the lesions in the C3H mice are essentially similar but milder than those produced in the rat by a low-protein diet.[4,6,11,12] Reports of the effect of low-protein diets in the mouse liver are limited. White and Mider [13] found no structural change in DBA strain mice fed a 3.0% casein diet with or without 0.5% cystine. Heller and Blackmore [14] observed rapid development of periportal fatty change in adult albino mice fed a low-protein diet (0.5% casein) adequately supplemented with choline and cystine. In some animals, the fat completely disappeared within 10 days, when the experiments were stopped. Our observations would indicate that the fat in the liver may remain for a much longer time, although the increased glycogen deposition in the periportal areas suggests realimentation of fat as seen in rats.[4] Although there was histological evidence of cytological damage with atrophy and regeneration of individual cells, no evidence of inflammation or early cirrhosis was apparent. In no animals was the degree of fatty change comparable to that seen in kwashiorkor.[1] The increased accumulation of fat in the liver with cystine supplementation in the rat is well known.[12]

Kidney.—C3H, Group 2; High-Protein Diet: The kidneys appeared grossly to be slightly enlarged. On section, many proximal convoluted tubules showed marked swelling of the epithelial cells, the cytoplasm of which appears clear and vacuolated with pyknotic basal nuclei (Figs. 4 and 5). The clear cytoplasm did not stain for fat and was PAS-negative. In some areas, the inner portion of the tubular cells appeared to have sloughed into the lumen to form acidophilic, proteinous, PAS-positive casts leaving only the basal portion of the cells, which often appeared amorphous and degenerated. In other tubules, casts were present without evidence of tubular damage. Although fixation in all groups was identical, occasional

Fig. 4.—Details of renal tubular change in C3H mice on high-protein diet showing marked swelling of the epithelial cells with apparent reduction of the lumen. The nuclei tend to be small, pyknotic, and basal in position. Hematoxylin and eosin; × 500.

Fig. 3.—Kidney from a C3H mouse on high-protein diet showing swelling of the epithelial cells and proximate convoluted tubules, many of which show poorly staining cytoplasm. Casts are present in the lumen of several tubules. Some of the tubules appear atrophic and collapsed with loss of cytological detail. Metaplastic change in the parietal layer of Bowman's capsule is prominent. Periodic acid-Schiff stain; × 240.

tubules showed advanced postmortem autolysis, so that well-fixed and autolytic tubules often were closely approximated. In the glomeruli, the entire parietal layer of the Bowman's capsule showed marked metaplasia to cuboidal or columnar epithelial cells morphologically similar to the cells of the proximal convoluted tubules (Fig. 4) and of greater extent than in mice on Purina Chow or 12% casein.

Kidneys from Hauschka on 20% casein and CFW mice, Group 8, did not show these tubular changes. In 2 CFW mice there was marked pyelonephritis and tubular damage associated with amyloidosis.

C3H, Groups 3 and 4; Low-Protein Diet: In these animals, the tubules were atrophic, and the parietal layer of the Bowman's capsule was almost invariably of the flattened pavement type. In some animals there was slight focal calcification of the tubules, especially in the medulla, and less frequently of the renal artery and its branches (Table 2).

The findings in the CFW and Hauschka mice were similar, and no amyloid was present.

Comment: The effect of dietary and injected protein in causing hypertrophy of the rat kidney has been previously described.[16-19] Baxter and Cotzias [17] have reported tubular lesions similar to those described here following gelatin injections but did not see such lesions following caseinate injections. Renal hypertrophy has been reported in rats on a high-casein diet, but the tubules have been described as normal.[16] However, when unilateral nephrectomy is carried out, tubular degeneration and necrosis occur.[18,20] The exact nature of the tubular enlargement is uncertain but is possibly related to increased function.[16,18] The wide range of renal lesions often found incidentally in the mouse has been summarized by Dunn.[22] It is possible that some of the tubular damage seen in chronic pyelonephritis found so frequently with increasing age in this species may be potentiated by the dietary protein content and not be due only to inflammation. The rapid postmortem autolysis is of interest and is probably related to tubular swelling. Similar rapid postmortem autolysis has been described in vitamin-E-deficient rats.[23] It was not observed in either CFW or Hauschka mice on a 20% casein diet.

The metaplastic change in the Bowman's capsular epithelium is found in normal mice,[22] predominantly in aging males [24,25] and also in pregnant females [24] or females injected with testosterone propionate.[26] Gorer [25] reports its occurrence in greater frequency in mice of DBA strain, particularly in the young mice with various infections. The absence of this metaplasia in the low-protein group could be owing to a relative lack of male hormones due to partial atrophy of the testes or to the general inanition due to low-protein diets. The marked response in the 20% casein group probably indicates that the capsular cells are responding in a similar manner to the tubular cells.

Salivary Gland and Pancreas.—The submandibular glands in C3H mice on low protein showed moderate to marked atrophy

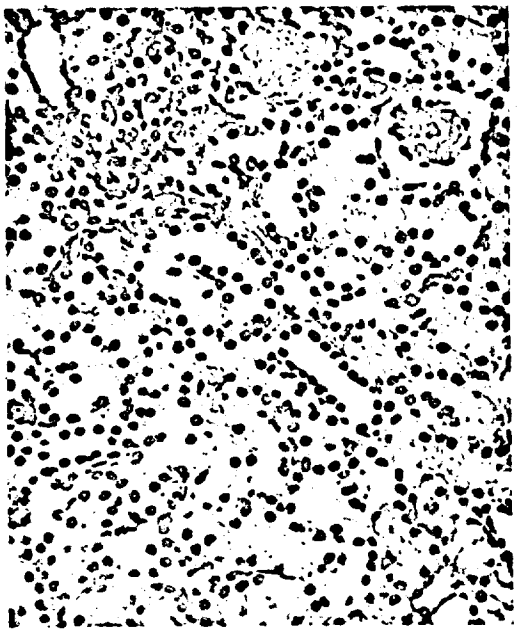


Fig. 6.—Kidney of C3H mouse fed on low-protein diet showing moderate tubular atrophy and flattening of the epithelium of the glomeruli, compared with previous figure. Hematoxylin and eosin; × 240.

Fig. 7.—Submandibular gland from C3H mouse on high-protein diet. Notice the typical tall acidophilic cells lining the ducts. Hematoxylin and eosin; × 240.

of the ductular glands which became lined by vacuolated low cuboidal cells, devoid of secretory granules (Figs. 7 and 8). The exocrine element of the pancreas in these animals also showed some degree of atrophy with partial loss of zyomogen granules (Figs. 9 and 10), but the extreme atrophy seen in human kwashiorkor was not observed. On the other hand, in animals receiving the cystine supplement, the salivary

Fig. 8.—Submandibular gland from C3H mouse on low-protein diet. Marked atrophy of the ductular epithelium is present. Hematoxylin and eosin; × 240.

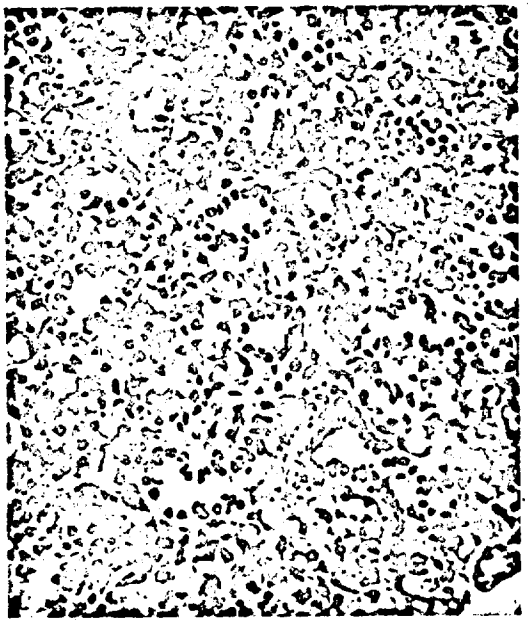
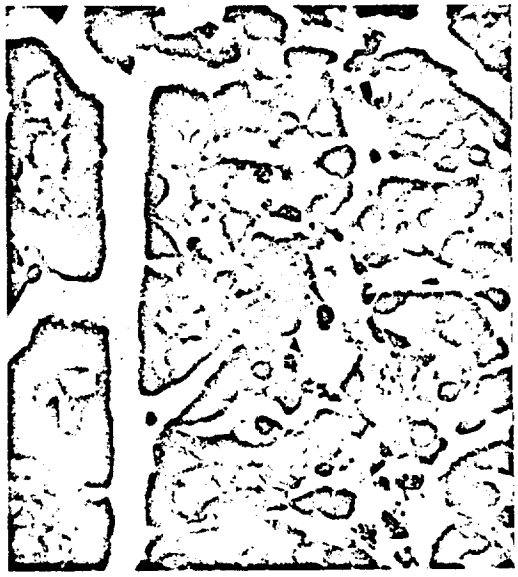


Fig. 9.—Almost normal pancreas in a C3H mouse on a cystine-supplemented low-protein diet. Hematoxylin and eosin; × 240.



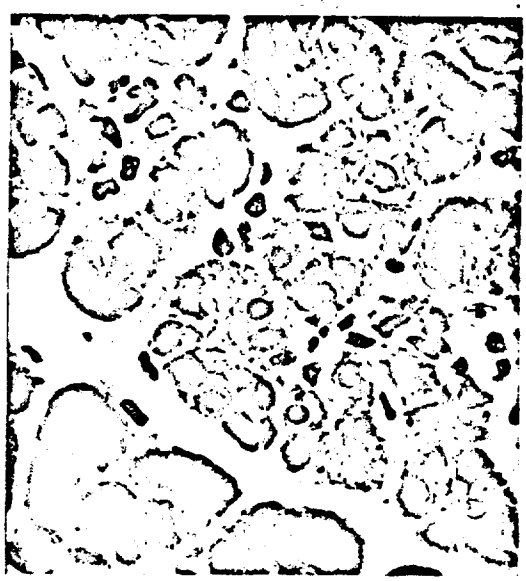


Fig. 10.—Pancreas from C3H mouse on low-protein diet showing slight atrophy. There is a slight reduction in the cell size, and the differential staining between the inner and outer portions of the acinar cells has disappeared. Hematoxylin and eosin; × 240.

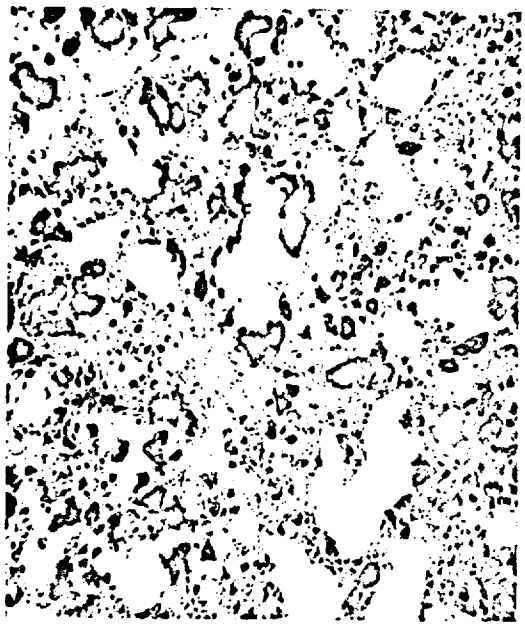


Fig. 11.—Lung from C3H mouse on 12% casein diet given carbon tetrachloride. There is marked calcific deposition in the alveolar walls with numerous large calcified masses in the alveoli. Hematoxylin and eosin; × 70.

glands and pancreases were essentially normal. In the C3H protein-restricted group, the degree of salivary atrophy was much less, and changes in the pancreas was negligible. Changes in the CFW and Hauschka shown were minimal, and only one showed significant salivary gland atrophy. Similar changes in pancreas and salivary gland have been described in the rat [11] on deficient diets.

Sex Organs.—In C3H mice on low-protein diet, the testes, vesicular, and other accessory sexual glands were markedly atrophic. Histologically, the testes showed noticeable diminution in spermatogenesis with thinning of germinal epithelium of the seminiferous tubules. However, there was no thickening of basement membranes or fibrosis. The accessory sex glands were distinctly smaller in size with low cuboidal epithelium. The changes in the testes in the CFW and Hauschka mice were much less prominent.

Brain.—The brain in some of the C3H mice on low-protein diet showed small calcific deposits and only occasionally pyknosis of the nuclei of few neurones. The latter change, however, was not consistent nor remarkable.

Other Organs.—The parathyroids and adrenal glands showed no significant abnormality in any of the animals apart from nonspecific atrophy in the low-protein group. The thyroid gland in many animals both from the low- and high-protein groups of all strains showed large vesicles formed by confluence of several smaller ones and lined by a low cuboidal of flat epithelium giving the appearance of colloid acini.

Calcification in Cardiovascular Systems and Lungs.—Calcific lesions were evident in the heart, lung, kidney, and larger blood vessels in some animals from all groups (Tables 2 and 3). The pathological changes were similar to those described by Highman and Daft [27] (Figs. 11-13). Calcification was more extensive in mice on a low-protein diet. The most marked calcification was found in the C3H on hepatic carcinogens.

Comment: White and Mider [13] and White et al.[28] fed a 3.9% casein diet to DBA mice and noted calcification in the aorta after a mean latent period of 106 to 121 days. This calcification appeared largely preventable by addition of 0.5% cystine to the diet. Similar results have been obtained in Strain A mice.

TABLE 3.—*Calcification in Mice on 12% Protein Diet with Carcinogens* *

Experiment	Duration in Days	Total	Animals with Calcification	Calcification: Heart	Aorta and Other Blood Vessels	Kidney	Lung	Liver
Control (Group 6)	100-	3M:1F	0	0	0	0	0	0
	201-	3M:2F	2M	1(±)	0	1(+)	0	0
	251-	2M:4F	2M:2F	2(+)	1(+)	3(±)	0	0
	301-324	9F	6F	4(+)	1(+)	6(+)	3(+)	0
Carcinogen Group (Group 7)	100-	12M:8F	9M:5F	5(+±)	4(++)	7(±)	6(+)	7(+)
	201-	12M:13F	12M:11F	16(+±)	14(+±)	19(+)	17(+±)	4(+)
	251-	19M:14F	15M:12F	19(++)	17(+)	17(+)	19(++)	5(+)
	301-324	12M:13F	13M:13F	15(+±)	8(+±)	21(++)	15(++)	6(+±)

* Figures in parenthesis indicate average degree of calcification.

However, Highman and Daft have demonstrated that calcification is not completely prevented by cystine.[27] Although more common in the low-protein than in the high-protein groups for animals up to 100 days, calcification was best seen in the C3H mice on 12% casein (Table 3) and was most marked in those animals given various hepatic carcinogens for long periods. Calcification was found equally with all agents including carbon tetrachloride. In this group, the weight curve was similar to the controls, indicating that retardation of growth alone is not responsible for the phenomenon. Highman and Daft [27] also used carbon tetrachloride in association with a low-protein diet in their studies on arterial calcification, and tissue destruction is probably a significant etiological factor. Morphologically, the arterial calcification shows similarities to Monckeberg's degeneration. Whereas the arterial and renal calcification appears essentially metastatic in nature, that in the heart would appear to be dystrophic.[29] Table 2 shows no obvious correlation between the degree of calcification and the pooled serum

Fig. 12.—Extensive calcification of pulmonary artery in a Hauschka mouse on low-protein diet. Hematoxylin and eosin; × 45.

Fig. 13.—A C3H mouse on low-protein diet showing extensive calcification of degenerated myocardial fibers. Hematoxylin and eosin; × 240.

calcium levels. Unfortunately, no calcium levels were done in the carcinogen group which showed the most severe calcification.

There was some reduction in the number of bone trabeculae in the long bones in animals on low-protein diets, and metaphysical changes were found similar to those reported in guinea pigs by Silberberg and Silberberg in starvation.[30]

Amyloidosis.—Amyloidosis was found in none of the C3H mice in the present study. Three CFW mice on 20% casein diet developed generalized amyloidosis involving the liver, spleen, kidneys, adrenals, and small intestine as described by Wong et al.[21] Similar amyloidosis has been observed by us in 60% of 55 Hauschka mice fed a 20% casein diet for periods up to 400 days.

Comment: It is well known that caseinate given by injection can readily cause amyloidosis in mice, but it would also appear that a high dietary content of casein may also be a causative factor.[21] According to Dunn,[22] the lesions are exaggerated by chronic infection, but this was not found by Wong et al.[21] In the additional group of Hauschka mice, while renal inflammation was more frequent in animals with amyloidosis, it would appear possible that renal amyloidosis potentiated the inflammatory changes rather than vice versa, and further studies are being made on this possibility.

Conclusions

The majority of the changes found in the present animals on a low-protein diet are in general nonspecific and similar in many ways to those in general inanition as described by Follis.[31] In no animals were the pathological features of human kwashiorkor fully produced. The degree of acinar atrophy in the pancreas was relatively slight in comparison to that seen in man, as was the degree of the liver fatty change. While the addition of cystine increased the degree of fatty change in the liver, it also prevented changes in the pancreas, salivary glands, and serum proteins, although weight gain was minimal. Since kwashiorkor in man is most frequently observed at weaning, it is possible that a more comparable picture might have been produced by the use of younger mice, and this possibility is being further explored.

Summary

Eight groups of mice representing 3 different lines were fed on diets with varying levels of casein for 100 days. Although periportal fatty change and mild cytological changes were present in the liver, there was no evidence of fibrosis or cirrhosis. The addition of cystine to the diet in C3H mice increased the degree of fatty change but prevented atrophy of the pancreas and salivary glands. In no instance was the picture of human kwashiorkor produced. A high-casein diet in the C3H mice led to a moderate degree of renal tubular degeneration and necrosis. In the Hauschka and CFW lines, amyloidosis was observed, but degenerative tubule changes similar to that seen in the C3H mice were not observed. Other common lesions observed in experimental mice and their relation to diet are briefly discussed.

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Effects of Added Casein on Goitrogenic Action of Different Dietary Levels of Soybeans.* (27535)

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In at least 14 papers, from 1933 onward, it has been reported that diets containing soybeans (*Glycine max*) or soybean products cause enlargement of the thyroid gland in the rat, chick, guinea pig, rabbit or human. Greer (1,2) has reviewed many of these findings, and has suggested that soybean diets may be goitrogenic simply because they have a low iodine content. Some of the soybean diets reported as goitrogenic contained only a small proportion of beans. Hence in these studies it seems possible that the goitrogenic effect of the beans was not manifested, with the result that the thyroid enlargement seen could have been due solely to iodine deficiency. To shed light on these questions, one of the experiments in the present study was designed to measure and compare the goitrogenic effects of low and high dietary levels of soybeans.

An observation on which there has been unanimity is that heating of soybeans reduces or obliterates their goitrogenic property(3). Moreover, the best studied of the known toxic factors of the soybean, the trypsin inhibitor, is similarly reported to be heat labile(4). This similarity raises a question as to whether the two effects might have the same basis, and has suggested the remaining experiments reported here. These experiments were designed to determine whether protein supplementation of raw soybeans, as is known to reduce or eliminate the growth-retarding effect of the soybean trypsin inhibitor(4), has an effect on the goitrogenic property of soybeans.

Materials and methods. The soybeans used were obtained from an oil processing plant (courtesy of Dr. H. W. Howard, The Borden Co.). The beans, of a small, yellow, black hilum variety, were ground in the laboratory, with care to avoid frictional heating. Soybean meal made from the same lot of beans was also obtained. This meal is the residue from fat extraction of soybeans, after which the meal is "toasted" and ground.

The diets designated 35 and 35CAS (Table I) contained 35% by weight of raw soybeans; those designated 60 and 60CAS contained 60% of raw soybeans. To equate the fat and

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TABLE I. Effects on Thyroid Gland Parameters of Feeding Soybean Diets *ad Libitum* to Male Sprague-Dawley Rats for 7 Weeks Starting When Weaned at 3 Weeks of Age with Weights Averaging 48 g. Avg values, 10 rats/group.

Diet code	60MEAL*	35	35CAS	60	60CAS
Iodine in diet, μg/g	.005	.005	.003	.003	.003
Food consumed, g	601	460	637	603	590
Wt gained, g	194	95	208	165	187
Thyroid wt, mg/100 g					
Fresh	19.9	18.8	17.1	27.4	20.8
Stand. dev.	4.0	3.3	4.1	6.9	2.9
Dry	4.31	5.16	4.06	6.05	4.85
Stand. dev.	.95	.78	.99	1.23	.73
Iodine in pooled thyroids, % of dry wt	.0063	.0150	.0180	.0033	.0110

* Control.

crude fiber levels of these diets, 5% of corn oil (Mazola, Corn Products Refining Co.) and 6% of cellulose (Alphacel, Nutritional Biochemicals Corp.) were included in diets 35 and 35CAS.

Casein (Purified, Nutritional Biochemicals Corp.) was included in 2 of the diets; 17% in diet 35CAS and 15% in diet 60CAS. All diets included 4% of U.S.P. XIII No. 2 salt mixture (which contains no iodine salts) and 2.2% of a complete vitamin mixture triturated in glucose (Diet Fortification Mixture, Nutritional Biochemicals Corp.). The remainder of each diet was made up of corn starch (Argo, Corn Products Refining Co.).

As Table I indicates, the control diet was the diet designated 60MEAL. This diet differed from diet 60 only in that it contained, in place of 60% of raw soybeans, 49.6% of soybean meal plus 10.4% of corn oil to restore the fat level to that of whole soybeans. Extraction of this meal had subjected it to approximately 75°C for one-half hour, after which it was subjected to moist heat at 110° for 25 minutes. There is a consensus in prior publications(3,5,6) that this much heating destroys the positive goitrogenic property of soybeans. Hence diet 60MEAL may be considered to have no goitrogenic effect other than that produced by its low iodine content. To verify this assumption, an experiment (not reported here for the sake of brevity) was conducted with the same experimental design as described in the present report, comparing thyroid weights of rats fed diet 60-MEAL with those of rats fed the Remington diet(7), a standard formulation known to produce an uncomplicated iodine deficiency goiter(8). The fresh and dry thyroid weights of animals fed one or the other of these diets did not differ significantly.

All 5 diets were found to contain 0.003 or 0.005 μg of iodine per g, and hence provided less than 2% of the normal rat's iodine requirement (Table I). Iodine in diets and in thyroid glands was determined by the method of Chaney(9).

Male albino rats, descendants of the Sprague-Dawley strain (from S & K Animal Co., Jamesburg, N. J.) were weaned at 3 weeks of age when their body weights ranged from 41 to 55 g and averaged 48 g. Ten rats were assigned, by statistical randomization, to each experimental group. The animals were housed in separate metal cages with wire mesh bottoms, were provided *ad libitum* with water from an ion exchange column (Barnstead Bantam) and were fed one of the 5 diets *ad libitum* for 7 weeks, while records of individual food intake and body weights were kept. Then the animals were killed with ether and the fresh weights of their thyroid glands determined individually. Thyroid gland weights were expressed in terms of mg per 100 g of total weight (Table I) because the weight of the thyroid gland in this strain and sex of rats has been found to bear an approximately linear relationship to total weight, at weights ranging from 87 to 328 g (10).

Weight of dry matter in each gland was determined by drying to constant weight in a

convection current oven at 105°C. The thyroid glands of the 10 animals in each experimental group were finally pooled to provide enough material for iodine assays.

Results. The animals fed diet 60 (60% raw soybeans) had significantly heavier thyroid glands than those fed the control diet 60MEAL, whether judged by fresh weight of the glands ($P = 0.01$) or by dry weight ($P = 0.005$). The thyroid glands of animals fed diet 60 also had a lower iodine content than the controls.

The thyroid glands of animals fed diet 35 (35% raw soybeans) did not differ significantly from those of control animals in fresh weight, but were significantly heavier in dry weight ($P = 0.05$). Iodine assay of the thyroid glands of animals fed diet 35 gave the anomalous result of a higher iodine level than the controls.

Adding casein to diets containing 35% or 60% of raw soybeans increased the rate of body weight gain. Such additions of casein also entirely obliterated the thyroid gland effects of the soybean diets. Thus the thyroid glands of animals fed diet 60CAS were significantly lighter than those of animals fed diet 60 ($P = 0.02$, fresh or dry weights); and not significantly different from those of animals fed the control diet 60MEAL. As the effect of 35% of raw soybeans was seen only in increased dry weight of the thyroid gland of animals fed diet 35, it is not surprising to note that the sole significant effect of adding casein to a 35% soybean diet was on this parameter. Thus fresh thyroid weights of animals fed diets 35 and 35CAS did not differ significantly, whereas dry thyroid weights of animals fed diet 35CAS were significantly lighter than those of animals fed diet 35 ($P = 0.02$). Thyroid gland weights of animals fed diet 35CAS did not differ significantly from those of animals fed the control diet 60MEAL. Animals fed the diets with casein had thyroid glands containing more iodine than those of animals fed the corresponding soybean level without casein.

Discussion. The minimum proportion of raw soybeans in the diet capable of producing a goitrogenic effect might be expected to depend upon the goitrogenic potency of the particular beans used, as well as perhaps upon the species studied and the nature of the remaining dietary constituents. Sharpless *et al.* (5) reported that rats fed a 35% unprocessed soybean flour diet had thyroid glands about half as big, containing about twice as much iodine, as those fed a diet containing 75% of this soybean material. Halverson *et al.*(6) found no goitrogenic effect when a 10% raw soybean diet was fed to rats, but observed goiters when a 30% soybean diet was fed. Wilgus *et al.*(11) used chicks, considered to be more sensitive to goitrogenic influences than rats; but they fed expeller process soybean oil meal which would be expected to have low goitrogenic potency as it is heated vigorously during manufacture. These investigators could offer no explanation for their observation that 12% or 30% of meal in the ration produced equal enlargement of the thyroid gland, whereas 60% produced goiters twice as large. Their diets were reported to contain less than 0.1 μg per g of iodine. Thus it seems likely that their 12% or 30% soybean meal diets produced a simple iodine deficiency goiter, whereas 60% of soybean meal produced a positive goitrogenic effect.

These previous workers measured goitrogenesis solely by fresh weight of the thyroid gland. Hence it is of interest that in the present study the effect of 35% raw soybeans in the diet was apparent only in increased dry weight of the thyroid gland, whereas 60% of raw soybeans caused increases in both fresh and dry weights.

Casein has generally been reported to have an antigoitrogenic effect(12). This property has been held variously to be due to iodine in the casein(7) or not to be due to iodine(13). The antigoitrogenic effect of casein in soybean diets is clearly seen in the present experiments, where it appears not to be due to iodine introduced by the casein, as diets 35CAS and 60CAS were assayed to have iodine contents as low as, or lower than, diets 35 and 60.

The sole protein in diets 35 and 60 was raw soybean protein, known to have low biological value(4). Hence the addition of casein, a protein having high biological value, may have served to reduce the goitrogenic ef-

fect by providing proteins to aid humoral transport of thyroid hormones(14) or perhaps to aid in reabsorption of thyroid hormones from the gut in the enterohepatic cycle(15). These possibilities raise a question as to whether the soybean goitrogenic effect may be related to, or even identical with, the soybean antitrypsin effect.

Summary. The goitrogenic effect of raw soybeans was seen in significant increases of both fresh and dry weights of thyroid glands when a diet containing 60% of beans was fed to rats, and a significant increase in the dry weight only of the gland when a diet containing 35% of raw soybeans was fed. The goitrogenic effect of 35% or 60% of raw soybeans in rat diets was obliterated by adding casein to the diets. This result was not attributable to iodine in the casein.

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CASEIN (PURIFIED)

Protein (NX 6.38)	88.0 min.
Moisture	8.0 max.
Ash Phosphorous Fixed	2.0 max.
Ether Extractables, Mojonnier	1.5 max.
Reducing Substances (as Lactose)	0.35 max.
Acidity (as Lactic Acid)	0.1 max.
Heavy Metals (U.S.P. Test)	25 ppm max.
Particle Size	through 30 or 80 mesh
Odor	clean and characteristic
Color	light cream
pH (10% suspension in water)	4.8 min.

AMINO ACID CONTENT

Alanine	2.6
Arginine	3.6
Aspartic Acid	6.3
Cystine	0.3
Glycine	2.4
Glutamic Acid	20.0
Histidine	2.7
Isoleucine	5.4
Leucine	8.2
Lysine	7.3
Methionine	2.5
Phenylalanine	4.4
Proline	10.1
Serine	5.6
Threonine	4.3
Tryptophane	1.1
Tyrosine	5.6
Valine	6.4

VITAMINS

	Micrograms per gram
Biotin	0.02
Folic Acid	0.15
Niacin	1.05
Pantothenic Acid	0.42
Pyridoxine	0.76
Riboflavin	7.53
Thiamine	0.25

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SODIUM CASEINATE

TYPICAL ANALYSIS:

PROTEIN (N x 6.38) (dry basis)	92.5 - 94.5%
MOISTURE	5.0%
ASH	4.0%
FAT	1.5%
SODIUM	1.3%

AMINO ACID CONTENT:
(calculated)

	Per Cent
ALANINE	2.7
ARGININE	3.7
ASPARTIC ACID	6.4
CYSTINE	0.3
GLYCINE	2.4
GLUTAMIC ACID	20.2
HISTIDINE	2.8
ISOLEUCINE	5.5
LEUCINE	8.3
LYSINE	7.4
METHIONINE	2.5
PHENYLALANINE	4.5
PROLINE	10.2
SERINE	5.7
THREONINE	4.4
TRYPTOPHANE	1.1
TYROSINE	5.7
VALINE	6.5

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TYPICAL ANALYSIS

CALCIUM CASEINATE

Typical Analysis:	Protein (N x 6.38)	90.0%
	Moisture	2.2%
	Ash	3.9%
	Fat	1.5%
	Calcium	1.6%
	Sodium	200 ppm
	Particle Size	thru 140 mesh
	pH (2% solution)	6.8

Amino Acid Content (calculated)	Alanine	2.7 Lysine	7.4
	Arginine	3.7 Methionine	2.5
	Aspartic Acid	6.4 Phenylalanine	4.5
	Cystine	0.3 Proline	10.2
	Glycine	2.4 Serine	5.7
	Glutamic Acid	20.2 Threonine	4.4
	Histidine	2.8 Tryptophane	1.1
	Isoleucine	5.5 Tyrosine	5.7
	Leucine	8.3 Valine	6.5

THE RELATION OF PROTEIN TO HEMOGLOBIN BUILDING*

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The evaluation of dietary proteins for hemoglobin formation in nutritional anemia, when amounts of copper and iron adequate for optimal regeneration are fed, has received little consideration. Indeed, the extensive use of liver preparations in the treatment of secondary anemias makes a systematic study of this problem desirable.

The hematopoietic action of tryptophane has been reviewed by Kotake (1). Fontes and Thivolle (2) claim to have increased the hemoglobin content of the blood of healthy dogs and rabbits by the injection of tryptophane and histidine. Rats that were fed a tryptophane-deficient diet were found to be anemic after a period of 10 weeks. When dogs were made anemic by repeated bleedings, a response was obtained by injecting copper and iron (3). However, Fontes and Thivolle obtained the most rapid hemoglobin regeneration when tryptophane, histidine, copper, and iron were administered simultaneously. In contradistinction to these results, Tochowicz (4) concluded that tryptophane and histidine did not play an important rôle in hemoglobin formation in either hemorrhagic or pernicious anemia. His assumptions were based on a careful study of the variations of tryptophane and histidine in the blood of patients suffering from hemorrhagic or pernicious anemia, as compared with the values found for healthy individuals.

Feeding experiments on rats and mice (5) with tryptophane-deficient diets showed that after 25 to 30 days on such a regimen

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there was a decline in hemoglobin. With the addition of 0.2 per cent of tryptophane to such a diet the hemoglobin values returned to normal within a week or 10 days. Alcock (6) attempted to repeat the work of Fontes and Thivolle (2), making animals anemic by feeding a tryptophane-deficient diet. He used diets in which the protein was furnished by equal parts of zein and acid-hydrolyzed casein, or by the latter only. During a period of 5 months there was a loss in body weight of from 25 to 50 per cent, but there was no significant change in the hemoglobin values. In a second series, rats were made severely anemic by being fed an exclusive milk diet from the time of birth. The animals were then divided into two groups; the first group was fed a tryptophane-deficient diet without supplement, and the second group received the same diet plus 0.5 per cent of tryptophane. The rate of increase in hemoglobin was virtually the same in both groups, with the values approximating the normal at the end of about 3 weeks. The animals receiving tryptophane gained in weight, while the deficient group were emaciated and moribund after 3 weeks.

Extensive studies of the influence of diet and other factors on hemoglobin regeneration in hemorrhagic anemia have been made by Whipple and associates, the results being summarized in his Nobel prize lecture (7). Their work was carried out with dogs which were made anemic by repeated bleedings. The food factor to be assayed was then added to the basal diet fed these anemic animals. The increased hemoglobin formed on the supplemented diet above that on the basal diet was then calculated. They obtained a more rapid response with liver than with any other food substance. It should be pointed out, however, that the response in many cases was practically as good when iron alone was added to the basal diet as when liver was fed. The results of the work of Whipple and associates in the treatment of hemorrhagic anemia with liver have without doubt been an important factor in promoting the use of liver preparations for the treatment of various secondary anemias. Aside from hemorrhagic anemia, the extensive application of liver to the treatment of anemias should be looked upon with doubt, as the favorable response from its use may be due to its effect on the regeneration of constituents of the blood other than hemoglobin. The importance

of liver preparations is, of course, fully recognized in the treatment of pernicious anemia. Furthermore, in view of the excellent response obtained by adding iron alone, it seems highly probable that the hemorrhagic method of evaluating various foods in respect to hemoglobin formation is measuring, at least in part, the available iron. Certainly, if accurate information is to be obtained on the hematopoietic properties of foods, aside from the iron and copper they contribute, then adequate amounts of these elements should be fed to permit optimal hemoglobin formation.

The investigations reported in this paper were designed to ascertain whether the quality and level of proteins are factors in the rate of hemoglobin regeneration in nutritional anemia when the intake of iron and copper is adequate to permit optimal response.

EXPERIMENTAL

All of the animals used in these studies were young rats made severely anemic on an exclusive milk diet according to the technique described by Elvehjem and Kemmerer (8). At 5 to 7 weeks of age the hemoglobin values usually declined to between 3 and 4 gm. per 100 ml. of blood. In a few instances some of the animals had hemoglobin values slightly lower or higher. Blood was obtained from the tail and the hemoglobin was determined by the Newcomer method with a standardized disk. In order to follow closely the regeneration curve the hemoglobin was determined at 4 day intervals.

Diets—The diets employed are presented in Table I. During both the depletion period on the milk diet and while on the experimental diets the animals were fed *ad libitum*. Proteins from nine different sources were fed at a level intended to furnish approximately 17.5 per cent protein. Casein and liver were also fed at a level that furnished slightly less than 5 per cent of protein. The small amount of protein furnished by the vitamin addenda is not a complicating factor, as all groups of animals received the same supplement. Blood was obtained by exsanguination of healthy rats and evaporated on dextrin at a temperature of 50° in the proportion of 112 ml. of blood to 100 gm. of dextrin. Reprecipitated casein was used. Gliadin was prepared by extracting wheat gluten with 70 per cent ethyl alcohol. The

TABLE I

Percentage Composition of Diets

Diet No.	100-P	101-P	110-P	111-P	112-P	117-P	118-P	119-P	120-P	121-P	122-P	123-P
Dextrin	76.0	89.0	86.6	67.0	94.0	76.0	76.0	52.0	76.0	53.0	70.3	76.0
Blood												91.2
Casein	18.0	5.0										
Corn gluten meal										41.0		
Egg albumin							18.0					
Gelatin									18.0			
Gliadin						18.0						
Liver			7.4	27.0								
Soy bean oil meal								42.0				
Wheat gluten											23.7	
Salts*	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Cod liver oil	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Wheat germ oil	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Protein	17.35	4.82	4.76	17.43		17.07	15.87	17.87	17.64	18.22	18.86	15.74

* Phillips and Hart (9).

corn gluten meal and wheat gluten were obtained from a local commercial feed market. The egg albumin was prepared in our laboratories. The liver was obtained in a powdered form from Wilson and Company. The soy bean oil meal was a high grade product prepared by the expeller process.

In order to furnish ample amounts of the vitamin B factors a 25 per cent acidulated alcoholic extract of brewers' yeast was evaporated on the dextrin at a level equivalent to 2 per cent of yeast. 0.5 ml. of liver extract was fed individually each day. The liver extract was prepared from an anhydrous liver concentrate manufactured by Wilson and Company. The powdered liver was dissolved in 2 volumes of water. 5 volumes of ethyl ether and 5 volumes of ethyl alcohol were then added and allowed to stand for several hours with frequent shaking. The solvent was then decanted and the precipitate dissolved in water and made up to a volume so that 4 ml. were equivalent to 1 gm. of the original powder. This material was allowed to stand for several hours and then centrifuged, and then again made up to volume. That all of the vitamins were supplied in adequate amounts is evidenced from the excellent growth obtained on Diet 100-P containing 18 per cent of casein.

DISCUSSION

Effect of Added Iron and Copper—Originally it was assumed that the intake of iron and copper from the experimental diets would be adequate for optimal hemoglobin regeneration. The gains in hemoglobin values of anemic rats transferred to Diets 100-P and 101-P, containing 5 and 18 per cent of casein respectively, were virtually parallel. While the animals in the group fed 18 per cent casein practically doubled their weight in 3 weeks, those in the group fed 5 per cent casein just maintained their weight. Both groups required about 20 days for the hemoglobin values to return to normal.

When adequate amounts of iron and copper for optimum hemoglobin formation are added to milk fed to anemic rats, the hemoglobin values will return to normal within a week or 10 days. Since it required about double this time for rats transferred to Diets 100-P and 101-P to regenerate their hemoglobin, we suspected that the iron and copper intake was inadequate. With

the sudden change of regimens the animals may not have immediately accustomed themselves to a solid diet. Consequently, the intake of food during the first few days may have been subnormal, so that the iron and copper consumed were insufficient for optimum hemoglobin regeneration. In order to check this assumption 0.5 mg. of iron and 0.05 mg. of copper were fed individually each day. A solution of ferric pyrophosphate and copper sulfate, together with the liver extract, was fed in a Syracuse type watchglass. A few of the animals did not readily consume the supplements, in which case they were mixed with a small amount of the

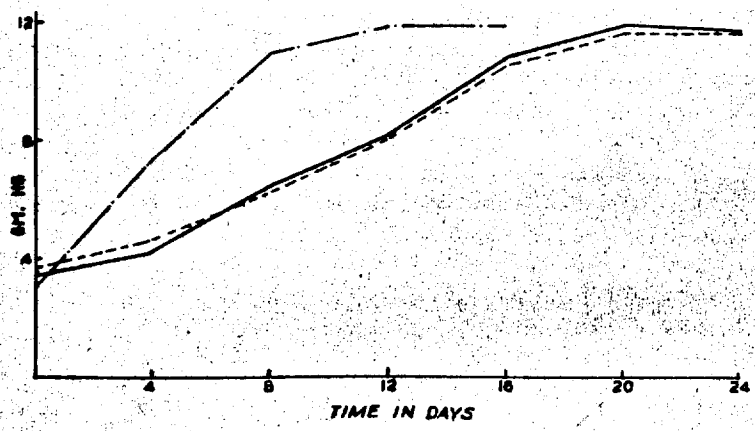

FIG. 1. The effect of Fe and Cu added to experimental diets. The continuous line represents four animals on 18 per cent casein; the broken line, four animals on 5 per cent casein; the dotted line, ten animals on 18 per cent casein plus 0.5 mg. of Fe and 0.05 mg. of Cu daily.

diet. This was eaten within a few hours and the feed was then replenished in the cups in the afternoon.

Feeding additional iron and copper above that contained in the diet greatly accelerated the rate of hemoglobin formation. Fig. 1 shows the effect on the rate of hemoglobin regeneration of feeding additional iron and copper. When 0.5 mg. of iron and 0.05 mg. of copper were fed individually each day to animals on Diet 100-P containing 18 per cent of casein, the hemoglobin values returned to normal within 8 to 12 days; whereas, without the supplements about 20 days were required in order to reach comparable values. Hence, it is evident that the feeding of additional

iron and copper above that contained in the diet is an important factor in the rate of hemoglobin formation. Subsequently, all of the animals, when transferred from the milk diet to the test diet, were fed additional iron and copper individually in the amounts previously mentioned. This procedure eliminated the iron and copper as a variable factor, as the intake was then adequate to permit optimal hemoglobin regeneration even in the most severe cases of anemia.

Quality and Level of Protein—Having eliminated all known factors that might affect the rate of hemoglobin formation, we then carried out studies to ascertain whether the quality and level of proteins influenced hemoglobin regeneration in rats that were made anemic on milk diets. There was considerable variation in the weight of the animals which ranged from 30 to 75 gm. when started on the test diets. However, an inspection of the results failed to reveal any relation between the initial weight of the animal and the rate of hemoglobin formation within a group. When smaller animals were placed on Diet 112-P containing no protein, except for the vitamin addenda, they frequently did not survive the 16 day period. Consequently, it was necessary to select the larger animals for feeding the diets that would not support body weight. Even in some of these cases, a few of the animals were moribund and extremely emaciated at the end of 16 days, especially those on the diet containing no protein.

The results of feeding twelve different diets are summarized in Table II. In each case the number of animals in the group, the average hemoglobin value, and weight at 4 day intervals up to 16 days are recorded. The animals fed the proteins that were adequate for growth generally made the most rapid gains in hemoglobin. Casein, liver, egg albumin, soy bean oil meal, and rat blood were of the same order in inducing hemoglobin formation. At the end of 8 days these groups showed hemoglobin values within the normal range.

Special mention should be made of Diet 123-P in which rat blood was used as a source of protein. Since Pommerenke *et al.* (10) had shown that dog plasma protein given by vein is utilized in body metabolism by the dog and that horse plasma is not similarly utilized, it was decided to use rat blood as a source of protein. It is doubtful that the utilization of blood when fed as

TABLE II

Effect of Various Proteins on Hemoglobin Formation and Body Weight

Protein	Diet No.	No. of animals	Hb, gm. per 100 ml. blood					Weight, gm.				
			Initial	4th day	8th day	12th day	16th day	Initial	4th day	8th day	12th day	16th day
Casein	100-P	10	3.19	7.38	11.15	11.96	12.10	48.8	65.1	74.2	84.4	96.4
Low casein	101-P	7	3.43	6.50	9.68	11.48	11.83	49.3	52.0	54.1	55.7	55.7
Liver	111-P	10	3.30	7.65	11.87	12.31	12.50	53.8	64.0	77.5	90.0	102.1
Low liver	110-P	7	3.31	5.86	8.68	10.12	11.05	55.4	56.0	56.7	58.0	56.1
Egg albumin	118-P	10	3.66	7.25	11.29	11.66	12.01	56.0	70.7	83.7	94.6	105.7
Soy bean oil meal	119-P	10	3.46	7.72	10.84	11.54	11.91	53.5	68.9	78.5	87.2	95.8
Blood	123-P	8	3.52	7.99	11.60	12.57	12.13	48.5	54.6	59.4	66.4	72.2
Corn gluten meal	121-P	10	3.69	6.69	10.05	10.81	11.25	50.2	53.4	55.3	58.8	60.6
Wheat gluten	122-P	10	3.60	6.40	9.37	11.23	11.71	47.7	51.0	52.9	53.2	54.0
Gliadin	117-P	7	3.24	6.51	8.15	10.27	10.38	51.6	50.6	49.7	49.3	47.7
Gelatin	120-P	7	3.48	5.95	7.99	9.50	10.40	52.6	51.3	46.1	45.0	42.3
No protein	112-P	7	3.41	6.05	8.05	9.13	9.56	59.7	54.4	49.1	46.7	43.8

a normal constituent of the diet differs whether obtained from the same or a foreign species. However, the use of rat blood obviates any question arising on this point in the interpretation of the results. Also, if the regeneration was much more rapid than with other sources of protein, it might be interpreted as a direct transference of dietary hemoglobin to the circulating blood.

Diet 123-P containing rat blood gave a faint salty taste, and because whole blood contains about 400 mg. of sodium chloride per 100 ml. of blood, it was decided to reduce by a corresponding amount the sodium chloride added to the diet. The performance of the animals on this diet was variable; some of the animals gained only a few gm., while others gained as much as 38 gm. in the 16 days. All of the animals showed hemoglobin values of 10.36 gm. per 100 ml. of blood or higher at the end of the 8th day. Thereafter, only slight increases were made in the hemoglobin values.

The animals fed the low casein and liver diets showed average hemoglobin values of 9.68 and 8.68 gm. per 100 ml. of blood respectively on the 8th day, but by the 12th day the values for both groups were above 10 gm. The groups fed corn gluten meal and wheat gluten had average values of 10.05 and 9.37 gm. respectively on the 8th day; by the 16th day the values for the corresponding groups were 11.25 and 11.71 gm. respectively. At the end of the 16 day period the group fed corn gluten had made an average gain of 10.4 gm. and the group fed wheat gluten, 6.3 gm. While the rate of hemoglobin regeneration is slower with the two groups that made only slight gains in weight, it is of interest to note that their hemoglobin values did return to within the normal range by the 12th day.

The groups of animals fed the diets containing gliadin, gelatin, and no protein, which failed to maintain body weight, showed a distinctly slower rate of hemoglobin formation. The average hemoglobin values for the two groups were in the vicinity of 8 gm. per 100 ml. of blood on the 8th day. By the 12th day only the group fed gliadin exceeded 10 gm. The relation of the biological value of the protein to the hemoglobin formation is indicated by the fact that the animals fed gliadin showed less loss in body weight over the 16 day period than did the other two groups, which likewise showed the lowest hemoglobin values up to the 12th day.

The performance of the animals on Diet 112-P, which contained no protein, is of particular interest. From the beginning these animals lost weight, but they gradually rebuilt their hemoglobin. The formation of hemoglobin was, of course, at the expense of body tissues. It is clear from this, as also from the performance of the animals fed gelatin or gliadin, that the requirements for hemoglobin are satisfied before those for growth. It is true that the average hemoglobin value of the group that received no protein was slightly less than 10 gm. per 100 ml. of blood at the end of the 16th day. Apparently the animal may maintain a slightly lower hemoglobin value if it is required to draw from its body tissues for building materials than if the necessary constituents are furnished in the diet.

The hemoglobin values on the 8th day, or the gain in hemoglobin values during the first 8 days, seem to be the most accurate and satisfactory criteria for comparing the effectiveness of the various diets for hemoglobin formation. This assumption is borne out by Fig. 1. Here the differences in the hemoglobin curves which are very striking on the 8th day become smaller on the 12th day and are nil by the 16th day. Even with the diets giving the slowest gains, the hemoglobin values may increase to as much as 10.40 gm. per 100 ml. of blood by the 16th day.

The group of animals fed Diet 111-P, in which the protein is furnished by liver, shows the highest average hemoglobin value on the 8th day. Diet 123-P containing rat blood comes second, and is followed in order by the egg albumin, casein, and soy bean oil meal. Corn gluten meal appears in an intermediate position. Diets 122-P and 101-P containing wheat gluten and 5 per cent casein respectively were slightly less effective in inducing hemoglobin formation. While the values obtained with liver were slightly higher than casein when fed at the higher level, on the lower level casein gave a slightly better response. If liver possessed greater hematopoietic properties than casein or other proteins, there is no reason why this action would not be evinced as readily on the lower level of feeding as on the adequate levels.

The hemoglobin values for the gliadin, gelatin, and no protein diets are distinctly lower on the 8th day than any of the other groups. This is as might be expected if there is any relation between the efficiency of a protein for promoting growth and

hemoglobin formation. These are the only three groups of animals that showed a decline in weight. With the no protein group there was only one animal with a hemoglobin value above 10 gm. per 100 ml. of blood on the 8th day; the lowest value was 6.20 gm.

The average gain in hemoglobin values and standard error of the mean, together with the change in body weight, are recorded in Table III. As body weight is a secondary consideration, only the average gain or loss over the 8 day period is recorded. On this basis of evaluating proteins for hemoglobin regeneration liver

TABLE III

Average Gain in Hemoglobin Values and Change in Weight over 8 Day Period

Protein	Diet No.	Hb per 100 ml. blood		Weight
		Mean ± σ*	Range	
		gm.	gm.	gm.
Casein	100-P	7.96 ± 0.24	6.83– 9.54	25.4
Low casein	101-P	6.25 ± 0.46	4.51– 7.82	4.8
Liver	111-P	8.57 ± 0.41	6.02–10.01	23.7
Low liver	110-P	5.36 ± 0.24	4.52– 6.15	1.3
Egg albumin	118-P	7.64 ± 0.38	5.93–10.19	27.7
Soy bean oil meal	119-P	7.38 ± 0.25	6.41– 9.15	25.0
Blood	123-P	8.08 ± 0.44	6.73–10.12	10.9
Corn gluten meal	121-P	6.36 ± 0.23	5.23– 7.53	5.1
Wheat gluten	122-P	5.77 ± 0.17	5.01– 6.61	5.2
Gliadin	117-P	4.91 ± 0.22	4.05– 5.75	−1.9
Gelatin	120-P	4.52 ± 0.49	3.23– 6.27	−6.5
No protein	112-P	4.64 ± 0.38	3.64– 6.26	−10.6

* σ represents the standard error of the mean.

shows the highest value, but is of the same order as rat blood, egg albumin, casein, and soy bean oil meal. Aside from rat blood these proteins have all given good growth. The no protein, gelatin, and gliadin diets were the least effective in inducing hemoglobin regeneration; they were also inadequate to support body weight. The corn gluten meal, wheat gluten, low casein, and low liver diets fall between the two previously mentioned groups. From the standpoint of hemoglobin formation they are distinctly below the proteins that permit good growth, and above those that do not maintain the animal. While the liver fed at

a level to furnish adequate protein again shows the highest value, it is to be emphasized that when fed on the lower level the average gain in hemoglobin is less than with the low casein diet. In view of these results one cannot ascribe to liver hematopoietic properties that are not possessed by casein or other proteins that permit rapid growth, so far as nutritional anemia is concerned.

A comparison of the average gain in hemoglobin values and change in body weights, as recorded in Table III, shows that there is a fair agreement between these factors. With the exception of Diet 123-P, rat blood, those groups that made the greatest gain in body weight likewise made the greatest gain in hemoglobin. This is not strictly true, but it follows that the liver, egg albumin, casein, and soy bean oil meal diets induced gains of about the same order and likewise fell into a superior group for hemoglobin regeneration. The groups fed gliadin, gelatin, and no protein which failed to maintain their weight also made the least rapid gains in hemoglobin. The diets which just maintained the weight of the animals or permitted slight increments were distinctly inferior to the adequate diets in respect to their hematopoietic properties. Inspection of the performance of the individual animals within the groups failed to reveal any such striking relationship between the rates of hemoglobin formation and the change in body weight which occurred between groups.

For further analysis of the data, the gain in hemoglobin value over the first 8 days of the group on 18 per cent of casein is taken as a standard. The significance of the difference between the average gain in hemoglobin of all groups as compared with the casein group has been determined statistically according to Fisher's t method (11) for small groups. The results of this statistical study substantiate the previous interpretations. The rate of hemoglobin formation of the group that was fed liver, Diet 111-P, is not significantly higher than in the group fed 18 per cent casein; likewise, the rate of gain in hemoglobin value of the rats fed soy bean oil meal is not significantly lower than that of the group fed casein. The rates of gain of hemoglobin of the groups fed gelatin, gliadin, no protein, wheat gluten, corn gluten meal, low liver, and casein are significantly lower than that for the group fed 18 per cent casein. From the data presented in Table III, giving the average gain in hemoglobin values and the

standard errors, together with the number of observations shown for the corresponding group in Table II, the significance of the difference between the means of any two groups may be calculated.

From the results reported in this paper it is evident that proteins that will permit good growth will allow optimum hemoglobin formation. In searching for an explanation of the apparent discrepancy between the results obtained by Alcock (6) and the observations recorded in this paper, we would draw attention to Fig. 1 showing the effect of feeding iron and copper in addition to that contained in the diet. Here it is seen that the additional iron and copper accelerate hemoglobin regeneration. As previously mentioned, Alcock found that the hemoglobin regeneration was virtually the same with animals fed a tryptophane-deficient diet inadequate for maintenance as for those on a diet to which tryptophane was added and which permitted growth. Alcock apparently depended on the iron and copper intake through the diet to furnish adequate amounts for optimum hemoglobin formation.

While we have not made an exhaustive study of the effect of a deficiency of all the essential amino acids, the results reported in this paper seem to warrant the general hypothesis that, when the dietary protein is either qualitatively or quantitatively inadequate for growth, the rate of hemoglobin regeneration is significantly retarded. Provided adequate amounts of iron and copper are furnished, animals suffering from nutritional anemia will draw upon body proteins for materials to build hemoglobin.

Normal Animals Fed Low Protein Diets—Having established that the rate of hemoglobin regeneration is affected by the level of dietary protein, we considered it of interest to study the effect of feeding low protein diets to normal rats over a long period of time. Bethell (12) reported that the dietary protein is a factor in maintaining normal hemoglobin values during pregnancy in both women and rats. Investigations on the relation of low protein diets to the estrous cycle (13) afforded an opportunity of obtaining at the same time information on hemoglobin.

Non-pregnant female rats were maintained on a diet containing 5 per cent of casein as the sole source of protein, except for the small amount that may have been present in the vitamin addenda. Hemoglobin determinations were made at regular inter-

vals throughout the 70 days the rats were on this regimen. There was no significant change in the hemoglobin values after the animals were transferred from a stock diet to a low protein diet, normal hemoglobin values being maintained throughout this 70 day period. Gliadin was then added to the diet of one group of animals and gelatin added to the diet of another group. The animals receiving gliadin increased in weight and resumed normal estrous cycles, while the group fed gelatin failed to respond in either respect. This alteration in the diets was without significant effect on the hemoglobin in both groups of animals. From these observations it is evident that non-pregnant rats fed low protein diets for long periods of time will maintain normal hemoglobin, provided adequate amounts of iron and copper are fed.

SUMMARY

Studies were made of the effect of nine different proteins on the rate of hemoglobin regeneration in nutritional anemia.

The proteins of liver, casein, egg albumin, and soy bean oil meal are consistently effective in building hemoglobin. When the intake of iron and copper is adequate to permit hemoglobin formation at the maximum rate, no special hematopoietic property in addition to their protein content can be ascribed to any of these materials. There is no evidence that liver is more effective for hemoglobin formation than are proteins of good quality. Rat blood permitted excellent hemoglobin regeneration, but the growth response was somewhat inconsistent.

Corn gluten meal and wheat gluten are poorly utilized by the animal for both the formation of hemoglobin and growth. Liver and casein fed at a level to furnish 4.8 per cent of protein permit hemoglobin regeneration at approximately the same rate as corn gluten meal or wheat gluten fed at a level to furnish about 18 per cent of protein. Animals fed gliadin or gelatin, which are inadequate for growth, rebuild their hemoglobin less rapidly than when fed a protein that permits growth.

It is clear that the formation and maintenance of normal hemoglobin values is more vital than growth and that the animal will utilize its body proteins for this purpose, provided adequate amounts of iron and copper are fed. When the dietary protein

is either qualitatively or quantitatively inadequate for growth, the rate of hemoglobin regeneration is significantly retarded.

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OBSERVATIONS ON PROTEIN DIGESTION IN VIVO

II. DIETARY FACTORS AFFECTING THE RATE OF DISAPPEARANCE OF CASEIN FROM THE GASTROINTESTINAL TRACT[1]

CARL PERAINO, QUINTON R. ROGERS, MINORU YOSHIDA, MOU-LIANG CHEN, AND ALFRED E. HARPER

Abstract

The effects of various dietary changes on the emptying of solids and nitrogen from the stomach and on the disappearance of nitrogen from the gastrointestinal tract of rats trained to consume a single meal daily are described. When the animals were fed 5 g of food the presence of casein in the diet caused a general deceleration of stomach-emptying and altered the shape of the stomach-emptying curve. The presence of 50% of casein in the diet did not result in an accumulation of nitrogen in the intestine much above the amount found when a protein-free diet was fed. When dextrin was the dietary carbohydrate the diet emptied from the stomach as a homogeneous mixture, whereas when sucrose was the dietary carbohydrate the casein emptied from the stomach more slowly than did the other components of the diet. Raising the dietary level of fat to 50% caused a general deceleration of emptying and abolished the above-mentioned carbohydrate effect. No delay in stomach-emptying due to the presence of casein in the diet was noted when only 1.5 g of diet was fed. As the level of food intake was raised the total quantity of nitrogen emptying from the stomach per unit time increased although a greater percentage of the amount ingested emptied from the stomach when the level of food intake was low.

Introduction

Although the physiology of digestion and absorption has been extensively studied in higher vertebrates, particularly prior to the first World War, 1914–1918, the existing knowledge concerning in vivo digestion and absorption is still quite inadequate, a fact which was brought out recently by Fisher in his monograph on protein metabolism (1). Many of the experimental procedures used in studies of digestion have subjected the test animal to unnatural physiological conditions (2); consequently the results obtained may not be representative of normal gastrointestinal activity. Furthermore, in the earlier studies, the test diets were frequently incomplete and the components that were present were usually in a crude state (3), thus raising the question as to whether the results obtained were unduly influenced by the lack of certain dietary components or by the presence of contaminants in the ingested food.

In more recent investigations of these processes intact animals and purified dietary components have been used. Geiger (4), and Geiger, Courtney, and Geiger (5), studied the digestion of purified dietary proteins by intact rats and concluded that the rate of disappearance of casein from the gastrointestinal tract was greater than that of zein, but less than the rates for meat and fish proteins.

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Contribution from the Department of Biochemistry, University of Wisconsin, Madison 6, Wisconsin. Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the National Live Stock and Meat Board, Chicago, Illinois, and Campbell Soup Company, Camden, New Jersey. Some of the crystalline vitamins were kindly provided by Merck Sharp and Dohme, Rahway, New Jersey.

In a study of the effects of certain carbohydrates on the gastrointestinal absorption of protein or carbohydrate in the rat, Dreisbach and Nasset (6) found that, 2 to 5 hours after the test meal had been fed, the quantity of protein recovered from the intestinal lumen was greater than the amount ingested. On the basis of these and other results (7) Nasset postulated (8) the existence of a homeostatic mechanism in which the gastrointestinal tract turns over a great deal of endogenous protein and that this "probably serves to even out temporary irregularities in the dietary supply of amino acids and to prevent gross changes in the amino acid pattern of the portal blood."

In contrast to these observations, Geiger, Humin, and Middleton (9) reported a low recovery of nitrogen from the gastrointestinal tract after feeding a test meal containing skim milk – protein to rats.

Gupta, Dakroury, and Harper (10) measured the rate of disappearance of a few proteins and an amino acid mixture from the gastrointestinal tract of the rat. Their procedure differed from that of Geiger *et al.* (4) in that a nutritionally complete diet was employed, 15% of which was the test protein (or amino acid mixture). The results of this study indicated that casein, beef protein, and an amino acid mixture disappeared from the gastrointestinal tract at similar rates. In the case of zein, the rate of disappearance was found to be significantly less than that of the previously mentioned nitrogen sources. Their observations also indicated that the amount of nitrogen recovered from the gastrointestinal tract 4 to 5 hours after a test meal was fed to the rats was much less than the amount ingested.

The findings of the workers cited above, as well as those of other workers, revealed the need for a more systematic approach to the study of the over-all processes of digestion and absorption. The present investigation was therefore undertaken in order to provide necessary basic information which would serve as a foundation for further studies of this subject. The specific objective of the work reported in this paper was to determine the effect of various dietary factors on the rate of disappearance of a single dietary protein (casein) from the gastrointestinal tract. The factors studied were: the type of carbohydrate, the dietary levels of protein and fat, and the level of food intake.

Methods

Male albino rats weighing approximately 200 g were trained (in individual suspended cages with screen bottoms) to consume their total daily ration within a single 2-hour period. They were fed at the same time each day so as to provide maximum uniformity in the training pattern. Initially the rats lost weight, but at the end of 2 weeks on this regimen they had regained their starting weights, and were considered to be suitable for experiment (10).

The training diet consisted of: casein 15%, salts (11) 4%, corn oil 5%, water-soluble vitamin mixture (12) 0.25%, choline chloride 0.15%, and dextrin 75.6%. Fat-soluble vitamins A, D, and E were included in the corn oil to provide the following concentrations per 100 g of diet: A, 400 I.U.; D, 40 I.U.; and E, 10 mg.

The experimental diets described in Table I were lacking in water-soluble and fat-soluble vitamins since preliminary investigations had shown that the results of these short experiments were uninfluenced by the exclusion of these nutrients from the diets.

TABLE I

Composition of experimental diets

Diet	% casein	mg N/g	Type of carbohydrate	% fat
A	0	<1	Dextrin*	5 corn oil
B	0	<1	Sucrose	5 corn oil
C	15	21	Dextrin	5 corn oil
D	15	21	Sucrose	5 corn oil
E	30	42	Dextrin	5 corn oil
F	30	42	Sucrose	5 corn oil
G	50	70	Dextrin	5 corn oil
H	50	70	Sucrose	5 corn oil
I	15	20	Dextrin	25 lard
J	15	20	Sucrose	25 lard
K	15	20	Dextrin	50 lard
L	15	20	Sucrose	50 lard
M	0	<1	Dextrin	50 lard

*Autoclaved cornstarch.

Preliminary trials had also revealed that a well-trained rat would consume 5 g of food within 20 minutes. Therefore, on the day of the experiment, zero time was fixed at 20 minutes after the animals had been offered the test diet, and 5 g was chosen as the standard weight of diet to be fed in all experiments except those in which the effect of the level of food intake was studied. During the 20-minute feeding period spilled food, collected on paper towels placed under the cages, was returned to the food cups before the time limit had been reached.

Rats were then killed by chloroform anesthesia at zero time and at various intervals (five rats at each interval) thereafter, as designated in the results. The stomach and small intestine were excised and the contents of each were washed into separate evaporating dishes, each of which was lined with a weighed sheet of nitrogen-free plastic wrapping material.[2] After the samples had been dried and weighed, the nitrogen content of each entire sample was determined by the Kjeldahl method, using mercuric oxide as the catalyst.

The method of preparing the samples for analysis, as described above, differs from the procedure described by Gupta *et al.* (10), who did not analyze the entire sample (except in their study of zein) but diluted the total contents to known volume, homogenized, and analyzed aliquot portions of the fine suspension. Quite good replication was obtained using this procedure, but it was subsequently found that, owing to the relative insolubility of the casein which tended to precipitate out in the homogenate, the values for total nitrogen of the stomach contents were somewhat low. During the testing of diets C and D, pH measurements were made on the contents of the stomach and small intestine. This was accomplished by making incisions at the cardiac and

[2]"Saran-Wrap", Dow Chemical Company.

pyloric ends of the stomach and inserting the electrode of a single-electrode pH meter into these openings. The natural openings of the stomach were held closed by hemostats during this process. Rough pH measurements of duodenal, jejunal, and ileal contents were also taken by slitting the small intestine lengthwise and touching the electrode to its inner surface at various points.

In the early stages of the present investigation attempts were made to prevent the experimental animals from eating their feces. Tail cups, the use of which is described by Barnes *et al.* (13), were tried, but it was found that under such conditions gastric emptying was greatly delayed in a large number of rats and was completely halted for as long as 24 hours in a few. Immobilizing cages, as well as more fanciful devices, produced similar effects. Attempts to adapt adult animals to these devices were unsuccessful.

Later experiments indicated that the maximum quantity of fecal nitrogen eaten by any rat was too small to introduce a significant error into the determination, and as a result attempts to prevent coprophagy were discontinued.

Results

I. Effect of Protein

The effect of the level of dietary protein on the rate of stomach-emptying is most clearly demonstrated by the results obtained with diets containing dextrin as the carbohydrate (Fig. 1). With sucrose as the carbohydrate the results are complicated by the differential rates of emptying of carbohydrate and protein as will be shown later.

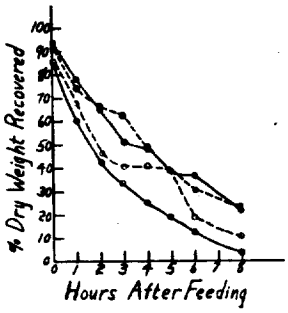


FIG. 1. The effect of dietary protein level on the rate of emptying of total solids from the stomach. The diets contain dextrin as the carbohydrate. Diet A ○——○ (protein-free), diet C ○ - - ○ (15% of casein), diet E ●——● (30% of casein), diet G ● - - ● (50% of casein).

The protein-free diet (A) emptied most rapidly from the stomach; the emptying curve is quite smooth and exponential in shape and the stomach appears to have been essentially empty 8 hours after the test diet was fed. In this experiment, and in all others in which a protein-free test meal was fed, there was no significant accumulation of nitrogen in the stomach at any of the time intervals tested. (The quantity of nitrogen recovered was always between 5 and 10 mg.)

In no case when a diet containing casein was fed was a smooth exponential curve for stomach-emptying obtained. Diet C (containing 15% of

casein) emptied from the stomach somewhat more slowly than diet A, the difference being more pronounced from the 3rd through the 5th hours after feeding. During this period stomach-emptying ceased entirely until after the 5th hour. Diet E (containing 30% of casein) passed from the stomach at a still slower rate than did diet C. Diet G (containing 50% of casein) emptied at essentially the same rate as diet E.

The effects of diets containing different levels of casein on the quantity of nitrogen in the intestinal contents at successive intervals after feeding are shown in Table II. Only the results obtained with diets containing dextrin are

TABLE II

Effect of variation in dietary protein level and fat level on the quantity of nitrogen in the small intestinal contents

Hours after feeding	mg N in intestinal contents				
	Diet A,* 1 mg N‡	Diet C,* 100 mg N‡	Diet E,* 200 mg N‡	Diet G,* 350 mg N‡	Diet K,† 100 mg N‡
0	8	9	12	17	10
1	7	12	12	14	9
2	8	13	12	10	9
3	11	15	11	11	9
4	10	9	11	9	11
5	11	8	7	11	10
6	9	7	8	10	14
8	9	8	8	9	13

*Contains 5% of fat.
†Contains 50% of fat.
‡Quantity of nitrogen in 5 g of diet.

shown because results obtained with sucrose as the carbohydrate were similar. The quantity of nitrogen in the intestinal contents remained consistently low both throughout the experimental period and irrespective of the percentage of casein in the diet. However, there was some tendency for more nitrogen to accumulate during the first 2 hours when the diet contained 50% of casein, although the difference was not statistically significant ($P > 0.05$). It was also observed that the total quantity of solids in the intestine remained relatively constant during these experiments, and did not rise significantly above values obtained with fasted animals ($P > 0.05$ at all intervals).

In these experiments the pH in the duodenum was approximately 6.8 while that in the jejunum and ileum lay between 7.0 and 8.0. These values remained constant in all experiments and at all time intervals tested.

Figure 2 provides a comparison of the total milligrams of nitrogen disappearing from the entire upper gastrointestinal tract (stomach and small intestine inclusive) at successive intervals after feeding diets containing different levels of casein. The quantity of nitrogen disappearing from the upper gastrointestinal tract within a given time interval was substantially greater when diet G (containing 50% of casein) was fed than when diet C (containing 15% casein) was fed. Although the curve for diet G appears to level off between the 7th and 8th hours after feeding, it actually continues its former steep ascent after the 8th hour. Only enough of the curve is shown to provide a comparison with

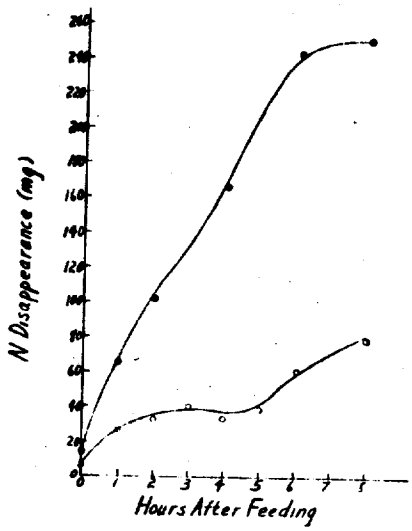


FIG. 2. The effect of dietary protein level on the rate of disappearance of nitrogen from the gastrointestinal tract between the cardiac sphincter and ileocecal junction. The diets contain dextrin as the carbohydrate. Diet C ○- - - ○ (15% of casein), diet G ●——● (50% of casein).

the curve for diet C. The curve for diet E (containing 30% of casein) would fall midway between these two curves, so despite the fact that diets E and G emptied from the stomach at about the same rate on the basis of the percentage of ingested nitrogen emptying per unit of time (Fig. 1), the total amount of protein that emptied was much greater in the case of diet G.

II. Effect of Type of Carbohydrate

In Figs. 3–6 diets A, C, E, and G are compared with diets B, D, F, and H with regard to their rates of emptying from the stomach. The only difference between the two series of diets is that the latter contained sucrose instead of dextrin as the carbohydrate.

Figure 3 shows that protein-free diets A (dextrin as carbohydrate) and B (sucrose as carbohydrate) emptied from the stomach at closely similar rates:

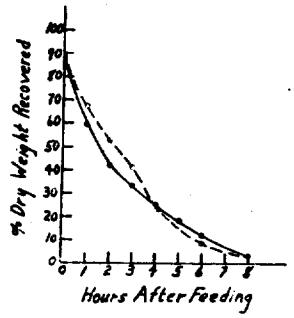


FIG. 3. The effect of type of carbohydrate on the rate of gastric emptying. Diet A ●—— ● (dextrin) (protein-free), diet B ○ - - ○ (sucrose) (protein-free).

at no time interval were the differences between them statistically significant ($P < 0.05$).

The curves in Figs. 4A and 4B show the rates of passage from the stomach of dietary nitrogen and total solids respectively. A comparison of the curves for diet C indicates that nitrogen and total solids emptied at the same rate when dextrin was the carbohydrate. When dextrin was replaced by sucrose, however (diet D Figs. 4A and 4B), the total solids emptied from the stomach

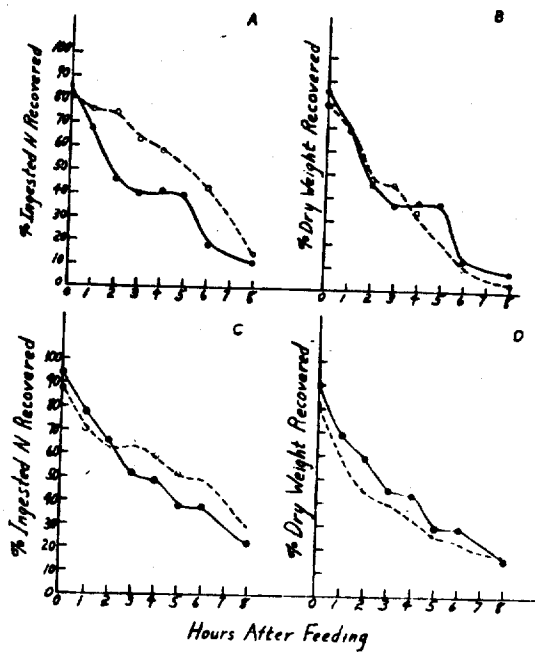


FIG. 4. The effect of type of carbohydrate on the rate of gastric emptying.
(A) and (B). Diet C ●——● (dextrin) (15% of casein), diet D ○ – – ○ (sucrose) (15% of casein).
(C) and (D). Diet E ●——● (dextrin) (30% of casein), diet F ○ – – ○ (sucrose) (30% of casein).

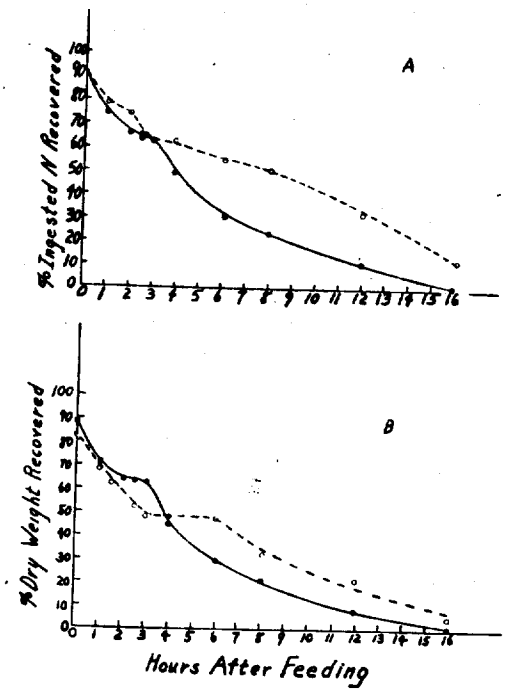
much more rapidly than did the nitrogen. The difference between these two curves is significant at all points from the 1st to the 8th hours after feeding ($P < 0.05$). In Fig. 4B the two curves are closely similar for the first 2 and last 2 hours of the experiment; but the delay in stomach-emptying during the intervening hours, which appears characteristic of diet C (containing dextrin), was relatively slight for diet D (containing sucrose).

Raising the dietary level of casein to 30% (diets E and F) produced the results shown in Figs. 4C and 4D. When diet E was fed, the dietary nitrogen and total solids passed from the stomach at similar rates (the percentage of nitrogen in the stomach contents, therefore, remaining constant) as had been previously noted with diet C in Figs. 4A and 4B. The rates of emptying of diets E and F (Fig. 4C) during the first 2 hours after feeding were not significantly different ($P > 0.05$). From the 3rd through 8th hours the nitrogen of

diet F (sucrose as carbohydrate) emptied from the stomach at a slower rate than did that of diet E (dextrin as carbohydrate). Although this difference is of doubtful statistical significance the consistency of its occurrence in repeated experiments lends it biological importance.

From Fig. 4D it appears that the total solids of diets E and F passed from the stomach at essentially the same rate, these results being similar to those shown in Fig. 4B for diets C and D. Figures 4C and 4D show that, as was previously observed with diet D (Figs. 4A and 4B), when diet F was fed the rate of disappearance of dietary nitrogen from the stomach was significantly slower than that of the total solids at all intervals between 0 and 8 hours after feeding ($P < 0.05$).

The effects of increasing the dietary level of casein to 50% (diets E and F) are shown in Figs. 5A and 5B. Comparison of the curves for diet E in Figs. 5A and 5B shows again that when dextrin was the carbohydrate the protein and



Figs. 5A and 5B. The effect of type of carbohydrate on the rate of gastric emptying. Diet G ●——● (dextrin) (50% of casein), diet H ○ – – ○ (sucrose) (50% of casein).

other components of the diet passed from the stomach at similar rates. Replacement of the dextrin in diet G with sucrose (diet H) again resulted in a more rapid exit from the stomach of the non-nitrogenous components of the diet.

There was no significant difference between the rates at which the nitrogen of diets G and H emptied from the stomach during the first 3 hours after feeding (Fig. 5A) ($P > 0.05$). However, between the 3rd and 16th hours

after feeding, the nitrogen of diet H emptied at a significantly slower rate than did that of diet G.

From Fig. 5B it can be seen that approximately 15% more of the total solids ingested had emptied from the stomach by the 3rd hour when sucrose was the carbohydrate than was the case when dextrin was the carbohydrate (diet G). This situation was reversed at the 6th hour, and thereafter the total solids of the diet containing dextrin emptied consistently more rapidly than did those of the diet containing sucrose, although the difference was of doubtful statistical significance.

The magnitude of the effect of carbohydrate on the emptying of protein is most clearly illustrated in Fig. 6. The curves in this figure represent the

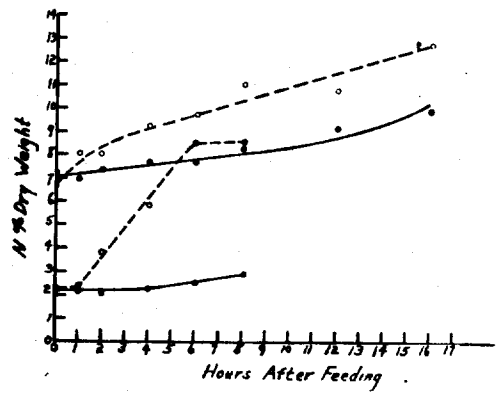


FIG. 6. The effect of type of carbohydrate on the nitrogen concentration of the gastric contents. The curves starting at 7% on the ordinate are for diets G and H, while the curves starting at 2% on the ordinate are for diets C and D.

$$\text{N\% dry weight} = \frac{\text{mg N}}{\text{mg dry wt.}} \times 100.$$

Sucrose as carbohydrate O- -O, dextrin as carbohydrate ● - - ●.

changes in the concentration of nitrogen in the stomach contents at intervals after feeding. Reference to the curves for diets which contained 15% of casein shows that when dextrin was the carbohydrate (diet C) the concentration of nitrogen in the stomach contents (N% dry weight) remained relatively constant throughout the experimental period. When sucrose was the carbohydrate (diet D), however, the nitrogen concentration had increased, by the 6th hour after feeding, until it was fourfold greater than that observed for the diet containing dextrin. This increase first became statistically significant at the 2-hour interval ($P < 0.05$).

The determination of pH as described previously yielded interesting results. When diet C, containing dextrin, was fed the pH of the contents of the antrum was 2.0 to 3.0 while that of the contents of the fundus was 5.0 to 6.0, and these values remained constant throughout the experiment. Of pertinence to these findings is the observation that the stomach contents were in the form of a discrete firm mass, the size of which decreased as digestion and gastric emptying progressed.

Replacement of dextrin by sucrose caused significant changes both in the pH and in the consistency of the stomach contents. The stomachs of rats that had eaten diet D appeared greatly distended with water and contained much less undissolved solid than was observed in the case of those that had eaten diet C. During the first 3 hours after feeding, a pH value of approximately 5 was obtained in all parts of the stomach of rats receiving diet D. A decrease to pH 4 was noted during the 4th hour, and by the end of the experiment, when only a small quantity of diet remained in the stomach, the pH had dropped to approximately 3.

III. Effect of Fat Level and Type of Carbohydrate

In a study of the combined effects of variations in the dietary level of fat and the type of carbohydrate on the passage of protein and total solids from the stomach, diets I and J (25% lard) and K and L (50% lard) were employed. Lard was substituted for corn oil in these experimental diets in order to avoid the use of a fat that was liquid and which, therefore, would tend to separate from the other components of the diet. In order to obtain a basis for comparison between diets containing lard and those containing corn oil an experiment was performed using a diet containing 5% of lard with dextrin as the carbohydrate. The resulting curves for the emptying of dietary nitrogen and total solids from the stomach were found to be superimposable on the curves for diet C in Figs. 2A and 2B, and for this reason are not presented. From these findings it was concluded that 5% of corn oil and 5% of lard had similar effects on stomach-emptying.

The curves for diet I (dextrin as carbohydrate) in Figs. 7A and 7B show that this diet emptied from the stomach at a substantially slower rate than did diet C which contained only 5% of fat (Figs. 3A and 3B). As was noted with diet C the total solids and dietary nitrogen passed from the stomach at closely similar rates. With sucrose as the carbohydrate (diet J) the emptying pattern was similar to that of diet D (Figs. 3A and 3B), i.e., the total solids in the stomach emptied at a more rapid rate than did the dietary nitrogen.

Figure 7A shows that the nitrogen of diets I and J emptied at closely similar rates for the first 3 hours after feeding, there being no significant difference between the curves during this period ($P > 0.05$). From the 3rd through 5th hours after feeding, the nitrogen of diet I emptied more rapidly than did that of diet J, the difference being statistically significant at the 3- and 4-hour intervals ($P < 0.05$). However, the total solids of diet J emptied at a consistently more rapid rate than was the case for diet I although the patterns of emptying were quite similar (Fig. 7B).

The effect on stomach-emptying of raising the dietary fat level to 50% (diets K, L, and M) is shown in Figs. 7C and 7D and Fig. 8. From Figs. 7C and 7D it can be seen that there was no significant difference between the rates of emptying of diets K (dextrin as carbodydrate) and L (sucrose as carbohydrate). The delay in emptying observed between the 2nd and 4th hours after feeding these diets is similar to that obtained with diet C (5% fat with dextrin, Figs. 3A and 3B), but there was a general decrease in the rate of emptying of the high-fat diets (Figs. 7C and 7D) compared with that of diet C.

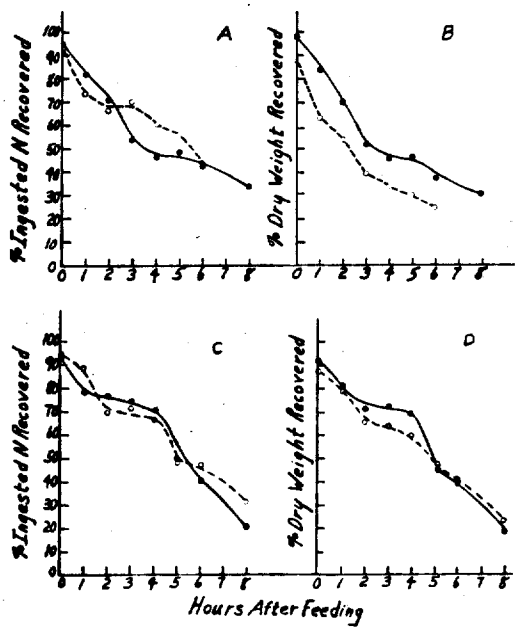


FIG. 7. The effect of dietary level of fat on the rate of gastric emptying.
(A) and (B). Diet I ●——● (dextrin) (15% casein, 25% fat), diet J ○ – – ○ (sucrose) (15% casein, 25% fat).
(C) and (D). Diet K ●——● (dextrin) (15% casein, 50% fat), diet L ○ – – ○ (sucrose) (15% casein, 50% fat).

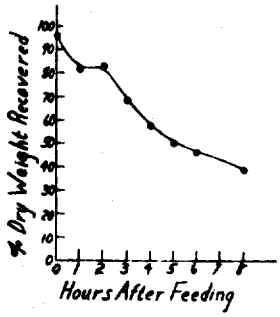


FIG. 8. The effect of dietary level of fat on the rate of gastric emptying. Diet M ●——● (dextrin) (protein-free, 50% fat).

Comparison of Fig. 8 with the curve for diet A in Fig. 3 reveals that protein-free diet M, which contained 50% of fat, emptied from the stomach at a significantly slower rate than did protein-free diet A, which contained 5% of fat. It is also notable that the emptying patterns of diets A and M failed to show a prolonged period of complete cessation of stomach-emptying, a phenomenon which was observed with high- and low-fat diets containing 15% of casein (diets K and C). However, a short period of cessation did occur between 1 and 2 hours after feeding the high-fat protein-free diet.

Comparison of the intestinal results for diets K and C (Table II) shows that although diet K contained 45% more of fat than did diet C the quantity of nitrogen in the small intestinal contents remained at the same low level for both diets throughout the experimental period. This low level corresponds to that observed for diet C, which contained no protein and 5% of fat.

IV. Effect of Level of Food Intake

The rates of emptying from the stomach of various quantities of diet C are compared in Fig. 9. When 1.5 g of diet C were fed, gastric emptying proceeded

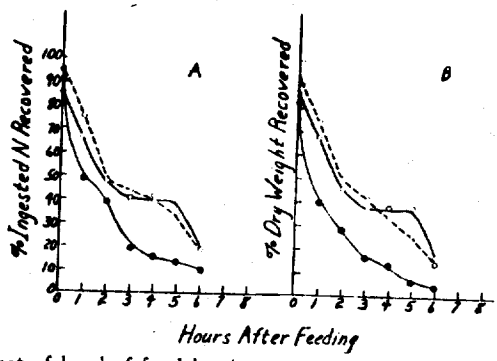


FIG. 9. The effect of level of food intake on the rate of gastric emptying of diet C. 1.5 g fed ●——●, 3.0 g fed ○ – – ○, 5.0 g fed ○——○

more rapidly than when 3.0 or 5.0 g were fed. The over-all rates of emptying at the latter two levels of intake were closely similar, although the delay in emptying, occurring between the 3rd and 5th hours after feeding 5.0 g of diet was less pronounced when 3.0 g were fed. This period of delay was totally absent when the level of intake was 1.5 g. Expressing these results as milligrams of nitrogen emptied rather than as percentage of ingested nitrogen discloses that the quantity of nitrogen passing from the stomach per unit of time increased with increasing level of food intake.

Discussion

I. Effect of Protein

From the curves presented in Fig. 1 it is evident that the inclusion of protein in the diet had a pronounced effect on the rate of passage of food from the stomach. The presence of protein caused the emptying process to be intermittent, with periods of deceleration (and in some cases of complete cessation) of emptying occurring at various intervals during the period after feeding. Maziyevskii and London (14) noted that periodic cessation of stomach-emptying occurred in dogs which had been given a test meal of starch. In the present investigation a similar phenomenon was found to be brought about by dietary protein. In addition, the added protein caused an over-all deceleration in the rate of emptying of the test diet from the stomach. This effect was increased somewhat when the level of protein in the diet was raised to 30%.

Among the many factors which must be considered in interpreting these effects are: (*a*) the alteration in the physical consistency of the diet due to

the increased casein content; (*b*) the increasing quantities of amino acids absorbed per unit of time with higher protein intakes; (*c*) the fact that higher levels of dietary protein in the stomach contents may have more completely buffered the hydrochloric acid secreted by the parietal cells, thus keeping the pH well above that required for the maximum digestive activity of pepsin. These three factors may in combination exert an inhibitory effect on stomach-emptying which varies with the level of protein in the diet. The lack of significant differences between the rates of emptying of diets containing 30% and 50% of casein suggests that the maximal inhibitory effect of protein on stomach-emptying is reached when the diet contains about 30% of casein.

The decelerating effect of added dietary protein on gastric emptying was also noted by Annegers and Ivy (15), who observed that a level of 55% of protein in the diet caused an over-all delay in stomach-emptying. These results, as well as those of the present investigation differ from those of Rosenthal and Nasset (7), who found that the presence or absence of protein in the diet did not significantly affect stomach-emptying. Their failure to observe a protein effect may be attributable to the fact that they fed only 1.5 g of diet as compared with the 5.0 g used in the present study. As will be mentioned later, the effect of the level of food intake on stomach-emptying is a variable which must be considered in studies of dietary effects on gastrointestinal processes.

The results shown in Table II reveal that when casein was the dietary protein, nitrogen did not accumulate in the intestinal lumen under any of the conditions of the present investigation but was absorbed extremely rapidly. The significance of this finding becomes apparent from examination of Fig. 2 which shows that the gastrointestinal tract was able to digest and absorb far larger quantities of dietary nitrogen (per unit of time after feeding) when the diet contained 50% of casein than when it contained 15% of casein. Thus the absorptive process was not the limiting factor in determining the rate of disappearance of dietary nitrogen from the gastrointestinal tract when the source of this nitrogen was casein. That the disappearance of the dietary casein from the small intestine occurs by means of absorption, and not by passage into the large bowel, is indicated by the observations of Mitchell and Bert (16) and Crampton and Rutherford (17), who reported a negligible increase in fecal nitrogen with a large increase in casein intake. It was postulated that even this slight increase may have been due to an increase in bacterial protein. The intestinal data of the present investigation are in agreement with the results of Gupta *et al.* (10), and with those of Rosenthal and Nasset (7). The conclusion of Rosenthal and Nasset (7) regarding the significance of the contribution of protein by the intestine to the chyme is, however, open to question. In the present experiments the capacity of the intestine to cope with a range of 0 to 350 mg of dietary nitrogen was tested, while Rosenthal and Nasset (7) tested only over a range of from 1 to 55 mg of nitrogen. Therefore their statement that "the lower the nitrogen intake the greater the percentage recovery" has little significance except in its support of the conclusions reached in the present investigation. The term "percentage" was not used in Table II because the

quantity of endogenous nitrogen constantly present in the intestine is equivalent to a much higher percentage of the dietary nitrogen when the protein intake is low.

Nasset's statement that diets containing protein cause greater stimulation of digestive secretions than do protein-free diets (18) is not supported by the results of the present investigation. Table II shows, as stated previously, that the feeding of diet C (15% of casein) did not result in a significantly greater accumulation of nitrogen in the intestinal contents than was the case when diet A (protein-free) was fed. Therefore, if intestinal secretions were stimulated to any great extent by dietary protein under the conditions of the present investigation, the secreted material must have been rapidly absorbed. Also, as shown in Fig. 6, the percentage of nitrogen in the stomach contents did not increase significantly (over the percentage originally present in the diet) with time after feeding diet C. This indicates that gastric secretions were not stimulated specifically by the dietary casein.

Admittedly, the foregoing conclusions are based on indirect evidence and more accurate information about this problem is required. More conclusive information concerning the contribution and turnover of the gastrointestinal secretions can be obtained using isotopic tracer techniques. Such an investigation is currently being considered.

II. The Combined Effects of Protein and Carbohydrate

In evaluating the results shown in Fig. 3 through Fig. 6 the physical differences between the two carbohydrates being tested should be kept in mind, i.e. that sucrose has a much lower molecular weight, and is much more water-soluble than dextrin. These physical differences did not have any noticeable effect on the rates of emptying of protein-free diets A and B from the stomach; however, when casein was present in the diet the rates of passage from the stomach of dietary nitrogen and total solids were influenced by the type of dietary carbodydrate. This is most clearly illustrated by Figs. 4A and 4B and Fig. 6, which serve as the basis for the following explanation of this phenomenon.

When a diet containing dextrin and casein was ingested these components formed a firm mass in the stomach and remained intimately mixed in the same proportion in which they were present in the diet. This is shown by the fact that the percentage of nitrogen in the dry stomach contents (Fig. 6) from rats fed diet C remained quite constant throughout the experimental period. Although the digestion of the protein in this mass must have proceeded slowly in the cardiac portion of the stomach owing to the relatively high pH there, normal gastric digestion could occur in the antrum where the pH was between 2 and 3, near that required for the optimum activity of pepsin. As the contents of the antrum were partially digested they apparently passed through the pyloris into the duodenum; meanwhile more food moved into the antrum where the process was repeated.

When diet D, containing sucrose as the carbohydrate, was fed, a separation of the diet into two phases occurred in the stomach. The sucrose dissolved in the water which was drawn into the stomach presumably because of the high

osmotic pressure exerted by this diet (19), whereas the casein failed to go into solution. Owing to the resulting high fluid content of the stomach contents there was no difference between the pH in the antrum and that in the fundus. The pH of the antrum was as high as that of the fundus for the first few hours after feeding. This would cause precipitation of the casein and would probably cause some inhibition of the gastric digestion of casein by pepsin. The fluid portion of the contents, however, was apparently able to pass through the pyloris into the intestine, leaving much of the casein behind. This is evident from the fact that the curves for the percentage of nitrogen in the stomach contents of rats that were fed diets containing sucrose rise sharply (Fig. 6). The high concentration of sucrose in the fluid passing into the intestine might also have caused a reduced rate of gastric emptying (20).

The above explanation appears to hold also for the results shown in Figs. 4C and 4D for diets E and F as well as those shown in Figs. 5A and 5B for diets G and H, although the previously noted effects were less pronounced with these diets presumably due to their higher casein content. Since the casein was added in place of carbohydrate, the contribution of carbodydrate to the over-all effect was undoubtedly diminished. The altered consistency of the diet caused by the added protein may also have been a contributory factor.

Rosenthal and Nasset (7) also observed effects of the type of dietary carbohydrate on stomach-emptying. They found that a diet containing dextrin emptied from the stomach more rapidly than did a diet containing fresh banana as the carbohydrate source. In earlier experiments Gupta *et al.* (10), using a diet similar to diet D, obtained a curve for the rate of emptying of dietary nitrogen from the stomach which fell more sharply than that obtained in the present study. The more rapid fall they observed can be accounted for partly by the sampling procedure they employed, which resulted in somewhat lower nitrogen recovery (see procedure), partly by the fact that the food intake of their rats was not constant and was somewhat less than 5 g, and partly by the fact that they studied fewer time intervals after feeding of the test meal, a procedure that tends to obscure irregularities in the time curve.

III. Effect of Fat Level

It is evident from Figs. 7A and 7B that the increase in dietary fat level from 5 to 25% caused a general deceleration in gastric emptying. However, the effects previously noted as being due to the differences in type of carbohydrate are still quite apparent and may be explained in the same manner as were the results shown in Figs. 4A and 4B; i.e., diet I remained as a homogeneous mixture throughout its digestion and passage from the stomach while the sucrose of diet J dissolved, and passed from the stomach at a more rapid rate than did the dietary casein. If pictured, the curves for the percentage of nitrogen in the dry stomach contents for diets I and J would follow a pattern similar to those for diets C and D in Fig. 7.

The inhibitory effect of a high level of dietary fat on stomach emptying is clearly illustrated in Figs. 7C and 7D as well as in Fig. 8. The findings of Farrell and Ivy (21) suggest that this effect may be due to the action of

enterogastrone. No effect of the type of carbohydrate on stomach-emptying was evident when the diet contained 50% of fat. A combination of three factors may have been responsible for the lack of carbohydrate effect with this level of fat. These are: a reduction in the physical differences between the diets due to the addition of fat (the consistencies of diets K and L were closely similar), a decrease in the carbohydrate content of the test diets, and the stronger influence on stomach-emptying of a fat-activated inhibitory mechanism. The rather substantial difference between the shape of the curve for diet K in Fig. 7D and that for diet M in Fig. 8 indicates that the effect of the presence of 15% of casein (added in place of dextrin) in diet K was not masked by the high level of dietary fat. London and Sivre (22) reported that fat emptied from the stomach of dogs at a slower rate during the first 2 or 3 hours after feeding than during the remaining period of digestion. Thus their data apparently resemble the results shown in Figs. 7C and 7D, although it is believed that the stomach-emptying pattern observed in the latter case was the result of the combined effect of protein and fat and was not due to the effect of fat alone.

As indicated by the results for diet K in Table II the presence of a high concentration of fat in the diet did not appear to influence either the speed of absorption of gastric digestion products or the quantity of intestinal secretion.

IV. Effect of Level of Food Intake

The results shown in Figs. 9 and 10 indicate that the level of food intake has a definite effect on the rate of emptying of food from the stomach. When the rate of emptying was expressed in the manner shown in Fig. 9, i.e., when percentage recovery is plotted versus time after feeding it is evident that a very low level of food intake (1.5 g) resulted in a more rapid rate of emptying. Also, the effects of the presence of dietary protein on stomach-emptying are not evident with this level of food intake; in fact, the curve for the percentage recovery of ingested dry weight at the 1.5 g level of intake (Fig. 9B) is quite similar in shape to the curve for diet A (protein-free) in Fig. 3. The over-all rate decreased as the food intake was increased up to 3.0 g, and the shape of the curve altered somewhat, with a fairly sharp decrease in the negative slope occurring at the 2-hour interval. This indicates that the quantity of food eaten in this case was such that the presence of protein in the diet had a noticeable effect on stomach-emptying. Raising the level of intake to 5.0 g caused no further change in the over-all rate of emptying. However, the effect of the presence of dietary protein on stomach-emptying becomes clearly evident at this level of intake. Therefore, the conclusion of Rosenthal and Nasset (7) that the presence or absence of protein in the diet has no effect on stomach-emptying is open to question. Although a greater percentage of ingested food emptied from the stomach per unit of time when 1.5 g of diet were fed than when 3.0 or 5.0 g were fed, the total quantity of food emptied from the stomach per unit time increased as the level of food intake increased (Fig. 10). This indicates that the stomach responds to the increased load associated with a

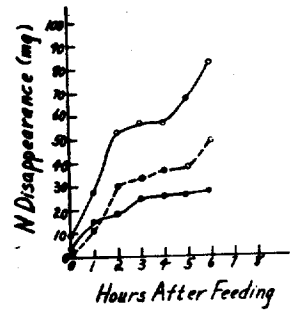


FIG. 10. The effect of level of food intake on the rate of gastric emptying of diet C. 1.5 g fed ●——●, 3.0 g fed ○ - - ○, 5.0 g fed ○——○.

higher level of food intake and that this factor can influence greatly the results of digestion studies of the type outlined in this paper.

Taken all together the results of these experiments indicate clearly that both the type and the quantity of diet consumed influence the rate at which the ingested substances pass into the body fluids.

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Antibiotic Inhibitors

I. The Effect of Certain Milk Constituents

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It has been observed by Edwards,[1] Deubler and Cole,[2] and Price et al[3] that minimum inhibitory and minimum lethal concentrations of many presently used antibiotics are markedly higher for both gram-positive and gram-negative organisms in skim and whole milk than in broth media. These observations were considered to be of greater importance than mere laboratory phenomena, since antibiotics are exposed to milk during mastitis therapy in dairy cattle. It is quite possible that the poor response to treatment obtained in some mastitis cases is attributable to the depressing effect of milk upon antibiotic activity. In addition, inaccurate sensitivity testing interpretations could result if tests are conducted in ordinary bacteriological media, since the relative activities of antibiotics in milk may vary considerably from their relative activities in these media. For these reasons, further investigation of this problem appeared to be warranted.

It seemed reasonable to assume that the observed differences in antibiotic activity could be attributed to either, (1) better and more vigorous growth of the organisms in milk, or (2) a factor(s) in milk inhibitory to antibiotics. The first possibility was suggested by the work of Linz and LeCocq[4, 5] who used this theory to account for the differences in streptomycin activity observed in nutrient and brain-heart infusion broths in tests with *Pseudomonas* and *Micrococcus* strains. This theory, however, did not seem applicable in the case of nutrient broth and milk, since indicative differences in growth rate and total cell crop were not apparent between milk and nutrient broth cultures for the *Pseudomonas aeruginosa*, *Micrococcus pyogenes*, and *Streptococcus agalactiae* strains tested by Price et al.[3]

Regarding the second possibility, a considerable amount of data is available to show that certain proteins and protein break-down products are antagonistic to antibiotics.[6–10] Salts of various metallic ions have also been shown to interfere with antibiotic activity.[11–13] In addition, specifically regarding differences observed for antibiotic activity in laboratory media, it was reported by Wallace et al[14] and by Rhymer and Wallace[15] that the greater activity of streptomycin in nutrient broth as compared to brain-heart infusion broth was due to an antagonistic factor in the latter medium.

Studies reported here were initiated to determine if any of the major milk con-

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* The trade name of Chas. Pfizer & Co. for oxytetracycline is Terramycin.
† The trade name of Lederle Laboratories Division, American Cyanamid Co., for chlortetracycline is Aureomycin.

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TABLE I
Inorganic Additives

Salts	Per cent
Potassium monohydrogen phosphate	0.195
Sodium (citrate), hydrated	0.250
Magnesium hydrogen phosphate, hydrated	0.150
Magnesium chloride, hydrated	0.150
Calcium chloride, hydrated	0.170
Calcium (citrate), hydrated	0.230
Potassium chloride	0.370
Calcium pyrophosphate	0.175
Sodium chloride	0.153
Milk ash	0.700

stituents is capable of inhibiting the antibacterial activity of several antibiotics (oxytetracycline,* chlortetracycline,† neomycin, polymyxin B, and dihydrostreptomycin).

EXPERIMENTAL PROCEDURES

Experiment 1. The culture employed in all experiments was a strain of *Ps. aeruginosa* arbitrarily designated as 1014. This strain was isolated from a case of clinical mastitis and produced a marked greenish pigmentation in both milk and broth. It displayed a moderate sensitivity to the antibiotics usually active against this species. Antibiotics studied included oxytetracycline hydrochloride, chlortetracycline hydrochloride, polymyxin B sulfate, neomycin sulfate, and dihydrostreptomycin sulfate.

The basal medium employed in the first phase of experiment 1 was Difco nutrient broth. Various salts, which are components of milk, were added at the concentrations shown in table I.

The salts and ash in table I were sterilized by autoclaving at 121 C. for 15 minutes, with the exception of calcium chloride, which was sterilized as a demineralized water solution by Seitz filtration and then added to sterile nutrient broth. This was found necessary, since sterilization of nutrient broth solutions of this salt by autoclaving produced a precipitate. Milk ash was prepared by heating skim milk at 700 C. for eight hours in a muffle furnace.

Several organic materials were also evaluated for their possible inhibitory effect by addition to nutrient broth at the concentrations indicated in table II. These

TABLE II
Organic Additives

	Per cent
Lactose	5.0
Casein powder (Pfanstiehl)	2.8
Hydrolyzed casein (Nutritional Bio Chem Corp.)	2.8
Calcium caseinate (Pfanstiehl-soluble)	2.8

materials were sterilized by autoclaving at 121 C. for 15 minutes after which sufficient sodium hydroxide was added to bring the *p*H to 6.8.

The minerals and organic materials listed in tables I and II were added at concentrations that approximated those obtained by analysis of milk as reported by Little and Plastridge,[16] with the exception of calcium chloride, which was added so that the cation content was equivalent to one-fourth the total calcium. This was based on their observation that only 25 per cent of milk calcium is in soluble form.

Antibiotic sensitivity tests were carried out by the common twofold serial dilution method. Final antibiotic concentrations ranged from 800 to approximately 0.1 μg./ml. Inoculum was prepared as follows: 5 ml. of an 18 hour nutrient broth culture was added to 1420 ml. of demineralized water and 1 ml. of this suspension pipetted into a flask containing 34 ml. of the test medium. This resulted in a final culture dilution of 1:20,000 when 0.5 ml. of the latter suspension was added to the 0.5 ml. of test medium containing the various antibiotic concentrations. After addition of the inoculum, tubes were shaken and incubated for 18 to 20 hours at 37 C. Minimum inhibitory concentrations for the broth media were determined by noting the presence or absence of turbidity; whereas minimum inhibitory concentrations for the tests in which milk or other turbid substances were used were determined by noting the presence or absence of greenish pigmentation and pellicle formation. Results thus obtained were verified by using de-fatted methylene blue stained smears. The minimum inhibitory concentration in these studies was considered to be the lowest concentration of antibiotic in which growth failed to occur. These procedures were employed for all antibiotic tube dilution sensitivity tests.

Experiment 2. In this experiment the effect of various milk fractions on antibiotic activity was determined by tube dilution sensitivity tests. Whole milk for fractionation was acquired from animals showing low bacterial counts. This milk was obtained by aseptic methods and pasteurized at 143 F. for 30 minutes. Sterility checks indicated that the milk was free of living organisms.

Reuninized milk was prepared according to the method described by Deubler and Cole.[2] After formation of the clot, the renninized milk was centrifuged at 3000 rpm for 15 minutes in a Servall angle head centrifuge and the supernatant removed. The volume of supernatant was determined and dehydrated nutrient broth powder added at the rate of 8 Gm./liter. The residue (clotted material) was then broken up and brought back to the original milk volume with nutrient broth. Following autoclaving, the *p*H of both fractions was adjusted to 6.8.

Sufficient concentrated hydrochloric acid was added to skim milk to obtain acid-precipitated casein and a casein-free supernatant. After centrifugation at 3000 rpm for 15 minutes, the supernatant was removed and nutrient broth powder added at the rate of 8 Gm./liter. The precipitated casein was then brought back to the original milk volume with nutrient broth. After autoclaving, the *p*H of both was adjusted to 6.8.

The four ion-exchanged milk samples employed in this experiment were prepared as follows: Amberlite IRC-50 (Rohm and Haas) was treated according to the method of Baker et al.[17] One hundred Gm. wet weight of the treated resin samples

were then added to each of four 120 ml. aliquots of milk for 15, 30, 60, and 120 seconds, respectively, so that graded quantities of calcium would be exchanged.

Experiment 3. In this study, milk cations were bound or complexed by suitable additives to determine the effect of this treatment on the activity of oxytetracycline. Various concentrations of potassium oxalate (giving a molar range from 0.01 to 0.05) and sodium citrate (giving a molar range from 0.05 to 0.25) were added to 50 ml. samples of whole milk as demineralized water solutions which had been previously sterilized by Seitz filtration. Liquid Versene (Bersworth Chemical Co.), the tetrasodium salt of ethylenediamine tetra-acetic acid in 34 per cent concentration, was added in volumes ranging from 0.005 to 0.05 ml./ml. of milk. The activity of oxytetracycline in treated milk samples was then determined by twofold serial dilution techniques.

Experiment 4. This trial was designed to measure the quantitative effect of calcium and magnesium ions on the activity of the test antibiotics. Efforts to employ a synthetic medium, such as that described by Weinberg[18] for this type of study, were unfruitful since addition of calcium chloride to such media resulted in a heavy precipitation. Presumably, this was due to the formation of insoluble calcium phosphate. Comparative studies employing both nutrient broth and a synthetic medium containing no calcium and only 0.002965 per cent magnesium sulfate were conducted to determine quantitatively the effect of magnesium additions. Results were obtained that showed slightly more than a twofold difference in antibiotic activity in these two media. Since this indicated that inhibitory substances were at a fairly low concentration in nutrient broth, all additional studies were carried out in this medium.

Various concentrations of calcium chloride and magnesium chloride (to give a range from 0.01 to 0.4 molar) were added to cylinders that had previously been placed on nutrient agar plates seeded with the test organism. Inhibitory activity of oxytetracycline in these plate diffusion studies was determined by measuring the radii of the inhibition zones after 20 hours incubation at 37 C.

Statistical Analysis. All data obtained in these trials were subjected to analysis of variance.[19] Least significant differences were computed either by the classical method of Fisher[19] or, for multiple comparison, by the method of Tukey.[20] Statements that an effect, or difference, was significant means at the 5 per cent level of probability for error, unless otherwise designated. Curves were fitted following procedures set forth by Bliss.[21]

RESULTS

Experiment 1. In the first trial the major constituents of milk were screened for antagonistic activity against the test antibiotics. Milk fat was not included, since previous studies by Price et al.[3] failed to reveal major differences in antibiotic activity in whole and skim milk. Table III shows the average minimum inhibitory concentrations (3 to 6 tests) for the various antibiotics in nutrient broth to which various milk constituents had been added at the indicated concentrations. It will be noted that all antibiotics tested were markedly less active in

TABLE III

Influence of Various Milk Constituents on the Minimum Inhibitory Concentration of Five Antibiotics for Pseudomonas aeruginosa *Strain 1014*

Nutrient broth plus	Oxytetracycline, μg./ml.	Chlortetracycline, μg./ml.	Dihydrostreptomycin, μg./ml.	Neomycin, μg./ml.	Polymyxin B, units/ml.
—	4.6	6.2	5.6	0.9	25.0
.195% dipotassium phosphate	6.2	9.0	6.2	0.8	25.0
.175% dicalcium phosphate	3.2	10.0	32.0*	1.6	40.0
.150% monomagnesium phosphate	19.0*	22.0*	16.0*	3.9*	32.0
.370% potassium chloride	4.8	6.2	36.0*	1.6	32.0
.153% sodium chloride	6.2	6.2	9.0*	1.1	12.5
.150% magnesium chloride	50.0*	50.0*	62.5*	10.5*	80.0*
.170% calcium chloride	25.0*	25.0*	100.0*	16.5*	88.0*
.250% sodium citrate	0.7†	2.2†	6.2	1.6	6.2†
.230% calcium citrate	6.2	10.0	62.5*	3.9*	25.0
.700% milk ash	25.0*	42.5*	50.0*	32.0*	80.0*
5.000% lactose	7.9	10.0	10.0	2.5*	64.0*
2.800% casein powder	4.4	6.2	31.0*	50.0*	10.0
2.800% hydrolized casein	6.2	9.0	50.0*	25.0*	15.5
2.800% calcium caseinate	25.0*	72.0*	200.0*	200.0*	200.0*
Whole milk	46.0*	140.0*	280.0*	200.0*	260.0*

* Significantly higher MIC than nutrient broth control for that antibiotic.
† Significantly lower MIC than nutrient broth control for that antibiotic.

whole milk than in nutrient broth and that many of the additives also significantly increased the minimum inhibitory concentrations of the antibiotics. Oxytetracycline and chlortetracycline were similarly affected by the additives, as were neomycin and dihydrostreptomycin. Polymyxin was essentially intermediate to these two distinct groups.

The importance of divalent cation interference can be noted immediately for oxytetracycline and chlortetracycline. Only those media containing added magnesium or calcium salts, milk ash, or milk itself interfered with the activity of these antibiotics. Identical results were obtained in a limited number of trials with the calcium salt of oxytetracycline (calcium dioxytetracycline).[22]

These same additives also induced a marked lowering of the activity of neomycin, dihydrostreptomycin, and polymyxin B. In addition, some interference due to the monovalent cations, potassium and sodium, was noted for dihydrostreptomycin.

However, for these three antibiotics, only a portion of the inhibition due to milk could be attributed to cations, since casein and/or lactose also caused a significant reduction in activity. The failure of polymyxin B to be retarded by the casein additives was the major basis for distinguishing this antibiotic from the neomycin-dihydrostreptomycin group. Response to lactose was fairly uniform for the three antibiotics with neomycin and polymyxin B showing a slight but significant depression, whereas reduction of dihydrostreptomycin activity approached signifi-

cance. It was of interest that the poorly ionizable salts of calcium (phosphate and citrate) failed to affect significantly the minimum inhibitory concentration (MIC) of oxytetracycline, chlortetracycline, and polymyxin B and caused less inhibition of neomycin and dihydrostreptomycin than did the readily ionizable chloride salts. Similarly, magnesium hydrogen phosphate had less effect than did magnesium chloride for all antibiotics tested.

The striking differences in MIC obtained, depending upon whether citrate was added as the sodium or calcium salt, should also be noted. When added as the calcium salt, only neomycin varied significantly from the nutrient broth control, while addition of the sodium salt caused significant increases in the activity of all antibiotics except dihydrostreptomycin.

Since cations, especially divalent, seemed to interfere markedly with the activity of all the antibiotics tested, further studies of milk cations seemed warranted.

Experiment 2. This trial was concerned with determining the inhibitory activity of various milk fractions for the test antibiotics. In addition, the MIC of the antibiotics were determined in milk samples that had been subjected to an ion-exchange resin for various periods of time. It can be observed in table IV that oxytetracycline, chlortetracycline, and polymyxin B were essentially similar in response to the various materials tested, as were neomycin and dihydrostreptomycin. The first group, as was previously demonstrated in experiment 1, was most inhibited by those fractions or milk samples having the highest mineral concentration. The rennin precipitate, apparently containing a great deal of calcium as calcium paracaseinate, was more inhibitory than was the rennin supernatant (whey). Conversely, the major interference due to the acid-treated milk was observed in the supernatant where one would expect to find the highest inorganic ion concentration. Significantly smaller concentrations of all three antibiotics were required to produce inhibition of the test organism in the acid precipitate than in whole milk.

TABLE IV

Average Minimum Inhibitory Concentrations of Five Antibiotics for Pseudomonas aeruginosa *Strain 1014 When Tested in Various Milk Fractions*

Media	Oxytetracycline, μg./ml.	Chlortetracycline, μg./ml.	Dihydrostreptomycin, μg./ml.	Neomycin, μg./ml.	Polymyxin B, units/ml.
Nutrient broth	3.2	3.2	6.2	1.1	12.5
Whole milk	25.0*	140.0*	200.0*	200.0*	200.0*
Rennin supernatant	18.0*	72.0*	72.0*	100.0*	36.0*
Rennin precipitate	100.0*	280.0*	200.0*	72.0*	400.0*
Acid supernatant	50.0*	100.0*	100.0*	200.0*	200.0*
Acid precipitate	9.0*	12.5*	800.0*	800.0*	36.0*
Ion exchanged milk (15 sec.)	6.2*	12.5*	100.0*	200.0*	100.0*
Ion exchanged milk (30 sec.)	6.2*	9.0*	72.0*	140.0*	72.0*
Ion exchanged milk (60 sec.)	3.2	6.2	50.0*	100.0*	25.0
Ion exchanged milk (120 sec.)	2.2	6.2	50.0*	50.0*	12.5

* Significant increase in MIC from nutrient broth control for that antibiotic.

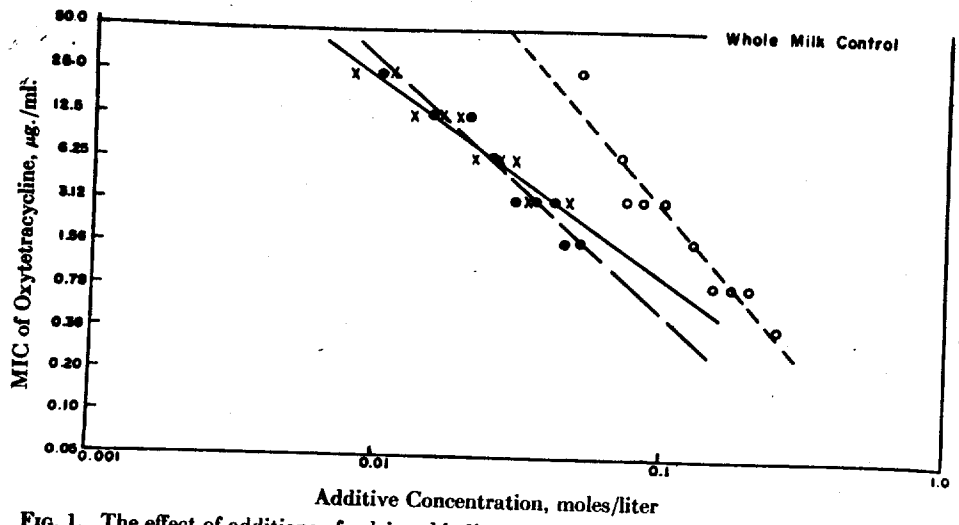

FIG. 1. The effect of additions of calcium binding agents to whole milk on the MIC of oxytetracycline for *Pseudomonas aeruginosa* strain 1014. X——— EDTA (tetrasodium salt); ●——— potassium oxalate; O——— sodium citrate.

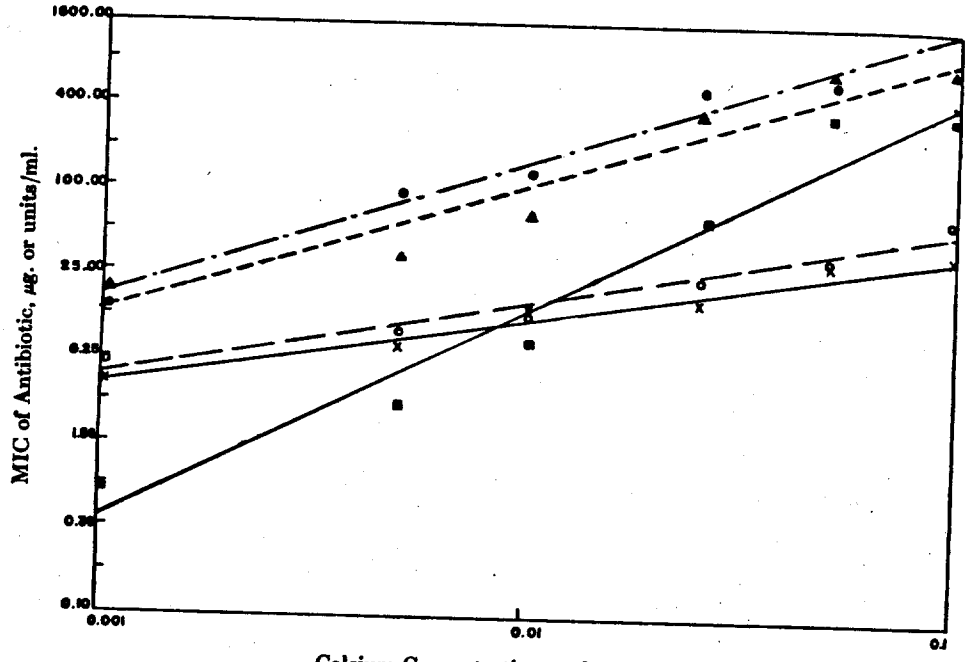

FIG. 2. The effect of calcium additions to nutrient broth on the MIC of various antibiotics for *Pseudomonas aeruginosa* strain 1014. The symbol keys and the MIC in the respective nutrient broth controls are: ■——— Neomycin, 0.33 μg./ml.; O———chlortetracycline, 3.12 μg./ml.; X———oxytetracycline, 3.70 μg./ml.; ●———dihydrostreptomycin, 5.2 μg./ml.; ▲———polymyxin B, 11.0 units/ml.

A striking removal of the inhibitory factor(s) for these three antibiotics was noted in the samples subjected to the ion-exchange resin. Activity not significantly different from that of the nutrient broth control was obtained after 30 to 60 seconds exposure.

Neomycin and dihydrostreptomycin were markedly inhibited by the supernatants and precipitates of both the rennin and acid-treated milk samples and, in contrast to these antibiotics, were more strongly inhibited by the acid precipitates than the acid supernatants. Although ion-exchanged milk samples significantly decreased the minimum inhibitory concentrations as compared to untreated milk, levels required were still considerably above those needed in nutrient broth.

Experiment 3. Further evidence that milk cations influence oxytetracycline activity was shown in this experiment. The effect of adding graded concentrations of Liquid Versene (LV), potassium oxalate, or sodium citrate to milk samples prior to use in serial dilution tests is shown in figure 1. A significant decrease in the MIC of oxytetracycline for *Ps. aeruginosa* 1014 was induced by addition of suitable amounts of these materials to milk. The increase in antibiotic activity obtained as the active cation is removed from milk is apparently a logarithmic function of the concentration of the cations as indicated by excellent fit of the lines.

Activity equivalent to that obtained in nutrient broth was observed when sufficient LV was added to effectively chelate approximately 102 mg. calcium per 100 ml. of milk. According to the analysis of cow's milk given by Little and Plastridge,[16] this would represent approximately 50 to 60 per cent of the total calcium of milk. Potassium oxalate, which was found by titration to be approximately equal on a molecular basis to the active ingredient of LV in combining capacity for calcium, was required in sufficient amount to bring about the theoretical combination of 99 mg. calcium per 100 ml. of milk in order to provide antibacterial activity equivalent to that obtained in nutrient broth. Sodium citrate, on the basis of a molecular combining capacity of approximately one-half that of potassium oxalate and the active ingredient of LV, had to be added at a level theoretically capable of binding 116 mg. calcium ions per 100 ml. milk to produce a comparable increase in activity.

Experiment 4. This study was designed to determine quantitatively the degree of interference of calcium and magnesium ions for the five antibiotics. The effect of calcium additions (as hydrated calcium chloride), ranging from 0.001 to 0.1 molar, is shown in figure 2. It can be noted that activity of all antibiotics was slightly reduced even when calcium molarity was as low as 0.001. For increasing concentrations, the logarithm of the MIC was found to be directly proportional to the logarithm of the calcium concentration. The slope obtained for neomycin was markedly steeper than those found for the other antibiotics. Polymyxin B and dihydrostreptomycin produced slightly steeper slopes than did oxytetracycline and chlortetracycline. Three distinct groups could be shown by a method based on that of Tukey.[20] Losses in antibiotic activity upon addition of 0.1 molar calcium were as follows: oxytetracycline, tenfold; chlortetracycline, eighteenfold; dihydrostreptomycin, two hundred ninetyfold; neomycin, one thousandfold, and polymyxin B, ninetyfold.

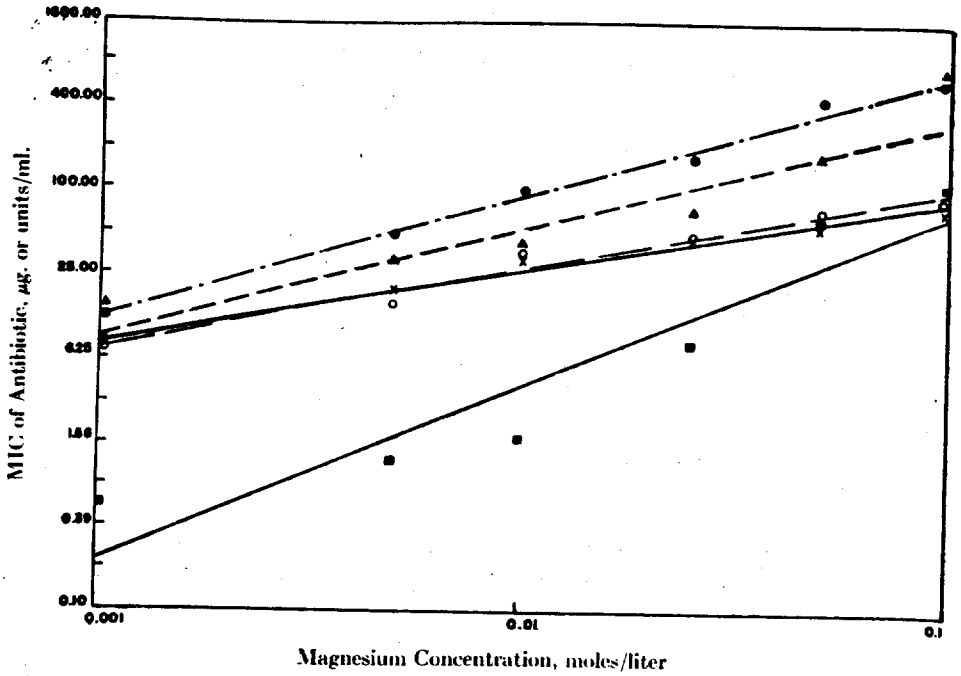

FIG. 3. The effect of magnesium additions to nutrient broth on the MIC of various antibiotics for *Pseudomonas aeruginosa* strain 1014. The symbol keys and the MIC's in the respective nutrient broth controls are: ■———Neomycin, 0.33 μg./ml.; O———chlortetracycline, 3.12 μg./ml.; X———oxytetracycline, 3.70 μg./ml.; ●———dihydrostreptomycin, 5.2 μg./ml.; ▲———polymyxin B, 11.0 units/ml.

The pattern observed for the various calcium levels was essentially similar for magnesium (as hydrated magnesium chloride). Data obtained with this ion are shown in figure 3. There were, however, some significant variations in the inhibitory activity of the two cations for the test antibiotics. Magnesium was found to be more inhibitory for oxytetracycline and chlortetracycline than was calcium. Conversely, the latter ion more effectively reduced the activity of polymyxin B and neomycin than did the former. Calcium was also apparently more inhibitory to dihydrostreptomycin than was magnesium (approached significance).

TABLE V
Ratio of the Moles of Cations Per Mole of Antibiotic Producing Growth Inhibition of Pseudomonas aeruginosa *Strain 1014 at Two Different Cation Concentrations*

Antibiotic	Molecular weight	Magnesium (molar concentration)		Calcium (molar concentration)	
		0.001	0.1	0.001	0.1
Oxytetracycline	497	63	603	108	1232
Chlortetracycline	515	71	515	103	837
Dihydrostreptomycin	743	64	163	77	82
Neomycin	1167	4720	1831	2461	190
Polymyxin B	1150	132	482	75	84

It was considered of interest to determine the number of moles of calcium or magnesium required for each mole of antibiotic to obtain a given MIC. These calculated values are shown in table V along with molecular weights for the various antibiotics as reported by Grove and Randall.[23] Only the values obtained with 0.001 and 0.1 molar ion solutions are shown as all other ratios fell between these extremes. Ratios obtained for calcium and magnesium were of the same order of magnitude for each antibiotic, considerable variation being shown between antibiotics. Correlation between molecular size of antibiotics and number of moles of the cations associated with them was not observed. Although oxytetracycline and chlortetracycline (similar molecular weights) required roughly comparable concentrations of calcium or magnesium ions, the larger molecule of dihydrostreptomycin required less. Despite the fact that inhibition of the large neomycin molecule was associated with a very high number of cations, the likewise large polymyxin B molecule was associated with a comparatively low number of calcium and magnesium ions.

Higher values were obtained at the higher ion concentrations with all antibiotics except neomycin, which had values at 0.001 molar calcium and magnesium that were considerably higher than those calculated for the 0.1 molar cation concentrations.

Experiment 5. In this trial, the effect of addition of calcium and magnesium

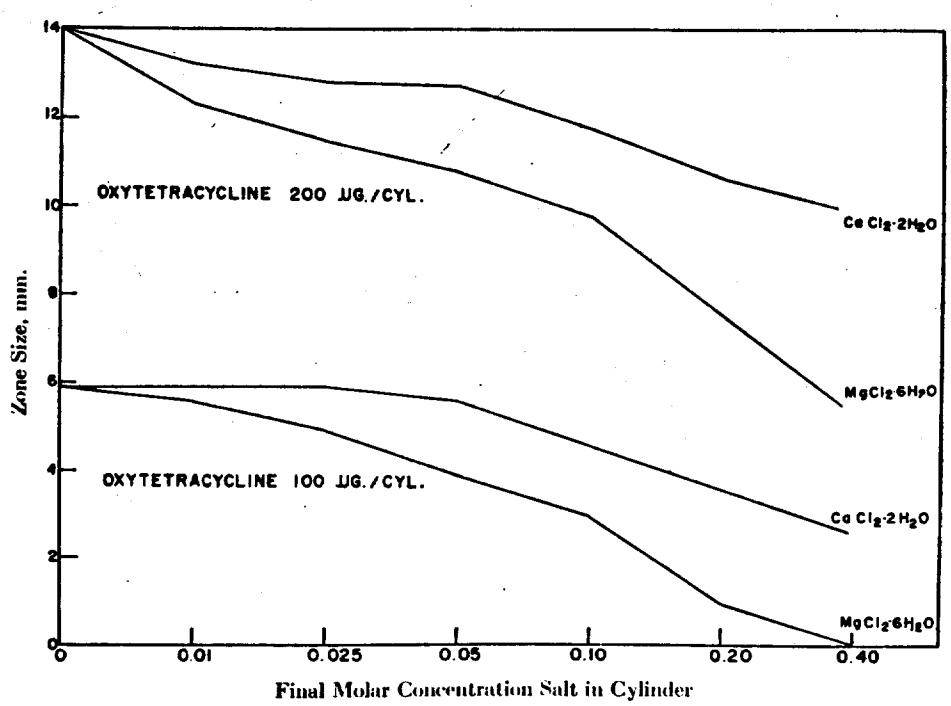

FIG. 4. The influence of graded levels of calcium and magnesium on the antibacterial activity of oxytetracycline in plate diffusion tests with *Pseudomonas aeruginosa* strain 1014.

ions to a constant level of oxytetracycline was determined by measuring zones of inhibition. Results of these studies are shown in figure 4 for both the 100 and 200 μg. levels of oxytetracycline. As was found with serial dilution tests in the previous experiment, equivalent molar concentrations of magnesium were found to be significantly more inhibitory than calcium. Antibiotic inhibition at both 100 and 200 μg. oxytetracycline was essentially a straight line function of zone radii versus logarithmic cation dosage, although, at 100 μg. oxytetracycline per cylinder, an initial plateau was obtained with calcium.

DISCUSSION

Apparently a great deal of the inhibition of antibiotic activity produced by milk can be attributed to casein and/or the mineral content (principally cations) of this medium. In addition, a smaller but statistically significant depression in activity was produced by lactose in the case of two antibiotics (neomycin and polymyxin B).

The five antibiotics studied could be separated into two distinct groups on the basis of their degree of inhibition by casein. Neomycin and dihydrostreptomycin were markedly inhibited in the presence of this protein, whereas oxytetracycline, chlortetracycline, and polymyxin B were unaffected. The ability of such complex substances to interfere with the activity of neomycin and streptomycin has been previously demonstrated. Schoenhard and Stafseth[24] reported that peptones antagonized the action of neomycin, while Foster and Pittillo[25] found that Basamine, Difco peptone, and media containing similar organic materials were inhibitory to a number of antibiotics including streptomycin. Green and Waksman[26] observed inhibition of streptomycin activity by neopeptone and peptonized milk.

Considerable data are available to show that certain cations present in milk are detrimental to the activity of these test antibiotics. Newton[11] has reported that calcium, magnesium, manganese and ferrous ions are inhibiting to the activity of polymyxin. Inhibition by magnesium occurred at molar concentrations as low as 0.0125 in tests against *Ps. aeruginosa*. Similarly, it has been demonstrated in a series of papers by Weinberg[18, 27, 28] that magnesium, calcium, manganese, ferric, and ferrous ions all are capable of causing inhibition of oxytetracycline activity with marked interference by magnesium occurring at concentrations below 0.025 molar.

Brody et al[29] found that the addition of magnesium reversed the inhibition by oxytetracycline and chlortetracycline of the oxidative processes in mitochondria. Similarly, Van Meter et al[30] found that magnesium reversed the ability of chlortetracycline to interfere with oxidative phosphorylation in animal tissues. Soncin[31] observed that 0.001 molar magnesium solutions were inhibitory to both oxytetracycline and chlortetracycline in studies with *Escherichia coli*. The magnesium ions prevented the antibiotics from interfering with respiration, glycolysis, and multiplication of the bacterial cells. According to Saz and Slie[32] chlortetracycline's ability to inhibit cell-free nitro reductase of *E. coli* was reversed by manganese. Similar reports are available to show that neomycin and streptomycin activities are reversed by various monovalent and divalent cations. Klein and Kimmelman[33]

found that 0.5 per cent sodium chloride reduced the effectiveness of streptomycin in tests against *Shigella* species. Sodium chloride, potassium chloride, and several other salts of these monovalent cations have been shown by Berkman et al[34] to interfere markedly with streptomycin activity with the degree of antagonism being directly proportional to the salt concentration. Green and Waksman[26] also found that sodium chloride was inhibitory to the antibacterial activity of streptomycin. Conversely, Donovick et al[35] failed to obtain a significant depression of streptomycin activity with potassium or sodium ions, although marked inhibition of this antibiotic was produced by magnesium and calcium ions. Strong reversal of neomycin activity was shown by Schoenhard and Stafseth[24] when tested in the presence of sodium chloride. The majority of the cation levels cited as being inhibitory for these antibiotics are lower than one would expect to find in milk.

It is apparent that only calcium and magnesium salts that readily ionize are capable of interfering with the activity of the antibiotics, at least, at the concentrations studied. This observation is supported by the failure of calcium phosphate, calcium citrate, or magnesium phosphate to cause marked decrease in the effectiveness of the various antibiotics. The decrease in minimum inhibitory concentrations noted when citrate was added as the sodium salt could possibly be attributed to removal of interfering ions, or more likely to a toxic effect on the organism induced by removal of essential ions.

The screening procedures employed in the first experiment could be interpreted as being only indicative that the milk minerals are actually functioning as inhibitors; however, the data obtained from succeeding experiments provide more substantial evidence that these minerals are actually inhibitors. The milk fractions, particularly those prepared by acid precipitation of the casein, show that the major inhibitory factor(s) for oxytetracycline, chlortetracycline, and polymyxin B is retained in the mineral-rich supernatant. This same fraction also produced a significant depression in the activity of neomycin and dihydrostreptomycin. However, the strongest evidence for interference due to milk cations was obtained by exposing milk samples to a potassium treated ion-exchange resin. This exposure resulted in highly significant decreases in the concentration of inhibitory factor(s). There appeared to exist a correlation between time of exposure to the resin and increases in antibiotic activity suggesting that concentration of cations was certainly a factor in determining the degree of their activity.

Evidence to show that cations are by far the most important and perhaps the only inhibitory factor for oxytetracycline was obtained in experiment 3. Addition of the tetrasodium salt of ethylenediamine tetra-acetic acid in increasing concentrations to whole milk resulted in an increase in antibiotic activity that followed a first order rate. This finding conflicted somewhat with that of Weinberg,[18] who was unable to demonstrate any influence of this chelating agent on multivalent cation interference with oxytetracycline activity under the conditions of his trials. Addition of increasing concentrations of sodium citrate and potassium oxalate also caused a reduction in minimum inhibitory concentration of oxytetracycline proportional to the logarithm of the concentration of these salts. The theoretical quantity of cation having to be removed in order to obtain a given increase in

antibiotic activity was remarkably uniform for these three calcium-binding agents. For example, to obtain a sixteenfold increase in activity, the three additives required the theoretical removal of from 99 to 116 mg. calcium per 100 ml. of milk.

A comparison of the relative effect of calcium and magnesium ions on the activity of the five antibiotics showed significant variations. The reasons for these variations are difficult to assign, but certainly must be a function of the antibiotics' ability to resist interference by ions.

Although magnesium is present in milk at approximately 1/5 the molar concentration of calcium, it is conceivable, in the case of oxytetracycline and chlortetracycline, that the former ion may influence their activity almost as much as the latter, the difference in molecular concentration being nullified to a certain extent by the greater inhibitory action of magnesium. For the remaining antibiotics, one would expect calcium to be more influential, since this ion more actively antagonizes their activities.

The fact that all antibiotics tested were inhibited by calcium solutions of molarity as low as 0.001 is especially interesting inasmuch as milk is not the only biological fluid in which this molar concentration is exceeded. In blood serum, for instance, the average calcium level for human beings ranges from 9 to 11 mg./100 ml. according to Anderson.[36] If one assumes an average value of 10 mg./100 ml., this would represent a calcium molarity of 0.0025, or two and one-half times a level shown to produce a substantial inhibition of antibiotic activity. Of course, it should be recognized that a certain portion of the serum calcium may be tightly bound and incapable of interfering with antibiotics.

There was apparently little correlation between the molecular weight of the antibiotics studied and the number of moles of ions required to produce a given degree of inhibition. Since molecular weight must be a rough measure of antibiotic surface area, one can conclude that cation antagonism is essentially independent of this measurement.

This finding would make it improbable that the mode of antibiotic inhibition by cations is physical adsorption of cation and antibiotic. Alternate possibilities are the preferential adsorption of cations on the bacterial cell surface or chemical combination of antibiotic and cations. If cation antagonism does function through adsorption on the bacterial cell surface, then the moles of inorganic ion adsorbed should be a function of the cation concentration and should follow some known isotherm. The isotherm of Freundlich was found to give an excellent fit in all instances where the logarithm of the MIC of the antibiotic was plotted versus the logarithm of calcium and magnesium ion concentration (figs. 2 and 3).

Freundlich's adsorption isotherm is the equation for physical or van der Waal's adsorption and is $x/m = k\ P^{1/n}$ (equation 1). In classical physical chemistry, x equals the moles of gas adsorbed, m the weight of the adsorbent, k and n are constants, and P is the gas pressure at equilibrium. By analogy, in the system being considered, x becomes the MIC, m the cell volume or weight, which is assumed to be constant, and P the concentration of the cations in solution.

Some discussion of the justification for the substitution of MIC for the quantity of cations adsorbed is indicated. If one theorizes that both antibiotics and cations

function through adsorption on the bacterial cell surface, and that cations are preferentially adsorbed, then the total quantity of antibiotic must equal the following: the amount adsorbed, plus the amount displaced by cations. The nonadsorbed antibiotic (in solution as a result of displacement by cations) must therefore be a function of the amount of cations adsorbed. This permits one to say that the amount of adsorbed or active antibiotic equals the total, minus that antibiotic measured as a function of the cation adsorbed (dependent on cation concentration). Since it is accepted that the MIC is a function of active antibiotic, the MIC then equals the total antibiotic minus the antibiotic measured as a function of the cation concentration. This line of reasoning, which shows that the MIC of the antibiotics is a function of the quantity of cations adsorbed, permits the substitution indicated previously.

When the logarithm MIC is plotted versus the logarithm of the cation concentration (equation 1), logarithm k then equals the intercept of the line with the ordinate, and logarithm 1/n equals the slope of the line. In this system, excellence of fit was probably obtained because of the highly dilute solutions employed.

The fit to this equation is certainly consistent with the hypothesis that adsorption of antibiotics on *Ps. aeruginosa* cells does occur and that these agents can be displaced by divalent cations. However, the fit is also consistent with the hypothesis that chemical combination of antibiotic and cations occurs. This latter hypothesis, of course, is dependent upon the formation of a weak chemical combination of cations and antibiotic which is capable of being driven toward completion by cation excesses of the order found in these experiments.

As the logarithm of the MIC is directly proportional to the logarithm of the active antibiotic concentration, in order to obtain the fit it is necessary to show that the logarithm of the MIC is also directly proportional to the logarithm of the cation concentration. Suppose that the antibiotic-cation complex ionizes to antibiotic (xA) and cation (yC). The solubility constant of such a complex would be

$$k = \frac{(A)^x (C)^y}{(A) \quad (C)}$$

If it is assumed that k is small, one can disregard the denominator, and then $k = (A)^x (C)^y$. Since k is a constant, $(A)^x$ must be a function of $(C)^y$. This would not in general give a linear plot because of the exponents. However, through conversion to logarithms on both sides of the equation, $x(\log (A)) = k'y(\log (C))$. Since x and y would be constant for a given complex, these can be combined with the constant of proportionality (k′) to obtain $\log (A) = k'' \log (C)$. Since log (A) varies with log MIC also, then log MIC varies with log (C). If all these assumptions are valid, they explain the fit to Freundlich's equation.

Many interesting possibilities are opened by these hypotheses but there are also immediate practical implications of inhibition of milk constituents by antibiotics. The observed decreases in activity, although admittedly relatively small in the case of oxytetracycline and chlortetracycline as compared to polymyxin B, dihydrostreptomycin, and neomycin, are sufficient to yield extremely erroneous inter-

pretations of bovine mastitis organism sensitivity tests. Such discrepancies could be overcome by use of milk as a sensitivity testing medium.

In regard to the actual in vivo results with bovine mastitis antibiotic therapy, the high percentage of cures obtained (although often temporary) is surprising. These results can be attributed almost certainly to the extremely high antibiotic concentrations present for a short time after administration of mastitis remedies. On the other hand, there has been a rapid diminution of *Str. agalactiae* mastitis cases with an increase (either real or relative) in cases caused by micrococci and gram-negative organisms. Unfortunately, antibiotics do not perform with the same efficiency against these organisms as they do against streptococci. Even if micrococci and gram-negative bacilli could be claimed to be more invasive, it would be difficult to believe that a certain percentage of the cases that resist treatment, and yet are caused by apparently antibiotic-sensitive organisms, are not due to the poorer efficiency of the antibiotics in milk.

The fact that antibiotic activity increases as the divalent cation concentration decreases opens several pathways for the development of future mastitis treatments. Simultaneous use of a highly efficient, inexpensive, and nontoxic binder of calcium and magnesium in preparations for treatment of mastitis might increase the effectiveness of presently used levels of antibiotics or might maintain the present effectiveness with lower concentrations of antibiotic.

SUMMARY

The antagonistic influence of milk on oxytetracycline, chlortetracycline, and polymyxin B can be attributed to the high concentration of inorganic ions (principally calcium and magnesium) present in this medium. Data obtained indicated that the readily ionizable salts of calcium and magnesium were more inhibitory than were poorly ionizable ones. Although inorganic ions were also found to be highly inhibitory for dihydrostreptomycin and neomycin, only part of the antagonism observed in milk for these antibiotics could be attributed to its cation content. This observation is based on the finding that casein also strongly interferes with the activity of these antibiotics. Confirmation that cations produce significant reductions in activity was given by the finding that antagonism of milk to antibiotics could be reversed by exposing it to cationic exchange resins, as well as by addition of calcium binding or chelating agents.

It was noted that magnesium salts were more antagonistic to oxytetracycline and chlortetracycline than were calcium salts; conversely, calcium salts were more antagonistic to polymyxin B, dihydrostreptomycin, and neomycin than were magnesium salts. For all antibiotics tested, the logarithm of the minimum inhibitory concentration was found to be a linear function of the logarithm of the concentration of calcium or magnesium ions. When the ratio of moles of cation per mole of antibiotic required to produce a given degree of inhibition was calculated from these data, it was found that there was no correlation between molecular weight of the antibiotic and number of moles of cation required.

It is concluded that knowledge of inhibition of antibiotics by milk constituents

may have great importance both in interpreting mastitis organism sensitivity tests and in the treatment of mastitis cases.

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The flavour of milk protein

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Summary. The flavour stability of casein and co-precipitated milk proteins has been examined organoleptically. Differences in flavour stability were correlated with processing conditions and the early stages of the non-enzymic browning reaction were implicated in the off-flavour development. The 'gluey' flavour present in stored casein could be removed and the flavour stability improved by treatment with activated carbon or Sephadex. However, a better solution to the problem was to avoid the development of off-flavour by careful choice of conditions of preparation, drying and storage to minimize heat damage and browning. Conditions were established under which milk protein could be expected to have flavour stability during at least 6 months storage at ambient conditions.

Casein is one of the most nutritionally complete proteins as well as being one of the cheapest animal proteins. It has a wide potential use in foodstuffs owing to this high nutritional value and also to its emulsifying and stabilizing properties. Casein is used, for instance, in hamburger and sausage meat, breakfast cereals, dessert mixes and toppings, coffee whiteners, concentrated baby and invalid foods, and in low calorie diets. Unfortunately, such uses are restricted at present by an unpleasant gluey or 'stale' off-flavour and the major consumption of casein is not in the food industry but in paper coating, in the preparation of adhesives, and in the production of artificial fibres.

Cayen & Baker (1963) showed that a fairly tasteless sodium caseinate could be prepared from a well-washed freshly precipitated wet curd by freeze-drying or tunnel-drying. In industrial practice, fresh casein is usually bland in flavour but acquires the gluey off-flavour on storage. Several patented processes claim improved flavour characteristics, including the preparation of calcium caseinate following a hot-water wash stage (Srinivasan, 1961), partial enzymic degradation (Wingerd, 1962) or oxidation by iron and ascorbic acid (Wingerd, Bauer & Damisch, 1962). These processes are effective to some degree on the laboratory scale but none has been accepted commercially—presumably because they are time-consuming or expensive or both.

Although there are few references in the literature to gluey off-flavour in casein, several authors have recognized a similar flavour—'stale', 'gluey' or 'burnt feathers'—in dried skim-milk powder (cf. Henry, Kon, Lea & White, 1948) and in dried whole-milk powder (Supplee, 1923; Tillmans & Strohecker, 1924; Coulter, Jenness & Geddes, 1951). This off-flavour was believed to be associated with protein breakdown, especially when it was associated with storage at high moisture levels. Coulter *et al.*

(1951) concluded that the typical off-flavour of dried whole-milk powder was a composite of 2 distinct flavours one of which arose from oxidation of lipids and the other from the non-enzymic browning reaction; in high moisture conditions a burnt feather (gluey) flavour predominated. Radema (1954) found that tallowy flavours in stored whole-milk powder were associated with low moisture and high peroxide values whereas gluey flavours were correlated with high moisture, low peroxide value and high ferricyanide reducing power.

Buchanan, Snow & Hayes (1965) showed that casein and whey proteins could be co-precipitated by heat in the presence of added calcium ions, and Muller, Hayes & Snow (1967) prepared co-precipitates of varying calcium content (high 2·5–3·0 % Ca, medium about 1·5 % Ca, low 0·5–0·8 % Ca). Early consumer trials using high calcium co-precipitate suggested that it may have better flavour stability than casein. Laboratory ageing trials on casein and the 3 types of co-precipitate, all obtained from different factories, were inconclusive and differences could be explained most readily by variation in lactose content or heat damage by varying factory procedures.

For the present study, casein and co-precipitates were prepared on a pilot-scale under as nearly identical conditions as possible, and their flavour stability examined using accelerated ageing trials and evaluated as a function of lactose content, calcium content and acidity (all of which may influence the browning reaction) and of colour development and ferricyanide reducing value, which are measures of the extent of the browning reaction (Part 1). A further examination was then made of the effect of processing conditions on the reducing value and flavour of co-precipitated milk proteins (Part II). Finally, methods of removing gluey off-flavour from casein and for preparing casein of stable flavour were evaluated.

PART I. COMPARISON OF THE FLAVOUR STABILITY OF CASEIN AND CO-PRECIPITATED MILK PROTEIN

Experimental

Casein and co-precipitate. These were prepared using the plant and methods described by Muller *et al.* (1967) and dried in a fluidized bed using hot air at an inlet temperature of 50 or 70 °C.

Lactose. This was determined by the method of Lawrence (1968).

Calcium content. This was determined by the method of Sawyer & Hayes (1961).

Colour differences. These were determined by measuring colour intensity before and after heating for 5 h at 102 °C. An 'Agtron' model 'F22 Green' (Agtron CB, Monterey, California, U.S.A.) was used to compare reflectance of the sample in the 546 nm region with that of black and white standards and express it on a 0–100 scale.

Ferricyanide reducing values (FRV). The test protein (0·5 g) was made up to 30 ml with distilled water, 3 ml 5 % (v/w) acetic acid added and the procedure of Choi, Koncus, Cherrey & Remaley (1953) used to determine the FRV expressed in mg potassium ferricyanide/g protein.

Accelerated storage test. The sample was heated in an open container in an air oven at 80 °C saturated with water vapour, stored for 24 h in a closed container under refrigeration, then evaluated organoleptically at room temperature.

Organoleptic evaluation. One per cent solutions at pH 7 were prepared by dissolving the samples in water by addition of NaOH, and adding about 1% sodium tripolyphosphate in the case of the calcium co-precipitates. A laboratory panel of 8–10 judges scored the flavour of solutions using a scale 0–3 (0, clean flavour; 1, just detectable gluey flavour; 2, distinct gluey flavour; 3, strong gluey flavour). Two labelled controls (clean and gluey) were available and were also presented unlabelled with the samples. The results were evaluated by calculating the least significant difference at the 5% level. Samples were rated gluey if their score exceeded the score of the unlabelled control by this difference.

Results

Samples of the 4 types of milk protein (casein, high calcium co-precipitate, medium calcium co-precipitate and low calcium co-precipitate) were prepared from the same milk with 2 levels of washing and dried with hot air at 2 inlet temperatures. The flavour stability of each sample was examined using the accelerated storage test, and assessed in relation to the differing experimental variables and analytical data. The results are shown in Table 1.

Table 1. *Analytical data and flavour stability in milk protein*

Sample no.	Type	No. of washes	Drying temp., °C	Ca^{2+} content, mg/g	Lactose content, %	Colour difference, 5 h at 102 °C	Flavour after heating at 80° C for 0 h	20 h	40 h	FRV, mg/g
362 E	Casein	3	50	0·62	0·22	4·1	N	N	N	0·51
F	Casein	3	70	0·59	0·14	4·1	N	N	N	0·54
G	Casein	5	50	0·24	0·06	2·1	N	N	N	0·47
H	Casein	5	70	0·30	0·07	1·4	N	N	N	0·50
361 A	Hi Ca	3	50	28·5	0·85	11·3	N	G	G	0·71
B	Hi Ca	3	70	29·0	0·61	9·7	N	G	G	0·71
C	Hi Ca	5	50	26·6	0·07	4·4	N	N	N	0·33
D	Hi Ca	5	70	26·5	0·04	3·1	N	N	N	0·30
362 A	Med Ca	3	50	15·6	0·85	12·9	N	G	G	1·14
B	Med Ca	3	70	15·6	0·85	13·7	N	G	G	0·95
C	Med Ca	5	50	13·0	0·27	7·3	N	N	G	0·89
D	Med Ca	5	70	13·1	0·16	6·4	N	N	G	0·89
361 E	Lo Ca	3	50	3·8	0·59	13·1	N	G	G	1·19
F	Lo Ca	3	70	4·6	0·62	12·4	N	G	G	1·25
G	Lo Ca	5	50	2·9	0·22	7·5	N	G	G	0·99
H	Lo Ca	5	70	2·6	0·16	7·5	N	N	G	0·96

N = not gluey; G = gluey.

Effect of drying temperature. Comparison of pairs of samples dried at different temperatures showed no difference in flavour. However, it must be remembered that these drying conditions (fluidized bed with inlet air at 50 or 70 °C) were not as severe as those encountered in normal commercial practice.

Effect of Ca content. Simon, Wagner, Silveira & Hendel (1955) reported that calcium at a level of 8·4 mg/g inhibits the browning of dried potato flakes. There was little evidence of any effect of Ca content on flavour stability (Table 1), but some slight suggestion (cf. 361 A *vs.* 362 B; 361 B *vs.* 361 E or 361 F) that, at similar lactose levels, a higher calcium level allowed less colour development on heating. Several further samples

were prepared by addition of aqueous $CaCl_2$ and redrying but none showed significant variation in flavour stability.

Effect of acidity. The pH value of a 10% aqueous slurry of each of the 4 samples of each type of dried protein was constant. The values were: casein, 4·15; high calcium co-precipitate (Hi Ca), 6·60; medium calcium co-precipitate (Med Ca), 5·90; low calcium co-precipitate (Lo Ca), 5·20, but there was no obvious correlation between acidity and flavour stability.

Table 2. *Lactose level and flavour stability in milk protein*

Sample no.	Type	Lactose, %	Colour difference, 5 h at 102 °C.	Flavour after heating at 80 °C for: 0	20 h	40 h	FRV, mg/g
362 H 1	Casein	0·084	4·2	N	N	N	0·41
2	Casein	0·161	5·5	N	N	N	0·36
4	Casein	0·314	9·0	N	N	G	0·39
10	Casein	0·880	17·4	N	N	G	0·41
361 D 1	Hi Ca	0·110	3·6	N	N	N	0·33
2	Hi Ca	0·204	5·9	N	N	N	0·33
4	Hi Ca	0·423	8·9	N	N	G	0·36
10	Hi Ca	0·850	15·9	N	N	G	0·36

Effect of lactose. Table 1 shows that the best flavour stability of the milk protein was associated with the lower lactose levels. There were apparent differences between the different types of milk protein—thus casein with 0·22% lactose was stable, whereas Med Ca and Lo Ca with 0·22% lactose became gluey—but in all instances the samples of lower lactose content were more stable than those of higher. To investigate this relation further, varying amounts of an aqueous solution of lactose were added to casein (362H) and Hi Ca (361D) and the slurry redried. The flavour stability and analysis of these samples is shown in Table 2. Once again, lower lactose levels correlate with better flavour stability and, both for casein and Hi Ca, lactose levels below about 0·3% gave high flavour stability.

Correlation of colour and flavour stability. The relation between flavour stability and lactose level suggests implication of the browning reaction. If this is so, there may be a relation between colour development (which must be a measure of the amount of browning) and flavour development. It was decided to measure colour differences before and after heating for 5 h at 102 °C as this is the test used in commercial practice but, in fact, the colour difference was of the same order as that produced by 40 h at 80 °C. Tables 1 and 2 show that the correlation between colour difference and flavour stability was slightly better than that between lactose and flavour stability. It is apparent that the best flavour stability was obtained when the colour difference was below 6 units. The anomaly noted above for casein and Med Ca or Lo Ca of 0·22% lactose can be explained as the latter 2 samples had a higher colour difference, indicating that they browned more on heating.

Correlation of ferricyanide reducing value (FRV) and flavour stability. It is apparent from the results so far reported that the browning reaction plays a significant part in the formation of gluey flavour, and some measure of its extent was needed. Although the exact mechanism of the reaction is not known, FRV has been accepted

as an index of the formation and degradation of a protein-sugar complex in milk powder (Lea, 1947). Richards (1963) suggested that the reducing substances are the L-amino-1-deoxy-2-ketones formed as intermediates in the browning reaction. Table 1 shows that gluey off-flavour development was associated with high FRV and indicates that a value below 0·60 mg/g is necessary for good flavour stability. The high FRV for Med Ca or Lo Ca at low lactose level is indicative of incipient browning, presumably initiated during their preparation. The values of samples listed in Table 2 did not change with added lactose and were close to the values for untreated samples 361D and 362H.

Discussion

Development of the accelerated storage test. In initial attempts to develop an accelerated storage test, heating or irradiation with UV light were used. Gluey flavours were induced by both treatments but a more typical gluey flavour was produced by heating; UV irradiation produced a flavour described as gluey but also as burnt, unpleasant, or dirty. In prolonged heat treatment the protein tended to become insoluble but this insolubility could be reduced by saturating the oven with water vapour. There was little difference in flavour between samples treated in open or closed containers or under wet or dry conditions provided the initial moisture content was not above 10 %. If the moisture content was above 10 % unpleasant, dirty, burnt flavours and excessive browning were produced on heating in a closed container.

Cayen & Baker (1963) presented 10 % solutions of sodium caseinate at 40–50 °C to their panel of 3 judges. In the present investigation, a panel of 8–10 judges with experience of organoleptic testing was used. They found 10 % too strong and detected no significant differences between 1 % solutions presented at 20, 30 or 40 °C. Thus, 1 % solutions were presented at room temperature.

Limited experience suggests that samples which remained clean in flavour after 40 h accelerated storage would remain of good flavour for at least 6 months under ambient conditions. This corresponds to an increase in reaction rate of about 3-fold for a rise in temperature of 10 °C which is in good agreement with the figure of 2·5 found by Henry *et al.* (1948) for stored whole-milk powder.

Mechanism of off-flavour development. The best milk protein flavour stability was associated with a low lactose level, low colour development on heating and low production of ferricyanide reducing substances. These facts all support the theory that gluey off-flavour is developed during the early stages of the non-enzymic browning reaction between the milk sugar and the milk protein. Removal of most of the lactose —one of the reactants—reduces the extent of the browning reaction but it is not always sufficient to prevent it altogether. Samples of Med Ca and Lo Ca of low lactose content exhibited higher colour development and higher FRV owing to initiation of browning during their preparation, which included 30 min preheating at 90 °C, a treatment known to increase FRV and initiate the protein–lactose reaction (Choi *et al.* 1953; Kumetat & Beeby, 1957).

PART II. THE EFFECT OF PROCESSING CONDITIONS ON THE FERRICYANIDE REDUCING VALUE AND ON THE FLAVOUR OF CO-PRECIPITATED MILK PROTEINS

Processing may involve severe heating which could promote the early stages of the lactose–protein interactions. Part II describes an investigation of the effect of varying the initial preheating time, and 3 different methods of drying co-precipitates that had been washed for 3 different periods of time and therefore contained different levels of lactose.

Experimental

Co-precipitated milk proteins were prepared with the plant and by the general method of Muller *et al.* (1967). Lo Ca was prepared from milk heated for 10 or 30 min at 90 °C and Hi Ca was prepared from milk pre-heated for 1 or 30 min at 90 °C. In each case, samples were taken after 1, 3 and 5 washes and freeze-dried or dried in a fluidized bed with inlet air at 70 °C and cooled immediately or kept at 50 °C for 2 h.

Results

The analytical data obtained for the 4 samples of co-precipitate are compared with their flavour stability in Table 3.

Table 3. *Analytical data and flavour stability of milk protein*

Method of drying	Lactose, %	Colour difference	FRV, mg/g	Flavour	Lactose, %	Colour difference, 5 h at 102 °C	FRV, mg/g	Flavour
	Low calcium, 10-min preheat				Low calcium, 30-min preheat			
F	1·89	23·1	1·27	G	1·59	12·7	1·62	G
C	1·96	20·9	1·36	G	1·63	20·3	1·89	G
H	1·59	26·1	1·86	G	1·46	24·7	1·86	G
F	0·404	7·0	0·95	sl	0·336	4·9	1·40	sl
C	0·406	8·4	1·18	G	0·300	11·1	1·48	G
H	0·373	10·2	1·01	G	0·234	8·3	1·63	G
F	0·042	2·2	0·78	N	0·333	4·5	1·29	sl
C	0·043	3·8	1·09	N	0·075	8·4	1·63	G
H	0·062	3·3	1·09	sl	0·048	5·1	1·72	G
	High calcium, 1-min preheat				High calcium, 30-min preheat			
F	2·15	12·3	0·83	G	2·41	32·9	1·80	G
C	2·05	24·4	1·27	G	1·96	22·2	1·78	G
H	1·71	17·3	2·96	G	2·02	30·0	3·00+	G
F	0·124	4·2	0·53	sl	0·058	4·3	1·09	G
C	0·121	2·4	0·80	N	0·048	4·6	1·86	G
H	0·102	2·6	0·65	N	0·040	8·9	1·86	G
F	0·230	4·0	0·67	sl	0·051	4·0	1·12	N
C	0·052	2·7	0·59	N	0·046	4·9	1·72	G
H	0·038	2·7	0·47	N	0·045	5·5	2·07	N

F = freeze-dried; C = fluidized-dried and cooled; H = fluidized-dried and stored hot; G = gluey; sl = slightly gluey; N = not gluey.

Effect of drying conditions. There was no clear correlation between drying conditions and lactose level, colour difference or flavour stability. However, in most series, FRV increased with increasing severity of drying conditions.

Effect of lactose level. There was a general trend towards lower colour difference,

lower FRV and better flavour stability as the lactose level was decreased. Samples with less than 0·3 % lactose did not develop gluey flavour after 40 h accelerated storage at 80 °C unless they had been subjected to heat damage in the preheating.

Effect of preheating conditions. Although it is difficult to obtain a clear picture, there was a general trend to higher colour differences with longer preheating times. FRVs are a good indication of damage in preheating and in all cases the FRV increased with preheating time. When samples which should differ only in preheating treatment are compared, the best flavour stability was always associated with the shortest preheating time. Excellent examples of the effect of the incipient browning caused by preheating are the Hi Ca, 30 min preheat samples of lactose content 0·058, 0·048 and 0·040 %. Although these have satisfactorily low lactose level, their colour difference, FRV and rate of gluey off-flavour development were all high when compared with similar Hi Ca samples that had been preheated for only 1 min, even with considerably higher lactose levels.

Discussion

Owing to the complex nature of the flavour and the difficulties associated with the human element in organoleptic evaluation, no single chemical or physical test can be expected to correlate accurately with flavour development. Nevertheless, the FRV provides an excellent index of the amount of heat damage to the protein caused by processing conditions. A ferricyanide reducing value less than 0·60 mg/g protein combined with a colour difference of less than 6 units and a lactose content below 0·3 % ensure a protein of good flavour stability. The results of this study confirm the hypothesis advanced in Part I that the development of gluey off-flavour is associated with the early stages of the non-enzymic browning reaction: samples of the best flavour were obtained when processing conditions were chosen to minimize browning.

PART III. REMOVAL OF GLUEY OFF-FLAVOUR FROM CASEIN AND PREPARATION OF CASEIN OF STABLE FLAVOUR

As commercially produced, casein is not a pure protein but contains minor quantities of other compounds including up to 2 % lactose and up to 2 % fat. Cayen & Baker (1963) showed that casein of good flavour could be produced from a well-washed curd and it is possible that the minor constituents play a major role in the formation of gluey off-flavour. The practical problems caused by gluey flavour may be overcome in 2 ways—either by treatment designed to remove the off-flavour already present in stored casein or by preparation of a flavour-stable casein of acceptable shelf-life. Although the latter solution would obviously be the more efficient, many products would allow a treatment stage to be incorporated in their formulation—for example, if the raw material, casein, is converted to sodium caseinate before mixing with other ingredients.

Part III describes a study of flavour stability of caseins prepared with low fat and with low lactose content and records the effect of some selected treatments on the flavour and flavour stability of aged casein.

Experimental

Organoleptic evaluation. Accelerated storage trials were performed and evaluated as described in Part I. To compare the effect of treatments on flavour, judges were presented with a geometric concentration series (e.g. 0·1, 0·32, 1·0%, etc.) and the threshold concentration estimated as that concentration at which more than half the panel could perceive the gluey off-flavour.

Preparation of casein of low lactose content. Casein was precipitated in the normal fashion and given 3 additional washes before freeze-drying. A control 'lactose' casein was prepared without these additional washes.

Preparation of casein of low fat content. Four litres of skim-milk were centrifuged at 10000*g* for 15 min at 30 °C followed by 15 min at 5 °C and the upper layer of solid fat removed. To the milk at 35 °C was added HCl to pH 4·5. The precipitate was filtered and washed with 3 separate portions of 1 l distilled water acidified to about pH 4·5. The damp precipitate was soaked for 1 h in 500 ml freshly distilled ethanol, filtered and dried under vacuum, then washed with 3 separate portions of freshly distilled diethyl ether, filtered and again dried under vacuum. The casein was dissolved in water by addition of NaOH to pH 7, reprecipitated, washed 3 times and freeze-dried. A control sample of 'fat' casein was prepared in a similar manner but omitting only the initial centrifugation and solvent washing stages.

Treatment of gluey casein. The following treatments were investigated:

(i) *Vacuum distillation.* The casein was placed in a round-bottom flask connected to a vacuum line through a trap immersed in liquid nitrogen. The flask was heated in a water bath at 50–70 °C and evacuated at 5×10^{-5} Torr, for 4 h.

(ii) *Steam distillation.* In initial experiments, a 3% solution of sodium caseinate was distilled almost to dryness in a rotary evaporator at 15 Torr and 25 °C. The distillate was condensed at 0 °C and reconstituted with fresh freeze-dried sodium caseinate while the viscous residue was reconstituted with distilled water. In later experiments, the casein solution or slurry was distilled under vacuum through a vertical condenser at 0 °C into a liquid nitrogen trap using essentially the method of Forss, Jacobsen & Ramshaw (1967).

Attempts were also made in this manner to remove the flavour by freeze-drying from 1, 5 and 10% solutions of gluey sodium caseinate.

(iii) *Reprecipitation.* Gluey casein was dissolved to 1% in water by addition of NaOH to pH 7, and then reprecipitated with H_2SO_4, washed, filtered, and freeze-dried.

(iv) *Hot-water washing.* Gluey casein was stirred into distilled water at 60–65 °C (about 2 g water/g casein), held for 5 min and then filtered. This washing was repeated 4 times and the final residue freeze-dried.

(v) *Treatment with activated carbon.* 500 ml 3% gluey sodium caseinate was passed through a 5 in. × 1 in. column of activated carbon (E. Merck, AG, for gas chromatography, 0·5–0·75 mm particles). The eluant was evaluated as such and also precipitated, washed, filtered and freeze-dried as casein.

(vi) *Treatment with Sephadex.* Portions of 150 ml 5% gluey sodium caseinate were passed through a 12 in. × 2·5 in. column containing 500 g Sephadex G25; the eluant was evaluated as such or precipitated, washed, filtered and freeze-dried as casein.

Results

The concentrations at which more than half the panel could perceive the gluey flavour (threshold concentations) of treated stored casein are shown in Table 4.

The effectiveness of treatments

Vacuum distillation or freeze-drying of gluey casein had little effect on its flavour; the threshold concentration remained at 0·3%. It is noteworthy that the vacuum treatment almost entirely removed the gluey odour of the casein and this odour could be detected in the adjacent cold trap. Reprecipitation effected some improvement, but the casein was still noticeably gluey in flavour.

Table 4. *Threshold concentration of gluey flavour in treated and untreated casein*

Material and/or treatment	*Threshold concentration, %	Remarks
Gluey casein	0·3	Control
Gluey sodium caseinate	0·3	Control
Fresh freeze-dried casein or sodium caseinate	> 3·0	Control
Vacuum-treated casein or caseinate	0·3	Flavour not removed
Steam-distilled sodium caseinate	> 1·0	Flavour partially removed
Distillate from sodium caseinate	1·0	Distillate gluey
Freeze-dried sodium caseinate (1, 5, 10%)	0·3	Flavour not removed
Reprecipitated casein	1·0	Flavour partially removed
Filtrate from reprecipitation	1·0	Filtrate gluey
Washed casein	> 1·0	Flavour partially removed
Wash water	1·0	Wash water gluey
Activated carbon-treated casein	> 3·0	Flavour removed
Sephadex G 25-treated casein	> 3·0	Flavour removed

* Concentration at which gluey flavour was first detectable.

In steam-distillation experiments, the threshold concentration of the residue rose from 0·3 to 1·0–3·0% and the solutions were only slightly gluey. Moreover, when the distillate was reconstituted to 1% protein, with fresh sodium caseinate, it was markedly gluey. A similar improvement in flavour could be achieved by hot water washes and the wash water became gluey.

The most effective removal of gluey flavour was obtained by passing a solution of sodium caseinate through a column of activated carbon or Sephadex. In both cases the eluted protein was organoleptically identical with a solution prepared from fresh freeze-dried sodium caseinate.

Accelerated storage of casein preparations

Preparations of casein low in fat or in lactose were tested in the accelerated storage trial together with samples of casein prepared by those of the treatments which showed good removal of off-flavour. The results are shown in Table 5.

Effect of fat removal. Although the low fat casein was considerably less susceptible than commercial casein to the development of gluey flavour, there was little, if any,

difference between the low fat casein and its control. It seems likely that the presence or absence of fat plays only a minor role in off-flavour development and that the apparent improvement was due to removal of some other component, probably lactose, during preparation.

The effect of lactose removal. Well-washed casein of low lactose content did not become gluey after heating for 80 h at 80 °C. Although this effect of washing might have been due to the removal of some other minor constituent, re-addition of lactose to a stable sample of casein has been shown (Part I) to increase its susceptibility to the development of gluey flavour.

Effect of treatments. Washing with hot water and treatment with carbon or Sephadex all gave a product of good flavour, but the treated caseins varied in their susceptibility to redevelopment of off-flavour. The Sephadex-treated casein was best—almost as good as the best casein preparation—while the carbon-treated casein was not quite so good and the washed casein was quite susceptible to redevelopment of gluey flavour.

Table 5. *Accelerated storage tests on casein preparations*

Preparation, Casein	Lactose, %	Flavour stability, heating at 80 °C
Fresh commercial	0·450	Gluey at 20 h
Fat extracted	0·061	Not gluey at 100 h
Non-extracted (control)	0·186	Not gluey at 100 h
Low lactose	0·067	Not gluey at 80 h
Washed	0·037	Gluey at 20 h
Activated carbon treated	0·051	Gluey at 40 h
Sephadex treated	0·026	Not gluey at 60 h

Discussion

The effects of treatment on the flavour and flavour stability of casein support the hypothesis that gluey off-flavour develops during the early stages of the non-enzymic browning reaction. It is clear that stored, stale casein has 2 sources of gluey flavour—lower molecular weight breakdown products of the browning reaction already present and their precursors, presumably of much higher molecular weight. Treatments, such as washing or steam distillation, effect a temporary improvement in flavour of the stored casein by removing the breakdown products but the precursors are still available and flavour stability is poor. On the other hand, carbon or Sephadex treatment remove much of the precursor as well as the breakdown products and improve flavour stability as well as the immediate flavour. However, both treatments suffer practical disadvantages—carbon treatment must be carefully performed to avoid contamination with minute carbon particles which are flavourless but not aesthetically pleasing, while Sephadex treatment dilutes the solution by a factor of about 4.

CONCLUSION

Practical applications. While methods are available for removing gluey off-flavour from stored casein and improving its flavour stability, the best remedy is to minimize the development of the off-flavours by careful preparation of the milk protein. Gluey off-flavour development can be minimized only if strenuous efforts are made to avoid

heat damage and possible browning at all stages. Processing should involve only low temperature preheat treatment, careful washing to low lactose level, drying at minimum temperature and cooling before storage, careful grinding to avoid high temperatures and cool storage at low moisture levels. Although the lowest obtainable lactose level, colour development and reducing power are desirable, a milk protein of lactose content below 0·3 %, colour difference less than 6 Agtron units and FRV below 0·60 mg/g can be expected to remain of good flavour for at least 6 months in normal shelf storage.

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Volatile compounds associated with the off-flavour in stored casein

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Summary. Compounds associated with the 'gluey' off-flavour in stored casein have been isolated and identified by a combination of gas chromatography and mass spectrometry. Most of the flavour was found in the non-acid steam-volatile fraction which contained many compounds including n-alkanals, n-alkan-2-ones, furfural and other furan derivatives, benzaldehyde, dimethyl disulphide and benzthiazole. No single compound had the typical gluey flavour, which must be a complex sensation in which the quantities of minor components may determine the specific gluey character. o-Aminoacetophenone was present in the casein in subthreshold amounts. This compound has some gluey flavour and may have a synergistic effect in enhancing the total flavour.

The wide use of casein as a food protein has been inhibited by an unpleasant stale off-flavour which is characteristic of the product. This flavour has been described as 'stale', 'gluey' or 'burnt feathers'. A similar stale flavour has been observed amongst the flavours developed on storage by other milk products such as dried skim-milk (Henry, Kon, Lea & White, 1948), dried whole milk (Whitney & Tracy, 1949) and sterilized concentrated milk (Arnold, Libbey & Day, 1966).

Investigations of the gluey off-flavour in stored casein have not been reported but in some other milk products the milk protein seems to contribute a similar storage off-flavour. Nawar, Lombard, Dall, Ganguly & Whitney (1963) and Kurtz (1965) found evidence that the stale flavour in whole-milk powder had 2 elements—one volatile in vacuum distillation and the other non-volatile and tightly bound to the milk powder. Parks & Patton (1961) identified a range of volatile carbonyl compounds including n-alkanals (about 1 ppm), n-alkanones, furfural and benzaldehyde in stale whole-milk powder and Bassette & Keeney (1960) isolated a similar range of carbonyl compounds from stored skim-milk powder including alkanals at a level of 5 parts in 10^8. Shtal'berg & Kretovich (1953) found 50 ppm aldehydes in dried milk which exhibited a darker colour and an unpleasant gluey flavour. These groups of workers all concluded that stale flavour was due largely to the increasing quantity of carbonyl compounds—particularly aldehydes—formed during storage of the milk powder. Parks, Schwartz & Keeney (1964) isolated a less volatile compound, o-aminoacetophenone, from skim-milk powder and showed that it was an important component of the stale flavour.

In the present investigation the compounds formed in gluey, stored casein have

been isolated and identified. The mode of their formation and their relevance to the flavour defect is discussed.

METHODS

Samples of commercial casein and sodium caseinate were examined fresh and after storage for at least 6 months under ambient conditions.

Isolation of flavour components

Preliminary experiments revealed that most of the off-flavour was not removed by high vacuum molecular distillation of 'dry' casein (as received, containing about 10 % moisture). Most of the flavour could be removed by washing with hot water or by steam distillation; the treated casein became less gluey and the wash water or distillate became gluey. Consequently, 3 methods of isolation were evolved.

Method A—hot washing. Casein was washed 3–5 times with about twice its weight of distilled water at 60 °C and the combined wash water was distilled under reduced pressure. The distillate was adjusted to pH 9 and extracted with diethyl ether in a separating funnel (non-acid extract) and the aqueous layer acidified to pH 2 and re-extracted (acid extract). The 2 ether extracts were concentrated separately by distillation to small volume using a short fractionating column.

Method B—steam distillation. Five hundred grams of casein was steam-distilled with 3 l distilled water at a temperature < 25 °C and pressure of 6–10 Torr. The distillate was concentrated by reflux through a vertical condenser at 0 °C and about 6 Torr into a liquid nitrogen trap using a method very similar to that of Forss, Jacobsen & Ramshaw (1967). Frequently the steam distillation and reflux concentration were combined in a single apparatus. The reflux concentrate and the residual distillate were extracted at pH 9 and again at pH 2, as described for method A above.

Method C—reflux concentration. The volatile components were concentrated from a solution of sodium caseinate at pH 7 by distillation under reflux through a vertical condenser at 0 °C and about 6 Torr into a liquid nitrogen trap. A non-acid extract was prepared as in method A. Negligible quantities of acid extract were obtained in this procedure.

Identification of components

Portions of the concentrated solvent extract were separated by gas chromatography using several columns with different stationary phases.

Column 1. A stainless steel column (4 ft × ¼ in. o.d.) packed with 7·0 g of 60–80 mesh Embacel coated with 15 % diethylene glycol succinate plus 2 % phosphoric acid. This was used for the acid fraction and operated at a constant temperature of 100 or 125 °C or was programmed from 50 to 150 °C at 2 deg C/min with the carrier gas flow rate 20 ml/min of helium.

Column 2. A stainless steel column (10 ft × ⅛ in. o.d.) packed with 7·3 g of 60–80 mesh Chromosorb G-AW-DMCS coated with 1 % Carbowax 20 M-TPA. This column was programmed from 50 to 150 °C at 2 deg C/min and carrier flow rate 10 ml/min of helium.

Column 3. A stainless steel support-coated open tubular (SCOT) column (50 ft × 0·02 in. o.d.) coated with Apiezon L. The column was supplied by Perkin-Elmer

Helium flow rate was 8 ml/min and the column temperature was programmed from 40 to 180 °C at 2 deg C/min.

Column 4. A SCOT column (50 ft × 0·02 in. i.d.) as above coated with Carbowax 1540. Helium flow rate was 8 ml/min and column temperature was programmed from 40 to 150 °C at 2 deg C/min.

Any of the columns could be operated in the combined gas chromatography–mass spectrometry (GC/MS) system described by Stark, Smith & Forss (1967). For further resolution of components, fractions trapped from column 2 were rechromatographed in the GC/MS combination using columns 3 or 4. Identification of components was made by comparison of their mass spectral fragmentation pattern with published spectra (Cornu & Massot, 1966) and spectra of authentic compounds. Correspondence of retention data on column 3 and MS data for an isolated component and a known compound was considered sufficient for identification. In most cases, retention data on a different stationary phase and odour appraisal were also available for confirmation of identity.

Flavour tests

All stages of the isolation and identification procedure were monitored by flavour tests in an attempt to assess the importance of various fractions in the gluey flavour. Fresh bland sodium caseinate was added to samples of the distillates, residues, and trapped fractions, and to authentic compounds, singly or admixed to make solutions containing 1 % sodium caseinate. The flavour of each solution was assessed by a laboratory panel of about 10 persons selected because of their experience in organoleptic evaluation of dairy products.

RESULTS

Evaluation of isolation techniques

For the isolation of flavour components by steam distillation (method B) slurries were prepared from distilled water and 30-mesh casein (1), with the addition of NaOH (2), by mixing with a Waring Blendor (3), and by passage through a colloid mill (4). Each slurry was steam distilled and the distillate extracted with ether and analysed by gas chromatography. All the slurries gave qualitatively similar non-acid extracts but the best qualitative recovery was obtained—particularly for the more volatile components—by distilling slurry (1) prepared from casein as received. Reflux concentration from a solution of sodium caseinate at pH 7 was evaluated at concentrations of 1, 5 and 10 %. The best quantitative recovery (per g of caseinate) was obtained from the most dilute solution, but larger quantities of components could be obtained most conveniently from the 5 % solution. Comparison of methods A, B and C for isolation of the more volatile fraction showed that reflux concentration (method C) was slightly better than 2-stage steam distillation (method B), while hot washing (method A) was very inefficient. On the other hand, isolation of the less volatile fraction or the acid fraction was best accomplished by hot washing (method A).

Flavour

Samples of the wash water, distillate and distillation residue were examined organoleptically. It was found that the gluey flavour was distillable—the distillate

was gluey while the residue was not significantly gluey. Further reflux concentration showed that most of the flavour resides in the more volatile fraction.

Separation into acid and non-acid fractions revealed that the gluey flavour was in the non-acid fraction—indeed, if anything, the non-acid fraction was more gluey than the total extract. Reaction with 2,4-dinitrophenylhydrazine in H_2SO_4 removed about 80% of the carbonyl compounds but there was a less marked effect on the gluey odour. There was some reduction in intensity but individual judges varied in their comments from 'little effect' to 'almost complete removal'. Odour appraisal of the effluent gases from an exit splitter during the gas chromatographic separation showed that no single peak or part of the chromatogram was associated with the typical gluey odour although odours were numerous and varied. Similarly, on examination of trapped broad fractions, none was found to have the typical gluey flavour, which was present only when fractions from all regions of the chromatogram were represented.

It was concluded that the main gluey flavour resides in the more volatile fraction and that no single compound or class of compound is typically gluey. Gluey flavour must be a complex sensation resulting from a mixture of several different compounds.

Identification of components of the acid fraction and their relevance to gluey flavour

Components of the acid fraction, prepared by method A, were separated by gas chromatography on column 1. Mass spectrometry and infra-red spectroscopy proved that all components were straight chain alkanoic acids. The approximate composition of the acid fraction is shown in Table 1. In fresh casein the total quantity of acids was negligible but on storage it increased to about 20 μg/g.

Table 1. *Composition of the acid extract* from stored casein*

Alkanoic acid, no. of carbons ...	2	3	4	5	6	7	8	9	10	11	12
% in extract	1	1	2	0·5	43	0·5	35	0	14	3	0
Approximate level in casein, ppm	0·2	0·2	0·4	0·1	8·6	0·1	7·0	0	2·8	0·6	0

* See p. 216.

After removal of acids the extract was still gluey, as was the reflux concentrate prepared by method C, which contained negligible quantities of acids, which were relatively non-volatile under these conditions. The flavour of the lower alkanoic acids is well-known as a sharp, bitter, rancid-fat taste with a threshold in water of the order of 5 ppm (Patton, 1964). These properties suggest that the acids have little relevance to gluey flavour although they are major components of the casein extract and several are present in amounts close to their flavour threshold values.

Identification of components of the volatile non-acid extract and their relevance to gluey flavour

Portions of the concentrated volatile non-acid extract prepared by method C were analysed by combined GC/MS. A typical chromatogram is shown in Fig. 1 and the

identity and approximate composition of the mixture (estimated from the gas chromatogram) is given in Table 2. In addition, the presence of trace amounts of other compounds such as methyl butanal, alk-1-en-3-ones, oct-1-en-3-ol, nona-2,6-dienal, nona-2,6-dienol and higher homologues of furfural and furyl ketones could be deduced from the appearance of their characteristic odours at the relevant retention time. However, insufficient quantities were available for positive identification. The total quantity of compounds was about 5 μg/g for stored casein while fresh casein yielded only very much smaller amounts of a similar range of compounds.

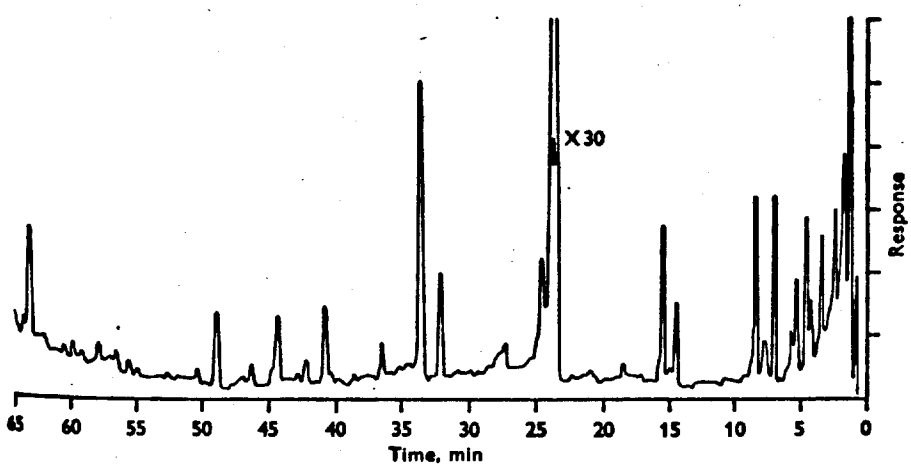

Fig. 1. Gas chromatogram of the steam-volatile non-acid fraction equivalent to approximately 40 g gluey sodium caseinate. Operating conditions described for column 3, p. 216.

The hydrocarbons and alkanols are unlikely to be of significance in the gluey flavour as both classes have weak flavours and relatively high flavour thresholds. The only components isolated at concentrations near or above their threshold concentrations (of the order of 0·005 ppm (Lea & Swoboda, 1958)) were the n-alkanals. These are well-reported components of oxidized and stale flavours, especially in systems containing lipids. They may be expected to have some significance in the overall gluey flavour even though their flavour character is not specifically gluey. The alkan-2-ones are also components of oxidized and stale flavours in lipids and could be important in gluey flavour but they have generally higher threshold values and were present in smaller amounts than the n-alkanals.

In organoleptic evaluation, benzaldehyde retained its almond, nutty flavour right down to its threshold and benzthiazole similarly retained its rubbery flavour. However, some judges thought furfural, methyl furfural and methyl furyl ketone were slightly gluey although most described them as weedy or bitter. All were isolated below their threshold value, even allowing for incomplete recovery, but they may well have had some additive effect on the flavour.

It was concluded that the flavour of the non-acid volatile fraction is due mainly to the n-alkanals with some contribution from the furan derivatives. Many of the minor components are strongly flavoured (e.g. diacetyl, dimethyl disulphide, methional,

phenylacetaldehyde) and could play some part by contributing to the specific gluey character of the flavour. Their importance cannot be assessed without laborious organoleptic evaluation of multi-component mixtures.

Table 2. *Composition of the volatile non-acid extract* from stored casein*

Compound	Retention time, min		MS	Odour confirmation	% in extract
	Ap L	C 1540			
Ethyl acetate	2·4	2·0	+	+	1·5
2,3-Butadione	2·4	·	+	+	1·5
3-Methyl butanal	4·3	3·2	+	+	1·5
n-Pentanal	4·6	3·5	+	+	1·0
Dimethyl disulphide	6·9	5·5	+	+	4·0
Butanol	·	14·0	+	·	0·5
Pentanol	7·6	18·5	+	·	4·5
Hexan-2-one	7·9	·	+	·	tr
Hexanal	8·3	12·5	+	+	5·5
Toluene	8·6	·	+	·	1·0
Heptan-2-one	14·5	34·2	+	+	1·4
Heptanal	15·0	14·8	+	+	4·2
Furfural	15·0	34·2	+	+	1·4
Hexanol	·	28·8	+	·	2·5
Ethyl benzene	15·0	·	†	·	tr
Dimethyl benzene	16·5	·	†	·	tr
Methional	18·5	·	†	+	tr
Methyl furyl ketone	20·2	37·0	+	·	0·5
Octan-2-one	23·6	23·0	+	+	1·4
Methyl furfural	24·0	·	+	·	1·4
Benzaldehyde	23·8	38·2	+	+	34·5
Octanal	24·6	23·5	+	+	2·1
Benzonitrile	24·8	·	+	·	0·5
Terpene	31·0	·	†	·	tr
Nonan-2-one	32·3	31·0	+	+	2·4
Acetophenone	33·0	46·0	+	·	0·5
Nonanal	33·7	31·3	+	+	8·3
Phenylacetaldehyde	34·7	·	+	+	0·5
Decan-2-one	40·7	·	+	·	1·4
Decanal	42·3	·	+	·	1·0
α-Terpineol	44·2	49·5	+	+	1·2
Naphthalene	46·3	50·3	+	·	1·3
Undecan-2-one	48·9	44·5	+	·	1·4
Undecanal	50·4	·	†	·	0·5
Methylnaphthalene	55·5	·	†	·	tr
Dodecan-2-one	56·5	·	+	·	0·5
Dodecanal	58·0	·	†	·	0·5
Tridecan-2-one	63·5	56·7	+	·	2·5
Tridecanal	65·0	·	†	·	tr
Trimethylnaphthalene	73·0	·	†	·	tr
Benzthiazole	·	63·0	+	+	2·5
Phenol	·	68·2	+	+	1·5

* See p. 216.

† Not positively identified by MS but spectra similar.
+ Positive identification in MS.

Identification of components of the non-volatile non-acid fraction, and their relevance to gluey flavour

During the isolation of flavour components by method B, the residual steam-distillate, after removal of volatiles by reflux concentration, contains the non-volatile fraction. The non-acid non-volatile extract from stored casein was analysed

by combined GC/MS using column 2 and its composition is shown in Table 3. These components increased on storage, from negligible quantities in fresh casein to about 1·5 μg/g in badly deteriorated casein.

Only one of these components, o-aminoacetophenone, was examined organoleptically as Parks *et al.* (1964) found that it was an important constituent in the flavour of stale skim-milk powder. In 1% sodium caseinate solution, it had a threshold of 3-10 parts in 10^9 (i.e. 0·3–1·0 ppm on the casein) and the panel were divided in their opinions—some thought it gluey while others described it as fruity, like grapes. It was also observed that the flavour of a solution of slightly gluey caseinate was intensified by subthreshold concentrations of o-aminoacetophenone (about 1 in 10^9).

Table 3. *Composition of the non-volatile non-acid extract* from stored casein*

Compound	% in extract	Approximate concentration in casein, ppm
Lower boiling compounds†	37	0·6
2-Furfurol	25	0·4
Benzyl alcohol	10	0·2
Cresol	4	0·1
Phenol	8	0·2
Benzthiazole	4	0·1
o-Aminoacetophenone	4	0·1

* See p. 216.

† Including benzaldehyde, furfural, alkanals, alkan-2-ones and terpenes.

Since this compound was not isolated in quantities above threshold and, moreover, since volatile fractions were isolated which contained none, it is unlikely to make an essential contribution to the gluey flavour. However, it is clear from the flavour trials that it has some gluey character and can exert a synergistic or potentiating effect on the other components of the flavour.

DISCUSSION

The origin of the flavour components

The range of compounds identified suggests that there are at least 2 degradative processes operating during the storage of casein—lipid oxidation and non-enzymic browning. The acids, alkanals, alkan-2-ones and alkanols are all common products of lipid oxidation and are likely to result from the degradation of the small amount (1-2%) of milk fat remaining in commercial casein. Diacetyl and the furan derivatives are sugar fragmentation products occurring during non-enzymic browning while 3-methylbutanal, methional and phenylacetaldehyde can be formed during this reaction from leucine, methionine and phenylalanine.

The source of the major non-acid component, benzaldehyde, is unknown although it has been found as a product of the caramelization of sugars (Hodge, 1967). Small amounts of benzaldehyde are often found in dairy products but the present findings and those of Scanlan, Lindsay, Libbey & Day (1968) both suggest that it is formed during storage. Work in our laboratory has shown that benzaldehyde, as well as phenylacetaldehyde, is formed during reaction of phenylalanine/lactose mixtures.

Moreover, if the reaction is continued benzaldehyde becomes the major product and the amount of phenylacetaldehyde is reduced. This apparently indicates that benzaldehyde can be formed from the phenylalanine residue as well as the sugar molecule in non-enzymic browning.

o-Aminoacetophenone is likely to be formed by breakdown of tryptophan. Attempts to produce it by simple browning reaction of tryptophan and lactose have failed so far but tryptophan was easily degraded to o-aminoacetophenone in the presence of oxidizing agents such as periodate. Indole and other compounds were also produced. Thus, it is possible that the aminoacetophenone is produced by oxidation of the tryptophan coupled to the oxidizing lipid system, possibly in some free radical mechanism.

The results support the hypothesis put forward by Henry *et al.* (1948) and by Radema (1954) on chemical evidence that mechanisms of oxidation and browning are both operating in whole-milk powder and the final flavour depends on which reaction is dominant.

The involvement of the early stages of the non-enzymic browning reaction is supported by organoleptic evaluation of casein preparations of varying lactose content. Removal of most of the lactose inhibits the development of gluey flavour although Nakanishi & Itoh (1967) were able to form some carbonyl compounds by heating carefully purified casein, presumably by reaction of the protein with bound carbohydrate.

The flavour of gluey casein

Fresh commercial casein usually has a relatively bland, clean flavour and a steam distillate contains only very small amounts of flavour components. On storage, the flavour becomes gluey and the steam-distillate contains 25–30 μg of volatile components per g of casein. It is difficult to assess the relative amounts of the 3 fractions but an average breakdown might be 20 μg of acid fraction, 5 μg of volatile non-acid fraction and 1·5 μg of non-volatile non-acid fraction. The main efforts of identification have been concentrated on the volatile non-acid fraction as organoleptic evaluation indicated that most of the flavour was associated with this fraction. The acid fraction had no relevance but the non-volatile non-acids made some contribution to the overall flavour. This is in accord with the evidence (Nawar *et al.* 1963; Kurtz, 1965) that flavour components of high and low volatility are present in stored milk systems.

Most of the compounds identified have been isolated previously during one or other of the many investigations of dairy products and the specific gluey flavour seems more a result of a different quantitative balance of components than the effect of any one compound. The alkanals formed 20–25% of the non-acid volatile fraction—equivalent to about 1 ppm on the casein.

The results suggest that the overall gluey flavour is compounded of the flavour of n-alkanals and furan derivatives with some synergistic effect from o-aminoacetophenone. It is possible that other compounds isolated such as the higher alkan-2-ones, 2-furfurol, benzaldehyde and benzthiazole may make a contribution to the flavour. While more complex mixtures of these isolated compounds will, in time, be evaluated organoleptically, adding to our knowledge of the flavour, the work already done appears to point clearly enough to the chemical reactions which must be suppressed in practice if gluey flavour is to be eliminated from casein.

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Volatile Components in Casein After Exposure to UV Light

(1970) [En] [Division of Dairy Res., CSIRO, Melbourne, Australia]

Gas-chromatographic analysis showed that the predominant compounds responsible for off-flavours in UV-treated casein were indole and skatole, and to a lesser extent, unsaturated aldehydes and benzyl methyl sulphide. SPT

* Not available in Washington, D.C. area.

Acta path. et microbiol. scandinav. 70, 249-258, 1967.

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AUTORADIOGRAPHIC INVESTIGATIONS OF AMYLOIDOSIS PRODUCED IN MICE BY TRANSPLANTATION OF SPLEEN CELLS FROM CASEIN-TREATED SYNGENEIC DONOR MICE

By

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In a recent report we were able to demonstrate the regular development of spleen amyloidosis in normal C3H mice 2 to 6 days following the intravenous administration of 10^8 spleen cells from syngeneic donors previously hyperimmunized with casein (*Werdelin & Ranlov* 1966). After the cell transfer the recipient mice were treated with nitrogen mustard; serial killings 1, 2, 3, 4 and 5 days after the spleen cell transfer showed no amyloid formation in the recipient spleens until after 48 hours when some of the recipients developed thin, incomplete rings in the perifollicular regions of the Malpighian corpuscles. During the following days of nitrogen mustard treatment the incidence and severity of the amyloid lesions increased. At day 5 after the cell transfer all recipient spleens showed marked amyloidosis. In other organs amyloid could not be demonstrated. Control experiments employing 1) the transfer of spleen cells from normal, non-immunized syngeneic donors followed by nitrogen mustard treatment of the recipients, 2) treatment with nitrogen mustard alone, and 3) transfer of spleen cells from donors hyperimmunized with casein but omitting the nitrogen mustard treatment of the recipients, all failed to produce amyloid lesions in the respective recipients. Therefore, it was tentatively concluded that the production of amyloid in the recipient spleens depended on two consecutive events: 1) A prolonged casein stimulation of the donor prior to the cell transfer, and 2) a suppression of the transferred cells with nitrogen mustard after the transfer, thus illustrating the theory of a local cellular and biphasic pathogenesis of amyloidosis originally put forward by *Teilum* (1964).

In our above cited earlier experiments a prominent feature was the exclusive splenic localization of the resulting amyloid lesions in the

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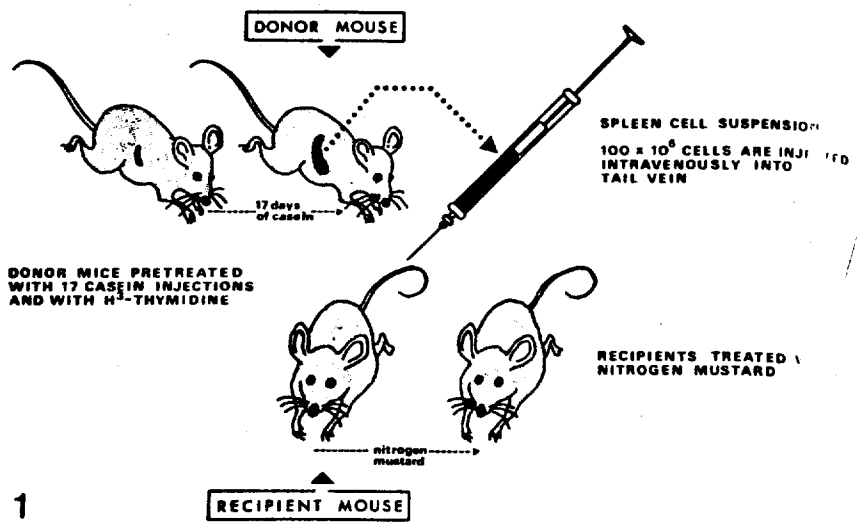

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Fig. 1.
General design of the experiment.

recipients. In addition, the amyloid deposits appeared more cellular than what is usually seen in experimental amyloidosis. Based on the assumption that the recipient amyloid was produced and precipitated *in situ* by the transfused spleen cells it was decided under similar conditions to transfuse isotopically labelled spleen cells from casein-treated donors to normal, syngeneic recipient mice subsequently treated with nitrogen mustard, in order to define the route of migration of the donor cells and their possible relationship to the amyloid deposits eventually developing in the recipients.

MATERIAL AND METHODS

38 closely inbred C3H mice were used. They were aged from 2 to 4 months and the sex distribution was equal. They were at random divided into a donor group and a recipient group. In Fig. 1 the main principles of the experiment are outlined.

Donors: 12 mice were given a total of 17 subcutaneous injections administered daily as ½ ml of a 5 per cent solution of sodium caseinate, 6 times a week. On the day after the last injection the donor mice were killed by cervical dislocation and the spleens were harvested and pooled. 72, 48 and 24 hours before killing each donor mouse received intraperitoneally 0.5 microcurie of H^3-thymidine per g body weight. 3 hours before killing the same amount of H^3-thymidine was administered intravenously. The H^3-thymidine (obtained from The Radiochemical Centre, Amersham, U.K.) had a specific activity of 5,000 millicuries per millimole and was dissolved in normal saline so that 0.5 ml contained 10 microcuries of H^3-thymidine.

Spleen cell suspensions: From each donor spleen a small piece was taken for microscopy. The rest was pooled in cold Ringer's solution and suspended by gentle manipulation in a loose-fitting Potter-Elvehjem homogenizer. The cell suspension was washed 3 times in cold Ringer's solution. It was adjusted so that 0.5 ml contained 10^8 nucleated cells. As judged by the trypan blue test the viability ranged from 85 to 90 per cent.

Recipients: 26 mice received each 10^8 nucleated spleen cells by intravenous injection in the tail. 6 mice died within 15 minutes after the injection and these are in the following referred to as group 0. The remaining 20 mice each received a subcutaneous injection of 0.05 mg nitrogen mustard (Erasol®) dissolved in ½ ml isotonic saline, whereupon they were further divided as follows:

group I: 5 mice killed 24 hours after cell transfer,
group II: 5 mice killed 2 days after cell transfer,
group III: 5 mice killed 3 days after cell transfer, and
group IV: 5 mice killed 4 days after cell transfer.

The recipients of the groups III and IV received an additional dose of 0.05 mg nitrogen mustard 48 hours after the cell transfer.

The recipients were killed by ether inhalation. Tissues were fixed in 10 per cent neutral formalin, paraffin-embedded, and sections were cut 5 microns thick. Amyloid was identified by its morphology in haematoxylin-eosin and P.A.S. stained sections and by its birefringence with Congo red under crossed polars.

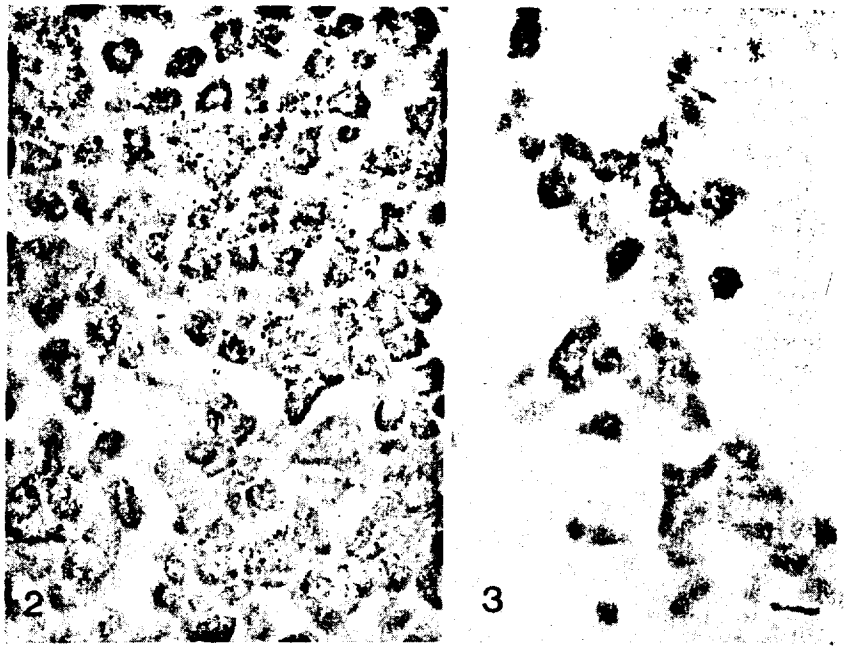
Autoradiography: The deparaffinized sections were coated with K_2 Nuclear Research Emulsion (Ilford), stored at 4° C, and developed after 20, 45, and 85 days of exposure. After developing the sections were stained with haematoxylin-eosin, methyl green-pyronin, and alkaline Congo red. A cell was considered labelled if 4 or more silver grains could be counted over its nucleus. A parallel series of not coated sections was prepared for P.A.S. staining.

RESULTS

Apart from group 0 no spontaneous deaths occurred among the recipient mice.

The distribution and incidence of amyloid deposits among donor mice and the various groups of recipient mice corresponded fairly well to those of our earlier reported, similar experiments (*Werdelin & Ranløv* 1966). In the *donor group* small amounts of amyloid were found in the spleens of 6 out of 12 mice, while no other organs showed amyloid lesions. Among the *recipients* no animals of the groups 0 and 1 showed amyloid lesions, while one out of 5 recipients in group II showed minute amounts of amyloid deposits in the spleen. In group III 4 out of 5 recipients contained amyloid in the spleen while in group IV all 5 recipients showed significant amounts of splenic amyloid. As a general rule the incidence and severity of the amyloidosis increased with the length of time after the cell transfer. As was the case in our earlier reported experiments the amyloid was found exclusively in the perifollicular zones of the spleens of the recipient mice.

Autoradiographic investigations: Most labelled cells in the *donor spleens* (Fig. 2) were found in the perifollicular regions. The majority of these were large cells with elongated, chromatine weak nuclei containing one or two nucleoli. A large proportion of these cells showed cytoplasmic pyroninophilia. Other labelled cells were lymphocytes of various sizes and a few megakaryocytes. Apart from the perifollicular zones the distribution of labelled cells throughout the splenic white and red pulps was rather uniform. The labelling of the donor thymus was less pronounced, most labelled cells being found in the thymic cortex. In the donor liver relatively more Kupffer cells were found labelled than were the parenchymal cells.



Figs. 2–3.

Fig. 2. Autoradiograph of perifollicular region of donor spleen (white pulp at top right). Labelled lymphoid and reticular cell, many of them showing cytoplasmic pyroninophilia. 45 days of exposure. Methyl green-pyronin (×1400).

Fig. 3. Autoradiograph of section of lung from recipient mouse killed 5 minutes after cell transfer showing accumulation of labelled donor cells in the capillaries. Exposure time 75 days. Haematoxylin-eosin (× 1400).

Figs. 4–7.

Fig. 4. Autoradiograph of spleen of recipient mouse killed 24 hours after transfer of hyperimmunized donor spleen cells labelled with H^3-thymidine. Labelled cells in the central areas of the Malpighian corpuscles (CA = central artery). 75 days of exposure. Alkaline Congo red (× 1400).

Fig. 5. Autoradiograph of recipient spleen 2 days after cell transfer. Labelled cells in the white pulp. 45 days of exposure. Methyl green-pyronin (× 1400).

Fig. 6. Autoradiograph of spleen from recipient mouse killed 3 days after cell transfer. Labelled cells are seen in the perifollicular region and in the red pulp. In addition, isolated silver grains have appeared over the cytoplasm of perifollicular reticuloendothelial cells. 45 days of exposure. Methyl green-pyronine (× 1400).

Fig. 7. Autoradiograph of recipient spleen 4 days after cell transfer. Labelled cells are seen in the perifollicular region and in the red pulp. In the same region newformed amyloid deposits have appeared (bottom). 45 days of exposure. Methyl green-pyronin (× 1400).

CA
4
5
6
7

In the animals of the *recipient group 0* labelled cells were found exclusively in the capillaries of the lungs (Fig. 3). No labelled cells could be recognized in the recipient spleens or livers. The labelled cells were of the same type as those seen in the donor spleens, many of them being pyroninophilic. Quite often they could be seen in clusters forming small emboli. In the recipient *group I*, 24 hours after cell transfer, practically all labelled cells had disappeared from the lungs and reappeared within the Malpighian corpuscles of the spleens. In each section usually 8–20 labelled cells could be found distributed over a single Malpighian corpuscle with a tendency to accumulate in the more central parts near the central vessel (Fig. 4). The labelling was confined to the nuclei. At this stage only an occasional labelled cell could be recognized in the perifollicular region and labelled cells were never found in the splenic red pulp. 2 days after cell transfer, in the recipient *group II*, labelled cells were still confined to the splenic white pulp, but now a tendency to accumulate in the perifollicular regions was apparent (Fig. 5). The majority of labelled cells were of the large, pale variety, many being pyroninophilic. At this stage labelling in the splenic red pulp was seldomly seen. In the lungs, the liver, thymus, intestines, and kidneys, extremely few labelled cells could be recognized. In the recipient *group III*, killed 3 days after the spleen cell transfer, the labelled cells seemed to have migrated further out, now being found in the splenic red pulp and in the perifollicular zone of the, admittedly, now somewhat desorganized and shrunken Malpighian corpuscle (Fig. 6). In 4 out of 5 animals of this group this localization coincided with the appearance in the same areas of small, new-formed amyloid deposits. These findings were repeated in the recipient *group IV*, killed 4 days after transfer. In these animals significant amyloidosis was found in all the spleens. The labelled cells were found in the same localizations as those of group III. As was the case in the other recipient groups practically no labelling was found over other tissue sections, neither was amyloid (Fig. 7).

In addition to the well-defined nuclear labelling hitherto observed now isolated silver grains appeared over the perifollicular regions of the recipient spleens. Most of these grains were apparently related to the cytoplasm of reticulo-endothelial cells, probably representing phagocytized nuclear material derived from the labelled donor cells.

DISCUSSION

The main observations of the experiment presented above are: Firstly, the major part of spleen cells from mice hyperimmunized with casein apparently migrate to the perifollicular regions of the spleen when injected intravenously into normal, syngeneic recipients subsequently treated with nitrogen mustard. This assumed migration is completed in the course of 2–3 days and seems to take place via the central vessel

of the Malpighian corpuscle. Secondly, the appearance of labelled cells in the perifollicular zone of the recipient spleen coincides with the appearance of amyloid deposits in the same region. Finally, the nitrogen mustard treatment necessary for the amyloid formation in the recipients seems to be accompanied by a significant destruction of the donor cells present in the recipient spleens.

In the literature dealing with the fate and the organ distribution of transfused lymphoid cells conflicting opinions have been expressed. These are mainly due to variations in genetical donor-host relations, variations in the type of donor cells employed, and, most often, variations in the intervals between the transfusion and the killing of the recipient animals. In general, though, most investigators agree that a major part of such transplanted cells after a certain length of time may be traced to the recipient spleens and, to a lesser extent, to the livers.

In the present experiment animals dying within 15 minutes after the transfusion showed practically all of the injected labelled cells concentrated in the capillaries of the lungs. This is in accordance with the findings of *Osogoe* (1950) and *Keohane & Metcalf* (1958) who found injected lymphoid cells accumulating in the capillaries of the lungs immediately after the injection, after which they were gradually released into the blood stream. In the present experiment 1 and 2 days after cell transfer labelled cells were found predominantly in the splenic white pulp, after 24 hours with a tendency to accumulate around the central vessel. This is in agreement with the observations of *Gowans* (1962). He found that between 3 and 12 hours after the transfusion of labelled lymphoid cells they could be found accumulated immediately around the central vessel in the white pulp of the recipient spleen. This was true regardless of the genetic relation between donor and host. In guinea pigs followed for 24 hours after the transfer of (allogeneic) isotopically labelled (Cr^{51}) lymphoid cells *Turk* (1962) found about one half of the activity remaining in the liver throughout the period, while the activity in the spleen increased steadily from 1 to 6 per cent. With a similar technique *Najarian & Feldman* (1965) studied the rate of isotope disappearance from the spleens of mice injected intravenously with H^3-labelled spleen cells from either syngeneic or allogeneic donor mice. In syngeneic recipients the calculated half life of labelled cells was 82 hours, in contrast to 40 hours in allogeneic recipients. In an allogeneic donor-host system *Hattler et al.* (1964) found the majority of injected, labelled spleen cells accumulating for 2–3 days in the recipient spleen. In a rather indirect way the fate of injected, allogeneic spleen cells was determined by *Haller* (1964). He found the mortality from "runting" cut in half by removing the spleen of newborn mice shortly after the injection of allogeneic adult spleen cells. His observations suggest that the injected spleen cells immediately settle out preferentially in the spleen of the new host from

which they initiate the lethal graft-*versus*-host reaction. From 4 to 21 days after the transfusion of allogeneic adult lymphoid cells to neonatal rats *Nowell & Defendi* (1964) found the injected cells proliferating more actively in the lymph nodes than in the spleen. In accordance with others (*Davies & Doak* 1960, *Howard, Michie & Simonsen* 1961, *Fox* 1962) their findings suggested the splenomegaly occurring later during the graft-*versus*-host reaction being mainly due to host cell proliferation. These observations suggest that the sojourn of injected lymphoid cells in the recipient spleen may be a temporary one perhaps limited to a few days. However, that the strain combination may be decisive for the fate of injected parental cells must be emphasized. *Fox & Howard* (1963) found dividing cells of donor origin dominating the spleen after 3 weeks of graft-*versus*-host reaction in the strain combination C57BL → (C57BL × CBA)F_1. From these observations it appears that conclusions regarding the fate of injected lymphoid cells into syngeneic recipients should not be drawn without criticism from experiments employing allogeneic or xenogeneic strain combinations.

Murray & Murray (1964) found H^3-labelled thymus cells injected intravenously into syngeneic mice after a few hours sojourn in the capillaries of the lungs migrating primarily to the spleen, and, to some extent, to the bone marrow and the lamina propria of the small intestine. In many instances these authors found evidence of transformation into other cell types ("blast-like") and they refused to rule out the possibility of a direct incorporation of labelled material into phagocytic cells.

That the origin of the "lymphoid" cells transferred to syngeneic recipients may be decisive for their destination within the recipient was illustrated in an interesting report by *Parrot et al.* (1966). These authors describe the preferential localization immediately around the central arteriole of the Malpighian corpuscle (the "thymus-dependent area") of injected, labelled thymus cells. Similarly labelled spleen cells also accumulate in these areas but, in addition, are distributed at the periphery of the splenic follicles. Many more spleen than thymus cells enter the lymphoid tissues and the spleen appears to be the primary target. Earlier experiments by *Diderholm & Fichtelius* (1959) with guinea pigs showed that transfused P^{32}-labelled thymocytes and lymph node lymphocytes both could be traced to the red pulp of the recipient spleens, mainly perifollicularly, though thymus cells seemingly could be recovered in the spleen to a greater extent.

Migration to the thymus of transfused lymphoid cells seems to be an uncommon finding. *Galton & Reed* (1966) injected H^3-labelled spleen cells from syngeneic donor mice into normal recipients. After 3 days the vast majority of labelled cells was recognized within the Malpighian corpuscles of the recipient spleens and, in addition, a few labelled cells appeared in the thymus glands.

Even in syngeneic combinations difficulties may arise. Recently *Celada* (1966) showed that mouse spleen cells, in the donor animal "primed" to form antibody against human serum albumin, failed to show the secondary response when challenged after transfer into intact mice of the same genotype. This barrier, which severely affected the donor cells' capacity to implant and/or to function in the syngeneic system, was found to be radiosensitive and to depend on the age of the recipient (being maximal after 2 months of age). In an allogeneic system *Hattler et al.* (1964) found that donor cells presensitized with an indifferent antigen had survival times similar to normal donor cells.

In the present material the relationship in time and topography between the appearance of labelled cells and amyloid deposits in the recipient spleens seems suggestive of a, direct or indirect, participation of these cells in the local production of the amyloid substance. Evidence that amyloid is formed *in situ* by cells belonging to the reticuloendothelial system was first presented by *Teilum* (1956, 1964). The present and our earlier experiments allow no conclusions to be drawn regarding the type of cells involved in this presumed local production of amyloid. The majority of labelled cells found in our recipient spleens were apparently of the "reticular" variety but may not necessarily be identical with the large, pale cells observed in the donor spleens, as transformation into lymphoid type cells or *vice versa* under these conditions is known to occur (*Diderholm* 1961). Further, less than 20 per cent of the nucleated donor cells were labelled under the conditions of the thymidine treatment and, moreover, the composition of this labelled cell population did not reflect that of the whole transfused spleen cell population.

SUMMARY

sotopically (H^3) labelled spleen cells derived from donors hyper-nmunized with casein were transferred intravenously to normal, syn-eneic mice subsequently treated with nitrogen mustard. The recipients ere killed in series: 0, 1, 2, 3 and 4 days after the cell transfer. From to 4 days after transfer all of the recipient mice developed splenic myloidosis. Autoradiographic investigations revealed that the major art of the hyperimmunized donor spleen cells apparently migrated to e perifollicular regions of the recipient spleen. This assumed migra-on was completed within 2-3 days and seemed to take place via the entral vessel of the Malpighian corpuscle. The appearance of labelled ells in the perifollicular zone of the recipient spleen coincided with the ppearance, in the same region, of new-formed amyloid deposits.

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IMPROVED GROWTH IN RATS ON IODINE DEFICIENT DIETS

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In previously reported work of this laboratory on the results of iodine deficiency in the rat (Remington and Levine, '36), the low iodine diet employed has been as follows: wheat gluten 20, yellow corn meal[1] 76, calcium carbonate 3, sodium chloride 1, irradiated yeast 0.2. Young rats maintained for 35 days on this diet show the following changes in the thyroid: 1) enlargement, 2) hyperplasia, 3) edema, 4) almost complete loss of colloid, and 5) marked loss of iodine. Since these changes are fully prevented by the addition of iodine (1 to 2 γ per day) either as iodides or certain iodine-bearing foods (Remington, Coulson and Levine, '36) they are considered as specific consequences of lack of this element.

This diet falls far short of satisfying the ideal for the study of deficiencies, i.e., full adequacy in all known essentials except the one being investigated. Glaringly evident are the unbalance between Ca and P, and the deficiency in vitamins of the G(B_2) group. Probable deficiencies also exist in inorganic elements, and in the quality of the protein. It does not support normal growth. Rats 4 weeks of age, averaging 60 gm. in weight, when placed on the diet for 5 weeks, with or without added iodide, grow only to 110 to 130 gm., as compared with 180 to 200 gm. (average for both sexes) for rats on our colony ration. Attempts to rear rats on this diet to full size and sexual maturity have not been successful.

[1] Whole yellow corn meal as sold for chick feeding.

Obviously, if one desires to answer such questions as to whether or not normal growth can be obtained in iodine deficiency, or what is the effect of deficiency continued into adult life on the animals themselves, on the composition and histology of the thyroid, or on reproduction, the first step must be an attempt to improve the diet without notably decreasing its goiter-producing power. Many other problems affecting the utilization of iodine in the rat can be attacked in a quantitative way, if such a diet is possible. Difficulties have been the universal presence of traces of iodine in natural foods or concentrates prepared from them, and the presence of significant traces in many chemically pure salts. It should be borne in mind that a degree of contamination which will augment the iodine intake of the rat 0.1 to 0.2 γ per day can produce a detectable difference in the size of the thyroid and its iodine content. For instance, if a given supplement is to be added to the diet at a level of 2%, it should not contain more than 0.00005% (500 p.p.b.) iodine, since this amount would add approximately 0.1 γ per day to that unavoidably present in the basal diet. It is, on the other hand, quite probable that if a given supplement improves growth, and the economy of food utilization for growth, the daily intake of iodine necessary to maintain a given degree of thyroid abnormality may be altered, in which case our ideas as to iodine requirement (Levine, Remington and von Kolnitz, '33) will require revision, since these were developed on rats which made subnormal growth.

EXPERIMENTAL

The first step in improving the diet was to counteract its rickets-producing property by reducing the proportion of $CaCO_3$ from 3 to 1%, it having been shown that this change did not lessen the degree of thyroid enlargement. This change permitted the omission of irradiated yeast. The new basal diet (no. 327) is as follows: wheat gluten 20, yellow corn meal 78, $CaCO_3$ 1, NaCl 1. Experiments were then made in supplementing the diet in three ways, viz., 1) improvement in protein quality by substituting purified (vitamin-free) casein

for part or all of the wheat gluten; 2) improvement of vitamin and mineral supply by addition of small percentages (0.5 to 4%) of dried pig liver, or 3) by the addition of similar small percentages of dried brewers yeast.

The handling of the animals was essentially the same as that used in earlier experiments. Young rats, approximately 28 days of age and weighing around 60 gm., were placed on screens and maintained on the diets with distilled water for 35 days, after which they were killed by chloroform and the thyroids removed. Records were kept of the gain in weight and of the food consumed. Ten rats, either five of each sex or four of one and six of the other, were placed on each diet.

The glands on removal were placed in special nickel boats, and weighed in a stoppered weighing bottle. All of the glands from a given group were placed in the same boat, which was then dried to constant weight at 100°, for calculation of dry matter. Iodine was determined colorimetrically on the entire contents of the boat by the method of the writer (Remington, McClendon, von Kolnitz and Culp, '30).

Effect of casein

Results obtained when vitamin-free casein was substituted for part or all of the wheat gluten are shown in table 1. Five per cent of casein, substituted for wheat gluten, produces a significant gain in rate of growth, which still is only about half that of normal rats. Further increases in proportion of casein do not bring about any greater gains. When the thyroid is considered, however, it appears that with increasing proportions of casein there is a progressive decrease in thyroid weight, and an increase in dry matter and iodine content, the data conforming well to our curves (Remington and Levine, '36). Either the casein carries a significant amount of iodine, or its presence in the diet makes possible a higher degree of storage in the thyroid of the small amount of the element available in the diet. The effects observed can be attributed to increased iodine supply if the casein contains 200 to 300 parts per billion, a point which will be discussed later.

Effect of liver

Fresh pig liver was obtained from a distributor, sliced, partially dried on a steam table, ground through a meat chopper, the drying completed in an oven at 80°, then reground and stored in tightly sealed jars. The first series of experiments was run parallel to the casein experiments, with

TABLE 1

Effect of casein

DIET	327	328	329	330	COLONY
Wheat gluten	20	15	10	0	
Casein	0	5	10	20	Commercial dog biscuit
Corn meal	78	78	78	78	
$CaCO_3$	1	1	1	1	
NaCl	1	1	1	1	
Number of rats	10	10	10	10	10
Sex	4 M–6 F	4 M–6 F	5 M–5 F	3 M–7 F	5 M–5 F
Gain in weight (gm.)	51±2	68±4	74±4	67±4	120
Thyroid weight (mg.)	27.4 (19.1–37.7)	24.9 (18.2–36.1)	23.1 (18.2–27.9)	18.3 (15.1–21.1)	15.8 (11.9–20.2)
Thyroid weight per 100 gm. body weight (mg.)	23.9±1.0 (18.7–35.2)	18.9±0.6 (13.7–21.9)	17.1±0.5 (13.6–21.6)	14.1±0.4 (11.4–16.5)	8.8±0.4 (6.3–11.7)
Dry matter (%)	21.0	23.6	25.9	27.4	32.0
Iodine, dry basis (%)	0.0137	0.0297	0.0451	0.0595	0.2000
Total iodine (γ)	0.79	1.75	2.70	2.99	10.1
Degree of enlargement	2.4	1.9	1.7	1.4	

the same control group. Dried liver was added to the diet at levels of 1.5 and 3%. Both groups showed greater growth than the casein-fed rats, but unlike the latter, these had considerably larger thyroids, of lower iodine content, than the controls. Since work in this direction appeared to show promise, a second series was run (table 2), the liver being fed this time at levels of 0.5, 1.0, 2.0 and 4.0% of the diet, replacing in each case the same amount of gluten. In addition, a group

of rats was fed 2% of dried lean beef (chuck). In this series, maximum growth was obtained at the 2% liver level, as was also maximum degree of thyroid enlargement (3.7 times normal weight). Beef muscle also stimulated growth, but to less than

TABLE 2

Effect of liver. Series II

DIET	327	340	341	342	343	344	COLONY
Wheat gluten	20	19.5	19	18	16	18	Commercial dog biscuit
Dried liver	0	0.5	1	2	4	0	
Corn meal	78	78	78	78	78	78	
$CaCO_3$	1	1	1	1	1	1	
NaCl	1	1	1	1	1	1	
Dried lean beef	0	0	0	0	0	2	
Number of rats	10	10	10	10	10	10	
Sex	6 M–4 F	6 M–4 F	6 M–4 F	6 M–4 F	6 M–4 F	6 M–4 F	
Gain in weight (gm.)	66 ± 3	81±2	92±3	104±4	105±5	85±4	125
Thyroid weight (mg.)	30.4	34.0	42.3	52.1	46.1	37.3	15.8
Thyroid weight per 100 gm. body weight (mg.)	23.9±1.0	23.9±1.0	27.8±1.4	32.3±1.7	28.2±1.1	25.6±0.8	8.8±0.4
Dry matter (%)	22.4	21.8	21.3	20.9	21.1	22.4	32.0
Iodine, dry basis (%)	0.0064	0.0049	0.0032	0.0051	0.0034	0.0055	0.2000
Total iodine (γ)	0.44	0.36	0 .29	0.55	0.33	0.46	10.1
Degree of enlargement	2.4	2.4	2.8	3.7	3.2	2.6	.....

half the degree of liver, and the thyroids were not significantly larger than the controls (2.6 times normal against 2.4). In all cases the iodine content of the thyroid is so low that no importance can be attached to such differences as exist.[2]

[2] The total amount of iodine contained in the dried thyroids of ten animals was 3 to 5 γ, which cannot be estimated by present methods with any higher degree of accuracy than about ± 20%.

Effects of yeast

In the third series (table 3) dried brewers yeast, furnished through the courtesy of Dr. Harold Levine of the Pabst Brewing Company, was added to diet 327 in the proportions of 0.5, 1.0, 2.0 and 4.0% replacing an equal amount of gluten. Growth is improved by the yeast, most marked effect being observed at the 2% level, but no significant changes in thyroid weight, dry matter, or iodine content are observed. Neither was growth as good as with equal additions of liver.

TABLE 3

Effect of brewers yeast

DIET	327	345	346	347	348
Wheat gluten	20	19.5	19	18	16
Dried yeast	0	0.5	1	2	4
Corn meal	78	78	78	78	78
$CaCO_3$	1	1	1	1	1
NaCl	1	1	1	1	1
Number of rats	10	10	10	10	10
Sex	5 M–5 F	5 M–5 F	5 M–5 F	5 M–5 F	5 M–5 F
Gain in weight (gm.)	54±2	64±4	67±3	80±4	84±4
Thyroid weight (mg.)	22.1	26.1	25.6	28.1	27.9
Thyroid weight per 100 gm. body weight (mg.)	19.2±0.6	18.5±0.8	19.9±1.0	19.7±0.8	19.1±0.5
Dry matter (%)	24.1	25.2	24.8	24.9	24.8
Iodine, dry basis (%)	0.0084	0.0074	0.0083	0.0067	0.0086
Total iodine (γ)	0.45	0.49	0.53	0.47	0.60
Degree of enlargement	1.9	1.9	2.0	2.0	1.9

DISCUSSION

The four supplements fed; casein, liver, beef muscle and yeast, all improved the growth of the animals, but in different amounts. Rats receiving 5% of casein in place of the same amount of wheat gluten gained 17 gm. more than the controls, but this gain was exceeded by those receiving 1% of dried liver, or 2% of beef muscle or yeast. Since casein is a widely used and satisfactory protein in experimental diets, it does not seem likely that the greater improvement in growth resulting from much smaller additions of the other supplements

can be due to protein quality alone. It is possible that the vitamin content, particularly with regard to A and G, of the corn meal, is insufficient for rapid growth, and that this partial deficiency is compensated by the vitamins present in liver or yeast. Deficiency in iron is also a possibility, although rats on the basal diet do not become anemic within the experimental period employed. Even though the present investigation does not reveal specific deficiencies in the basal diet (327) it has achieved the desired end of producing normal, or nearly normal, growth on diets markedly deficient in iodine. An investigation of the effects of augmenting the vitamin content of diet 327 with carotene or flavin would contribute to the specificity of our knowledge as to how the liver acts, and might make possible a further simplification of the problem.

When it comes to the effect on the thyroid gland, i.e., degree of goiter as indicated by the criteria previously proposed: fresh weight, iodine content, and dry matter content of the gland, the response to different supplements is quite different. Casein added to the diet in progressively increasing amounts brought about a diminution in the severity of goiter in almost exact relation to the amount fed. Previous experience with adding iodides or iodine-bearing foods in quantities insufficient for full protection against goiter, enables us to calculate that if the effect is due to iodine in the casein, it should contain between 200 to 300 parts per billion. Two analyses of casein from the same source made several years ago revealed twelve and eighteen parts, respectively, an insignificant amount. It seemed possible therefore that unless casein itself is able to protect the thyroid against the results of iodine lack, either the earlier analysis must have been in error, or the present lot of casein differs in iodine content from previous ones. Accordingly, the particular lot of casein employed was analyzed, the chemist not being informed as to the purpose of the analysis or probable values.[3] Four determinations gave values of 250, 254, 259 and 267 parts per billion, amply con-

[3] Iodine analyses on dietary materials were made by Mr. Cecil L. Smith, on thyroid glands by Mr. E. J. Coulson.

firming the belief that the reduction in degree of goiter obtained with casein is due to iodine contamination, and rendering it unnecessary to postulate any specific protective action due to casein.

The marked increase in severity of goiter when liver is added to the diet was not expected, and is rather disturbing when considered alongside the earlier work of this laboratory in which it appeared that quantitative relationships exist between iodine intake and degree of hyperplasia of the thyroid. The dried pig liver contained about 150 p.p.b. of iodine, enough to contribute 0.04 γ per day per rat when fed at the 2% level. This additional intake of iodine should have had a slight protective action, probably too small to measure. Instead of such an effect, however, the degree of enlargement of the glands of rats receiving 2% liver is 3.7 times normal, compared with 2.4 for those receiving no liver.

It was suggested earlier in the paper that augmented growth might conceivably increase the iodine requirement of the rat, and hence bring about a more severe thyroid condition on the same iodine intake. Fortunately, the yeast series bears on this question. Rats receiving 2% of yeast made the same gain as those receiving 1% of liver, about 26 gm. more than the controls in both cases. The yeast contained less than 20 p.p.b. of iodine, hence its contribution of that element was negligible. Nevertheless yeast did what we had expected the liver to do, increased growth without affecting the degree of thyroid enlargement. If, then, rate of growth can be increased by 50% by the addition of 2% of yeast, which neither increases nor decreases appreciably the iodine content of the ration, and this improved growth is not accompanied by an increased severity of the thyroid condition, we cannot with surety attribute such increased severity to the effect of growth when obtained by means of liver.

To explain the effect of liver on the thyroid, two possibilities are presented: 1) the presence in the liver of some substance, the metabolism of which causes a drain on the thyroid mechanism, or 2) the presence of a specific thyroid-stimulating

hormone. In Marine's early observations on goiter in young brook trout fed largely on chopped liver, he wrote ('14): "It is also probable that the liver contains some substances in excess, in attempting to utilize which the animal exhausts other elements necessary for nutrition which are not present in the liver in sufficient amounts." This view is reflected

TABLE 4

Economy of food utilization for growth

DIET NO.	SUPPLEMENT	TOTAL GAIN	TOTAL FOOD	FOOD FOR GRAM GAIN
		Series I		
327	Basal	51	348	6.8
328	5% casein	68	335	4.9
329	10% casein	74	383	5.2
330	20% casein	67	382	5.7
331	1.5% liver	81	398	4.9
332	3.0% liver	87	417	4.8
		Series II		
327	Basal	66	385	5.9
340	0.5% liver	81	411	5.1
341	1.0% liver	92	437	4.8
342	2.0% liver	104	458	4.4
343	4.0% liver	105	475	4.5
344	2.0% lean beef	85	406	4.8
		Series III		
327	Basal	54	317	5.9
342	2.0% liver	99	411	4.2
345	0.5% yeast	64	332	5.2
346	1.0% yeast	67	348	5.2
347	2.0% yeast	80	368	4.6
348	4.0% yeast	84	346	4.1
Colony		120	482	4.0

in the first suggested explanation. Marine ('22) also states that "liver, particularly pig liver, was the most potent of a great variety of meats in causing thyroid hyperplasia in dogs and cats . . . ," but since his protocols have not been published, one cannot be sure that he has ruled out differences due to other deficiencies, such as iodine. Marine also believed that increased protein in the diet increases the tendency to goiter, but in the present work we have not appreciably varied

the percentage of protein, hence the experiments do not bear on this point.

All the supplements fed, in addition to improving growth, also increased the economy of food utilization for growth, as shown in table 4. If we disregard the small amount of fat contained in the liver and yeast, the caloric value of all diets was the same. The most efficient utilization of food was found in rats fed 2% liver or 4% yeast: 4.2 and 4.1 gm. of food, respectively, for each gram of gain. On the basal diet alone it required 5.9 gm., while a typical group on the colony diet (dog biscuit) consumed only 4 gm. of food for each gram of gain.

From the foregoing it appears that diet 342, containing 2% of dried pig liver, should enable us to reach the desired end of prolonging our experimental period through a normal life span of the animals. Rats have been raised to maturity on this diet, with a progressively greater degree of thyroid hyperplasia (up to 5 months), have produced normal numbers of living young and reared them without loss to weaning age, despite the fact that their thyroids were markedly hyperplastic, contained practically no colloid after 8 weeks, and only 3 to 5% as much iodine as those of rats on colony ration.

SUMMARY

The subnormal growth of young rats fed the goiter-producing diet of Levine and Remington is improved when a part of the wheat gluten is replaced by 1) purified casein, 2) dried pig liver, or 3) dried brewers yeast. Equal gains were produced with 10% casein, 1% liver or 2% yeast. The addition of 2% liver produced most satisfactory growth.

Purified casein carries sufficient iodine to render it unsuitable for use in goiter-producing diets. Yeast was without effect on degree of goiter but liver aggravated the condition.

A suitable diet for goiter studies on rats is wheat gluten 18, dried pig liver 2, yellow corn meal 78, calcium carbonate 1, sodium chloride 1. On this diet rats reach maturity and produce normal numbers of living young despite almost complete absence of iodine and colloid from the thyroid gland.

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Quantitative Determination of the Major Components of Casein Mixtures by Column Chromatography on DEAE-Cellulose [1]

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Abstract

Quantitative determination of the α_s-, β-, and κ-casein content of casein samples has been achieved by column chromatography of alkylated samples on DEAE-cellulose in the presence of urea. Resolution of the caseins is not complete, but is reproducible within 2 or 3%, and the estimated accuracy of the method is ±5%. In contrast to methods based on determinations of sialic acid content and of turbidity, the chromatographic method is applicable to casein mixtures of widely varying composition, and is not affected by varying amounts of carbohydrate per molecule of κ-casein or by the presence of minor components such as γ-casein and temperature-sensitive caseins.

Previous publications from this laboratory have presented methods for the estimation of κ-casein from sialic acid analysis (3), and of the sum of β- plus κ-caseins by turbidimetric measurements (6). κ-Casein is now known to consist of a series of components of variable sialic acid content (2, 4, 7), while the turbidimetric method is open to objection (1) and subject to errors caused by the presence of other caseins and by unidentified changes in casein components (unpublished observations).

The present paper describes an improved method, based on column chromatography, for the quantitative analysis of casein mixtures.

Materials and Methods

Tris-citrate-urea (TCU) buffer (pH 8.6, 0.05 M tris, 6 M urea). To prepare 20 liters of TCU buffer, 7,200 g urea (analytical grade) are dissolved in distilled water and the volume brought to about 19 liters; 100 ml tris-citrate solution (M tris, 0.13 M citrate, pH 8.6) are added, the pH adjusted to pH 8.6 ± 0.05, and the solution diluted to a volume of 20 liters. The buffer is filtered through a column (4 by 15 cm) of DEAE-cellulose to remove interfering impurities and stored in glass containers at 4 C.

Preparation of sample. A suitable quantity of freeze-dried casein, usually 250 mg, is alkylated by the procedure of Woychik and Kalan (10). The sample is dissolved in 20 ml 0.01 M imidazole 4 M urea buffer (pH 7.0), 0.02 ml β-mercaptoethanol added, and the solution stirred for 1 hr at 4 C. Iodoacetamide (167 mg) is then dissolved in the solution, which is stirred for another 15 min, and 0.05 ml β-mercaptoethanol is added to react with the residual iodoacetamide. The treated sample is finally dialyzed against three 300-ml lots of TCU buffer over a period of 24 hr.

Preparation of column and chromatography of sample. DEAE-cellulose (Whatman DE 11) which has been washed or regenerated by standard methods to remove impurities and fine material is equilibrated in TCU buffer by repeated suspensions until no change in buffer pH occurs. The slurry of cellulose and buffer is then run into a Sephadex Laboratory Column (K15/30, Pharmacia, Uppsala, Sweden) to form a column of DEAE-cellulose 1.5 by 27 cm. The cellulose column is packed by passing 300 ml TCU buffer through it under a hydrostatic head of about 150 cm. Immediately before use, another 100 ml of TCU buffer is passed through under a hydrostatic head of 75 cm. As soon as this buffer has entered the column the dialyzed sample is added slowly and collection of 5-ml fractions of column eluate is commenced. The sample dialysis sac and the small beaker used to hold the sample during its addition to the column are rinsed with 5 ml TCU buffer and the rinsings added to the column, plus 45 ml of TCU buffer. The column is then connected to the mixing chamber of a two-chamber gradient device (500 ml TCU buffer; 500 ml TCU buffer made 0.3 M with respect to sodium chloride) which feeds a linearly increasing concentration of sodium chloride to the column. The flow rate is 90 to 110 ml per hour, with a hydrostatic head of approximately 75 cm. Column preparation and chromatography are done at 4 C.

A total of 200, 5-ml samples of the eluate

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[1] Issued as N.R.C.C. no. 10484.

[2] Guest worker from Hannah Dairy Research Institute, Ayr, Scotland.

are collected, and the absorbance at 280 mμ, relative to distilled water, is determined on sufficient of these (each tube at the inflection points between elution peaks, and every second tube elsewhere) to define the elution profile (Fig. 1). On the basis of this profile, the 5-ml samples are combined to give five fractions, and the absorbance of the four protein-containing fractions is measured at 280 mμ relative to the reference sample (eluate tubes 195-200) (Fig. 1). The protein content of the fractions is calculated from the absorbance, the volume, and the absorptivity ($A_{1\%}^{1cm}$ 280 mμ). The absorptivities used are, for Fraction 1, 9.2; Fraction 2, 8.5; Fraction 3, 7.1; Fraction 4, 9.2; their derivation is discussed below.

Results and Discussion

For identification of proteins present in Fractions 1 to 4 (Fig. 1), and to determine purity of the fractions, the eluted materials were dialyzed to remove urea, concentrated by ultrafiltration, dialyzed for a further 18 hr, and freeze-dried. The proteins thus obtained were then subjected to polyacrylamide gel electrophoresis with and without being treated with rennin (Fig. 2). The patterns indicated that Fraction 1 (not shown) contained the slow-moving components such as temperature-sensitive casein and some *para*-κ-casein-like material. Fraction 2 contained κ-casein, probably some γ-casein, and small amounts of components with a mobility greater than that of β-casein. Because the elution profile for Fraction 2 was complex (Fig. 1), subfractions of this component were also subjected to electrophoretic examination before and after treatment with rennin. The patterns obtained confirmed that all portions of Fraction 2 were mainly κ-casein. Fraction 3 contained relatively pure β-casein and Fraction 4 contained α_s-casein plus some faster-moving components.

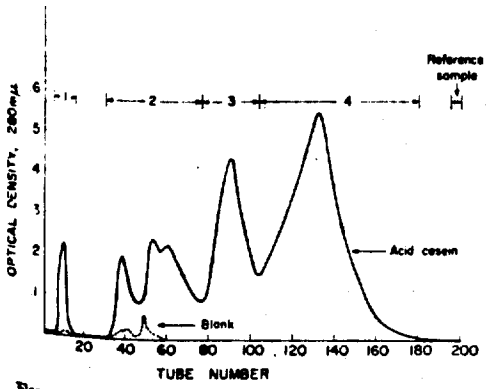

FIG. 1. Elution curve for a 250-mg sample of acid casein.

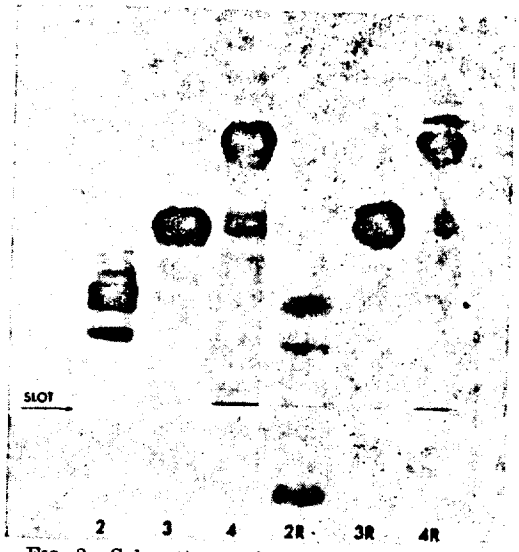

FIG. 2. Gel patterns of the three major eluted fractions, before and after treatment with rennin. Samples numbered as in Fig. 1. Rennin treatment indicated by R.

To quantify the fractionation, the absorptivities at 280 mμ of the protein in each fraction was determined in TCU buffer at pH 8.6 and the absorptivity calculated with allowance for the moisture content of casein. The amount of Fraction 1 available did not permit multiple determinations of the absorptivity, but a single determination on a combined sample from five chromatographic separations gave a value of 9.2. While this value may be not truly representative, its use will not affect the results appreciably, because the amount of Fraction 1 in casein mixtures is small. Values for the absorptivity of samples of Fraction 2 from five different fractionations ranged from 8.0 to 8.7, with an average of 8.5 ± 0.30. The corresponding values for Fraction 3 were 6.7 to 8.2 and 7.1 ± 0.55, and for Fraction 4, 8.9 to 9.5 and 9.2 ± 0.19.

During the development of the present method, a procedure based on chromatography in the presence of β-mercaptoethanol of reduced, but unalkylated, casein was tried. While such a procedure gave a good resolution of casein mixtures, it could not be made quantitative, because of interfering material eluted by the same salt concentration as Fraction 2. Attempts at removing the interfering material were unsuccessful, and the present procedure involving alkylation was, therefore, adopted to obviate the need for β-mercaptoethanol in the elution

TABLE 1. Replicate analyses of caseins.

Sample	Fractions 4	3	2	1
	(Per cent of total casein)			
Whole casein[a]	50.8	31.8	14.6	2.8
	49.8	33.0	14.9	2.3
	49.2	33.4	14.9	2.5
Serum casein[b]	27.4	46.6	24.8	1.2
	28.0	47.2	23.6	1.2

[a] Precipitated from milk at pH 4.6.
[b] Centrifuged serum warmed to 25 C, acidified to pH 4.6.

buffer. With the present procedure, no serious interference has been encountered but two minor components appeared in the elution profile of mock samples of imidazole buffer containing no protein. The first, which eluted at the void volume, apparently represented material extracted from the dialysis membrane (9). When dialysis membranes were washed in two changes of boiling water before use, this interfering component corresponded to less than 0.5 mg of protein and no correction was applied. The second component, eluted as a series of small peaks in the Fraction 2 region of the elution profile (Fig. 1), was probably a contaminant of TCU buffer. Correction factors calculated as κ-casein varied between 3.5 and 4.5 mg.

Since a quantitative method required the collection of all protein-containing eluate, some cross-contamination of the fractions was unavoidable. However, the gel patterns (Fig. 2) suggest that cross-contamination was slight, and the reproducibility of the procedure was satisfactory (Table 1). The accuracy of the values has not been determined but is probably within the limits of variation for determinations of the absorptivities.

To compare results obtained by the chromatographic method with those by the methods based on sialic acid (3) and turbidimetric (6) determinations, five caseins (see Reference 5 for their preparation) were analyzed. With four micellar (sedimented) samples, values (Table 2) for α_s-casein are in reasonable agreement, although the values obtained by chromatography suggest there may be a small decrease in α_s-casein content with decreasing micelle size. The results for κ-casein confirm the reported (5, 8) increase in κ-casein with decreasing micelle size, but the increase is less than that found previously. The errors in κ-casein values by the sialic acid method were reflected in the earlier values for β-casein, but a decrease in β-casein content with decreasing micelle size is also confirmed by the chromatographic results. With the serum or nonsedimented casein, values for κ-casein by the two methods are similar, but those for α_s-casein and for β-casein are not in agreement and it is very probable that the previous methods (3, 6) were unreliable when applied to this type of sample.

Calculations of κ-casein content from an experimentally determined[3] factor of 2.45 SH-groups per 25,000 g of purified κ-casein gave results 10 to 15% higher than those obtained by column chromatography, but the relation between sulfhydryl content and κ-casein by column chromatography was constant within the estimated error limits (Fig. 3); this suggests that the two methods measured the same component of these mixed caseins. The relation between sialic acid content and κ-casein by column was not constant (Fig. 3) and must, therefore, have been affected by some other property of the system. A possible explanation is that the larger the caseinate micelle, the smaller the carbohydrate content of the constituent κ-casein. Calculation of the apparent

[3] R. Jenness, private communication.

TABLE 2. Composition of centrifugally fractionated milk caseins.

Centrif. time[a]	α_s A[b]	α_s B[b]	β A	β B	κ A	κ B	Groups per 25,000 g[c]	Sialic acid
(min)	(Per cent of total casein)						(SH)	(%)
0-7 1/2	45	47	43	34	12	16	.40	.28
7 1/2-15	47	46	38	32	15	18	.45	.35
15-30	46	45	34	31	20	20	.52	.46
30-60	47	42	25	29	28	26	.68	.65
Not sedimented	11	39	51	23	38	33	.96	.88

[a] All centrifugations in Spinco no. 30 head, 30,000 rpm, times from start of centrifugation to time of turning power off, refrigerated during final (60-min) centrifugation.
[b] A. As determined by earlier methods (3, 6). B. As determined by the present chromatographic method. Note that these data do not total 100%, because Fraction 1 component is not included.
[c] Data from sulfhydryl analyses kindly provided by Dr. R. Jenness, Minnesota. SH-groups and sialic acid are reported for the freeze-dried material, uncorrected for moisture.

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Casein and Factor 3 in Dietary Necrotic Liver Degeneration; Concentration of Factor 3 from Casein. (19610)

KLAUS SCHWARZ. (Introduced by Floyd S. Daft.)

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It is a general experience that standard vit. E-free test diets which contain 10% or more of so-called "vit. free," *i.e.*, alcohol-extracted casein cannot be used for the production of dietary necrotic liver degeneration in rats. The deficiency does develop, however, when the animals are raised on rations containing only 4 to 6% of the "vit. free" casein(1,2). A purified, alkali-treated Hammarsten* casein ("casein VI"), on the other hand, had been used over a period of years at levels of 15 and even 30% for the production of dietary necrotic liver degeneration in experiments which led in 1944 to the detection of the protective activity of vit. E(3,4). It was stated at that time that "the most important condition for the development of the liver damage is to be seen in the nature of the casein used," and that "the occurrence of protective activity in completely dissimilar materials demonstrates that several different substances must exist which have an effect. . . ." (3). Evidence accumulated thereafter for a protective substance which was removed during the preparation of casein VI and which was different from cystine or from vit. E remained unpublished.† Recently, however, we reported the presence of such a factor in certain yeasts(6). The active principle, designated factor 3, prevents the development of dietary necrotic liver degeneration and is found in a wide variety of natural sources.‡

* "Hammarsten" casein (O. Hammarsten 1877, l. c. 7.) is prepared from freshly obtained casein by repeated acid precipitation from dilute alkaline solution and final drying by ethanol and ether.

† See discussion in: Liver Injury, Transactions of the Eighth Conference, Josiah Macy, Jr., Foundation, New York, 1949, p49.

‡ To be published separately.

TABLE I. Protective Effect of Casein and Casein Preparations.

Exp.	Supplement	No. of animals	Dead due to liver necrosis	Avg reciprocal survival time (×100)	Activity, units/g of supplement
A*	0	8	8	2.00 ± .19‖	—
	Crude casein (3%)	9	2	.32 ± .25	9
	"Vit. free" casein a (3%)‡	10	2	.19 ± .12	9.6
B*	0	9	9	2.19 ± .19	—
	"Vit. free" casein b (3%)‡	10	3	.72 ± .41	7.2
	Amino acid mixture (3.6%, ≅ 3% casein)§	10	10	2.03 ± .31	0
C*	0	9	9	2.10 ± .09	—
	Alcohol extr. obtained during casein b production (≅ 5%)	10	10	2.22 ± .13	0
D*	0	9	9	2.74 ± .22	—
	"Vit. free" casein b (3%)‡	10	7	1.19 ± .31	6
	Hammarsten casein prepared from casein b (3%)	10	9	1.98 ± .31	3
E†	0	9	9	2.51 ± .24	—
	Bacto Casitone (2%)‡	10	6	1.1 ± .32	9

* Prophylactic method. † Repletion method (supplementation started 24th day). ‡ Commercial sample. § Composed according to figures given by R. J. Block and D. Bolling, The Amino Acid Composition of Proteins and Foods, C. C. Thomas, Springfield, Ill., Ed. 1945, p. 303. ‖ Mean ± stand. error.

TABLE II. Purification of Active Substance from Bacto Casitone. (Tests by repletion method.)

	Dry wt, mg/g of starting material	Activity: u/g of starting material	Activity: u/g of dry wt	Approximate conc.
Bacto Casitone	1000	10.5	10.5	1 : 1
Charcoal filtrate*				
Charcoal eluate	356	10.5	29.6	2.8 : 1
Alcohol precipitate from charcoal eluate	261	3.7	14.4	1.4 : 1
Alcohol filtrate	68.4	3.8	56	5.3 : 1
Precipitate from water sol. of alcohol filtrate	12.2	4	320	30 : 1
Water filtrate after separation of precipitate	88†	1.6	27	2.6 : 1

* Not tested. † After neutralization.

In this paper evidence is presented for the occurrence of factor 3 in crude and purified samples of casein and of enzymatic casein digests. The 30-fold concentration of the active substance from an enzymatic casein hydrolysate is described.

Experimental. a) *Animal experiments.* Details concerning the animal experiments were reported previously(5,6). Male Sprague-Dawley rats of the National Institutes of Health colony were used. The animals were weaned at 21 to 24 days of age, at which time their average body weight was 41 g. Litter-matched groups of 10 rats of equal average weight were formed. In each experiment one group was fed the basal ration as a control. The basal diet consisted of 30% torula yeast, 59% sucrose, 5% salts, 5% vit. free lard and a vitamin mixture(5). From this ration the experimental diets were obtained by the addition of supplements at the expense of sucrose. Tests for protective activity were performed either by a prophylactic method (Exp. A-D, Table I), *i.e.*, supplementation was started immediately after weaning, or by a repletion method (Table II), in which case supplementation started after a period of 23 days on the basal diet(6). To express the speed of development of the deficiency, the average of the reciprocal survival time was employed and compared, as described before(3,5). With increasing dietary levels of supplements containing factor 3, the reciprocal survival time decreased. A straight line relationship was observed over a wide range of dosage.‡ Protective effects were evaluated in units (u), one u being defined as the amount of factor 3 required per day per animal to effect a 50% reduction of the average reciprocal survival time as compared to the unsupplemented lit-

termate control group. In other words, one u of factor 3 per day per animal doubled the (virtual) average survival time of the rats. It was found, for example, that 1 u was contained in about 0.1 g of Bacto Casitone.§ It is recognized that the test method used does not represent a perfect biological assay procedure. It permits, however, an estimation of the approximate factor 3 activity of natural materials and preparations therefrom. Repeated tests yielded results consistent within a range of ± 15%.

b) *Concentration procedure.* One thousand g of Bacto Casitone were dissolved in 6 liters of water, the pH was adjusted to 8.7 by the addition of 6 N NaOH, the temperature of the solution was brought to 25°C and 1000 g of charcoal (Darco KB) were added with gentle stirring. The pH dropped below 8 and was brought back to 8.7 by 6 N NaOH before filtration (Buchner funnel). The filtrate was again adjusted to pH 8.7, diluted with 2 liters of water and treated a second time with charcoal as described above. The combined charcoal adsorbates were washed with 2 liters of water with stirring, the pH was kept at 8.5 by the addition of 6 N NaOH. After filtration the charcoal was stirred with 5 liters of ethanol at 50-55°C for 30 minutes. The alcohol elution was repeated. The combined ethanol eluates were evaporated under nitrogen to a sirup which was made up to 1 liter with water ("charcoal eluate"). Nine liters of ethanol were added slowly to the "charcoal eluate" with constant stirring. The precipitate was redissolved in 1 liter of water and precipitated again with 9 liters of ethanol. The mother liquors of the first and second precipitations were combined, cleared from turbidity by filtration and concentrated to a sirup which was taken up in 200 cc of hot water ("alcohol filtrate").

The water solution of the alcohol filtrate was kept at +4°C for 24-48 hours. The heavy, creamy precipitate which formed was separated by filtration through a Buchner funnel ("precipitate from water"). This fraction was about 30 times as active as the starting material, as shown below.

§ Difco Laboratories, Detroit, Mich.

Results. Factor 3 activity of casein and preparations. Addition of 3% crude casein and also of various commercial samples of alcohol-extracted "vitamin free" casein at a 3% level to the torula ration greatly inhibited the development of necrotic liver degeneration (Table I, Exp. A, B, and D). The 2 samples of "vit. free" casein of different origin showed an activity similar to that of crude casein. Substitution of an equivalent amount of amino acids, imitating the composition of casein, was without effect (Exp. B).

The alcohol extract obtained during the commercial production of "vit. free" casein was tested and found to be inactive (Exp. C). This shows that factor 3 is not removed to any extent from casein by continuous alcohol extraction.

When "vit. free" casein was purified by the procedure originated by Hammarsten(7), *i.e.*, by solution in alkali and reprecipitation with acid, about half of the "factor 3" activity was eliminated (Exp. D).

Commercial enzymatic casein digests, such as are widely used as ingredients in bacteriological media, showed an activity similar to that of casein, for example, Exp. E, Table I.

It is obvious from these findings that crude and also commercial alcohol-extracted "vit. free" casein contain a component actively protecting against the deficiency. That the effect must be attributed to a factor other than vit. E or cystine, the 2 established protective substances, was derived from the following considerations: "Vit. free" casein does not contain vit. E since such casein is a standard component of rations used for the production of vit. E deficiencies. The quantity of cystine (and methionine), on the other hand, which is supplied by the addition of 3% of casein to the ration is too small to be of consequence. It was found that levels of 0.2% or more of cystine in the diet are required to influence the course of necrotic liver degeneration under our experimental conditions(5).

More direct proof for the existence of factor 3 in casein and for its distinctness from vit. E and from cystine is provided by the fractionation of the active material.

Concentration of factor 3. In enzymatic casein hydrolysates, factor 3 appears to be, at

least in part, present in an easily accessible, low molecular form. Concentration of factor 3 was, therefore, initiated from an enzymatic casein digest as starting material. The animal test was used as assay procedure. The first 3 steps of concentration, described in detail above, have been used repeatedly with only slight variations. Results of a typical run are given in Table II. In order to avoid losing activity in the first step of the concentration process, the material was absorbed on relatively large amounts of charcoal from which the active substance could be removed by repeated treatment with hot ethanol. A loss of activity was not apparent during this process; the charcoal eluate contained about one-third of the original dry weight. Precipitation of inactive impurities, mostly peptides, by ethanol was employed as the next step. Half of the activity remained in solution, the other half was lost in the precipitate, which possibly contains a bound form of factor 3. From the "alcohol filtrate" further purification of factor 3 was provided by the separation of a portion less soluble in water. This product, comprising some organic and inorganic cations, amino acids, and other organic acids shows a sulfur content of less than 0.5%. It is 30 times as active as the starting material and is being used for the further purification of factor 3.

Discussion. The results show that casein contains factor 3. So far no essential difference between the active principle concentrated either from yeast or from casein has been encountered(8). The question, however, whether the active substance from these 2 sources is completely identical remains to be clarified. Both crude and "vit. free" casein compare favorably with the best known sources for factor 3, for example, with yeast K(5). It is apparent that alcohol extraction, the method used for the production of "vit. free" casein, does not remove factor 3. The customary vit. E free diets do not lead to necrotic liver degeneration, evidently because protection is afforded by the amount of factor 3 present in the "vit. free" casein.

Acid precipitation of casein from alkaline solution ("Hammarsten" procedure(7)) partly removed factor 3. This might explain why "casein VI" diets could be used for the induction of the deficiency in the older experiments. At that time "casein VI" had been prepared from a repeatedly reprecipitated "Hammarsten" casein by additional treatment with alkali in the heat and subsequent acid precipitation(3). For the preparation of casein VI, the purity of the starting material appears to be a decisive factor. Several attempts to produce casein VI from alcohol-extracted "vit. free" casein were unsuccessful(8-10).

A potential discrepancy might be seen between the protective effect elicited by the addition of 3% casein to the torula diet and the older findings that diets containing 4, 6, or even 10% casein produced the deficiency. This might be explainable on the basis of differences in the factor 3 contents of casein samples employed at various times. Whether other reasons are involved, however, has to be clarified by further experimentation. It is obvious, however, that the protective effect of casein on dietary necrotic liver degeneration is not due simply to the improvement of the protein supply; it is of a more specific nature.

Summary. 1. Samples of crude and "vit. free," alcohol-extracted casein, and also enzymatic casein digests, were found to contain considerable amounts of factor 3, which protects against dietary necrotic liver degeneration. This explains why the deficiency does not develop on customary vit. E free casein diets. 2. A method is described for the concentration of factor 3 from an enzymatic casein hydrolysate, affording a 30-fold purification in 3 steps, with an over-all recovery of about 38%. This concentrate is being used for the further purification of factor 3.

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Adenosine Triphosphatase Activity of the Vascular System.* (19611)

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The contractile mechanism which specializes skeletal muscle for quick movement is presumably the same as that which enables smooth muscle to maintain its tonus state. It has been shown that the energy-rich phosphate bonds of adenosine triphosphate are the sole immediate source of energy for skeletal muscle contraction. In addition, evidence points to the fact that ATP plays a central role in the maintenance of smooth muscle tone and in the initiation of its contraction. Dubois and Potter(1) devised a method to determine the amount of adenosine triphosphatase (ATPase), the enzyme which is responsible for ATP hydrolysis, in various tissues. Krantz, Carr and Bryant(2) showed that the acutely-acting vasodilators of the nitrite-nitrate series inactivated ATPases present in the aorta of rabbits. The interference with enzyme activity occurred with therapeutic levels of the vasodilators. Thus it appears possible that these vasodilators may exert their action through the ATP-ATPase mechanism as the target enzyme system in arterial tissue.

Owing to this observation we considered it of interest to study the ATPase activity of various segments of the vascular bed. Furthermore, the normal variations within a single species of animal appeared important. In addition, the variation of the vascular ATPase activity of different species was determined, and the effect of age upon the enzyme concentration in the vascular wall was estimated.

* The expense of this investigation was defrayed in part by a grant from the National Institutes of Health.

Method. The principal studies were carried out on male mongrel dogs approximately in middle life. The animals were maintained on a balanced laboratory ration for a period of several months until a good nutritional state was established. The animals were anesthetized with an intravenous dose of pentobarbital sodium, and the vessels removed immediately after anesthesia ensued. It was previously determined that this barbiturate did not affect the ATPase activity of the vessels. All determinations were carried out within one-half hour after tissue removal. It was determined that during this period the ATPase activity did not undergo diminution. This same procedure with suitable modifications, to adapt it to the various species, was employed in all animals studied, omitting barbiturate anesthesia. Rats were used for the studies concerning the influence of age on the enzyme owing to the ease of obtaining animals of definite age. The ATPase activity of the tissues was determined by the method of Bell, Carr and Krantz(3). This method employs the tissue incubation of DuBois and Potter, the precipitation of the phosphate as magnesium ammonium phosphate recommended by Griswold(4), and the use of ammonium sulfate as suggested by Lowry and Lopez(5). The recovery of phosphate from tissue by the method is approximately 90%. The reproducibility of the method on a single specimen of tissue is shown by a standard deviation of 0.18 unit and a coefficient of variability of 3%. The values are expressed in ATPase units as adopted by DuBois and Potter, *i.e.,* the number of gam-

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PROTEIN FEVERS[1]

WITH SPECIAL REFERENCE TO CASEIN

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Although the blood always contains a variety of proteins as normal constituents, the introduction of a foreign substance of this class into the circulation either by direct injection into the blood stream or by some other parenteral path whereby digestive changes are avoided, is likely to bring about a physiological reaction in the organism. The response may be mild and undetectable except by very refined methods; or it may vary in degree from a barely perceptible fever to severe shock symptoms, involving fever, chill, dyspnea, paralysis, and even death. Such a so-called non-specific reaction is sometimes elicited by the injection of bacterial suspensions, vaccines, serums, milk, etc., and is of great physiological interest. Moreover, it is the basis of a type of therapy which has been used very extensively during the last few years. The many beneficial effects reported in diseases which failed to respond to other methods of treatment have given this therapy practical value in the medical world, and make its scientific study particularly interesting and worthwhile at the present time.

Arthritis especially is said to have been quite successfully treated by the injection of such non-specific agents as typhoid vaccine or milk. Cecil (6), for example, reported that 40 per cent of the cases of rheumatic and toxic arthritides recovered completely in two to ten days as a result of the effects of typhoid vaccine injections. Alhswede (1) described a favorable influence upon acute and chronic staphylococcus infections of the skin and upon gonorrheal complications, from the injection of germ-free milk solutions. Culver (8) used gonococcus protein, and Dehio (9) used deuteroalbumoses with success in gonorrheal infections. Certain cases of eye disease have yielded excellent results by treatment with milk injections. The above mentioned cases serve merely as examples of the many diseases which have been reported to be favorably affected by this mysterious "protein therapy."

[1] The data in this paper are taken from the dissertation presented by Florence B. Seibert for the degree of Doctor of Philosophy, Yale University, 1923.

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As has been pointed out, many different and apparently unrelated agents—vaccines, serums, milk, etc.—may favorably affect the same disease, and on the other hand, one substance may cause improvement in many different diseases. Therefore, the symptoms which are more or less manifested in all of these cases are properly spoken of as the non-specific reaction. Until the work of Roemer (18) in 1891, a viewpoint of strict specificity was held and suggestions contrary to this idea were swept aside without consideration by the medical world. But when this investigator reported marked effects upon tuberculous animals with injections of pneumobacilli and bacillus pyocyaneus, and when Rumpf (19) almost at the same time presented favorable results in a series of typhoid patients treated with subcutaneous injections of pyocyaneus vaccine, the existence of a non-specific therapeutic effect became not only possible but even promising.

The symptoms of this non-specific reaction are manifold, but all of them are not manifested in every case. The chill is one of the most characteristic symptoms to be noticed and it may set in as soon as fifteen minutes after the injection or as late as eight to ten hours thereafter, depending upon the agent used. Following the chill, there is generally a marked febrile reaction, the temperature rising in man sometimes to as high as 103° to 104°F. and reaching its maximum in from three to four hours after the injection. The occurrence of this maximum may be considerably postponed when certain agents are used, especially by the intramuscular route. Coincident with the increase in temperature there is an increase in pulse rate and blood pressure. Shortly after this, profuse sweating commences and the accompanying peripheral dilatation reduces the blood pressure and pulse rate. Other symptoms, such as nausea, vomiting, headache, feeling of malaise, herpes, diuresis and albuminuria may or may not be experienced. In the severe shock reaction a fall in temperature, violent dyspnea, abdominal collapse, paralysis and even death may occur. The metabolism is increased as evidenced by the increased nitrogen excretion. The permeability of the capillaries is increased for a short time. As might be expected, there is an alteration in the blood picture. The erythrocytes, blood platelets, sugar, fibrinogen, thrombokinase and leucocytes have all been observed to increase. The variations of the leucocytes in particular have been extensively studied by many investigators who have usually detected a leucopenia after the injection, followed in turn by a leucocytosis. Moreover, the albumin-globulin ratio of blood proteins is found to be disturbed. On the day after these symptoms are experienced a general feeling of euphoria sets in and gradually the patients improve until in many cases a cure is effected.

Whether or not the discomfort of such a general reaction is necessary to the achievement of the remarkable therapeutic effects reported is an

important question—one as yet unanswered. According to Brooks and Stanton (5) unpleasant symptoms are not essential. They observed improvement although there were no systemic reactions in cases of arthritis treated with the "lower fractions" of digestion products of ox fibrin. However, the great majority of investigators and clinicians lay much emphasis upon the necessity of a sharp general reaction in order to obtain satisfactory therapeutic results.

Assuming therefore the desirability of such a reaction, a careful study of the individual symptoms as well as of the factors producing the symptoms is essential. In this investigation the febrile reaction has been chosen as the specific symptom for study, since the heat regulation of an organism is extremely delicate and very susceptible of quantitative measurement, and also since its disturbance is one of the earliest appearing and most prevalent symptoms following the injection of foreign protein.

How the heat regulating mechanism of the body is maintained or disturbed is an elusive and much debated question among physiologists, but that it is upset when certain foreign substances are introduced into the blood stream, is a recognized fact. Non-specific therapy is particularly concerned with the nature of these disturbing substances and with the conditions under which they cause the disturbance.

Historical. Influential factors were first defined by Krehl (12) in a remarkably comprehensive study of many unrelated substances that were able to produce fever. He emphasized the importance of species, the number of previous injections an animal has received, and the dosage and mode of administration. Moreover, the state of health of the individual animal is of considerable importance in determining the extent to which the reaction is manifested. But most important of all is the substance itself which is injected.

Petersen (17) cites such agents as blood serums, proteins, protein split-products, enzymes, tissue extracts, vaccines, bacterial extracts, colloidal metals, yeasts, salt and sugar solutions, formulin, solusin, hetol, iodids, turpentine, antipyretics, and even distilled water. Although they would appear at first sight to be unrelated, yet the question arises whether or not it will be possible to find a chemical nucleus or physical property common to all of them, which is capable of upsetting the heat regulating mechanism.

Since the time when Kühne (13) found by analysis that the tuberculins, then so extensively used, consisted largely of protein cleavage-products, and since Krehl (12) obtained febrile responses with a most heterogeneous variety of substances many of which did not contain protein, the question of whether or not the cause of the fever is protein has been a fundamental one. The problem today remains almost in its original form. It is true that the study of milk therapy has disclosed some interesting evidence but even this is most fragmentary.

The first serious investigation with milk used as a fever-producing agent was undertaken by Besredka (4) in 1900, but not until 1916 was it used as a satisfactory therapeutic agent, when Schmidt (23) and Saxl (21) began to report favorable clinical results. From then on numerous investigators have used milk with marked success. Many have suggested that the casein in the milk produces the beneficial effects, but few have attempted to test purified casein. Lindig (14), however, made casein

from milk and injecting this in sodium bicarbonate solution produced in patients with septic, gonorrheal or tubercular diseases a chill and headache, a marked rise in temperature after the third injection, and then decided improvement. Similar results have been reported by Zimmer (34), Arweiler (3), Taege (25) and Voehl (29). But to other investigators it appeared that casein may not be the important therapeutic factor in the milk. Müller (16), for example, prepared a solution of milk free from casein and still obtained a reaction. Therefore he concluded that endotoxins from the bacteria in the milk were the cause of the rise in temperature rather than the casein itself. Similarly, Uddgren (26) and Ryhmer (20) believed the fever must be due to bacterial products, since with fresh sterile milk they obtained scarcely any reaction. Furthermore, Aman (2) laid emphasis upon the importance of the "antagonistic colloidal activity" of the salts in the milk, and made a preparation "albusol" which was said to be milk freed from bacterial products and salts and rendered neutral to litmus. This gave no reaction.

Besides casein very few other proteins have been investigated with this question in mind. von den Velden (30) mentioned using ovalbumin and serumalbumin with very little success. With the exception of these few isolated cases little attempt has been made to determine whether or not the protein itself is the agent which is able to produce a fever in an animal.

The fact that similar fevers were being reported following the injection of salt solutions and salvarsan solutions gave this entire problem a broader aspect and made the question more fundamental. For example, Schaps (22) reported temperature fluctuations in children following the injection of as little as 1 cc. of physiological saline, grape sugar, and lactose. Englander (10) claimed to have cured typhoid patients in fourteen to sixteen days by the intravenous injection of salt solutions. On the other hand, other investigators such as Weiland (32) and Wechselmann (31) were reporting the absence of febrile reactions with saline, sugar and salvarsan solutions.

A review of an earlier paper on "Fever producing substance found in some distilled waters," which is presented in this JOURNAL, will show clearly that the fevers obtained with sugar and the various salt solutions as well as with water are due to a bacterial contamination of the solution. Is it not possible that this same contamination adheres to the protein under examination, rendering it fever-producing? With this point in mind, it was the purpose of the present investigation to study the effect produced by a variety of more or less purified proteins and in addition, of some individual proteins highly purified. The fact that a protein cannot be inactivated by purification will not necessarily mean that the reaction is not due to contamination rather than to the protein as such, since there may be a strong affinity between protein and the contaminating substance. Furthermore, the possibility of denaturizing the protein molecule in the process of purification must be carefully considered. With the premise that the fevers here reported are not due to any individual reactions on the parts of the animals themselves; or to any of the factors in the technic such as rate of injection, method of filtration and manipulation of the animals; or to hemolytic factors, or to the hydrogen ion concentration of the solutions, or to contaminating impurities such as dissolved gases or glass,

specific inorganic ions, cork extracts; or to the common solvent water if properly prepared,—an attempt was made to study the fever-producing power of pure native proteins.

Technic. The rabbit was used throughout and the technic employed has been fully described in an earlier paper on "Temperature Variations in Rabbits," presented in this JOURNAL. In every case the protein was dissolved with freshly distilled water and the solvent also was injected into two animals as a control experiment on the same day on which the protein was studied. If this test was not negative, the experiment was repeated. Some of the proteins studied were prepared at the Connecticut Experiment Station and were given through the courtesy of Dr. T. B. Osborne and Dr. H. B. Vickery, to whom thanks are expressed; the others were prepared by one of the authors.

Experimental data. The following proteins in 5 mgm. doses, except the majority of the alcohol-soluble proteins, gave a marked positive reaction in two animals each. From table 1 it can be seen that two lots of wheat gliadin, made in different laboratories and one lot of hordein produced no fever in rabbits. Both zein and rye gliadin gave only one slightly positive reaction out of five experiments. With millet seed protein one negative and one positive reaction were obtained and the alcohol soluble protein from milk, described recently by Wells and Osborne (33) gave only positive reactions. The fact that negative reactions were found only in the alcohol-soluble proteins points to a fundamental significance attached to the method of preparation or to a group property, and adds evidence to results reported later.

Casein, however, is the protein which has received the most attention in this investigation, since it is the one which is concerned largely in milk- and casein therapy. So far no one has reported the preparation of a pure casein which will give a negative reaction when injected into rabbits. But the authors have deemed it advisable to determine whether or not such a preparation could be made.

Casein was prepared according to Van Slyke's method by precipitating with acid the protein at its isoelectric point during rapid stirring. It was found necessary to separate the protein by centrifugation and then it was dried with 70 per cent and 95 per cent alcohol and finally with ether. The analysis showed: ash = 0.86 per cent, moisture = 8.45 per cent, fat = 2.02 per cent.

This casein preparation A, dissolved in sodium bicarbonate, gave a fever when injected in 50, 5 or 1 mgm. doses, and practically the same amount of temperature rise in all three cases. This fact cannot be without significance and makes doubtful the importance of the casein itself as the fever-producing element. All of the experiments with the various casein preparations are summarized in table 2. It is interesting to note that through-

TABLE 1

DATE OF EXPERIMENT	PROTEIN INJECTED	TEMPERATURE VARIATION		NUMBER OF ANIMAL	SOLVENT USED	pH OF SOLUTION
		+	−			
		°C.	°C.			
1-26-23	Squash seed globulin	1.6	—	28	10 per cent NaCl	6.3
	Squash seed globulin	0.9	—	33	10 per cent NaCl	6.3
1-26-23	Blue lupin globulin	1.4	—	24	10 per cent NaCl	6.3
	Blue lupin globulin	1.3	—	32	10 per cent NaCl	6.3
1-29-23	Cotton seed globulin	2.5	—	22	N/500 NaOH	>8.0
	Cotton seed globulin	1.4	—	33	N/500 NaOH	>8.0
1-29-23	Phaseolin	1.3	—	26	N/500 NaOH	>8.0
	Phaseolin	1.4	—	31	N/500 NaOH	>8.0
1-29-23	Lactalbumin	1.4	—	24	N/500 NaOH	>8.0
	Lactalbumin	1.0	—	32	N/500 NaOH	>8.0
1-29-23	Amandin	2.4	—	27	N/500 NaOH	>8.0
	Amandin	2.1	—	36	N/500 NaOH	>8.0
2- 2-23	Glycinin-soy bean	2.7	—	27	N/500 NaOH	>8.0
	Glycinin-soy bean	2.7	—	38	N/500 NaOH	>8.0
2- 2-23	Excelsin	1.5	—	26	N/500 NaOH	>8.0
	Excelsin	2.3	—	30	N/500 NaOH	>8.0
2- 2-23	Maize glutelin	1.7	—	28	N/500 NaOH	>8.0
	Maize glutelin	1.2	—	24	N/500 NaOH	>8.0
2- 2-23	Cannabin	0.6	—	31	N/500 NaOH	>8.0
	Cannabin	2.2	—	22	N/500 NaOH	>8.0
2- 2-23	Legumelin-vetch	1.4	—	33	N/500 NaOH	>8.0
	Legumelin-vetch	2.7	—	23	N/500 NaOH	>8.0
1-26-23	Conglutin	0.8	—	25	10 per cent NaCl	5.3
	Conglutin	0.8	—	36	10 per cent NaCl	5.3
6- 1-22	Gliadin-wheat A	—	0.35	12	N/50 NaOH	>8.0
	Gliadin-wheat A	—	0.35	13	N/50 NaOH	>8.0
12-15-22	Gliadin-wheat B	—	0.6	25	N/50 NaOH	>8.0
	Gliadin-wheat B	—	0.3	27	N/50 NaOH	>8.0
	Gliadin-wheat B	—	0.4	28	N/50 NaOH	>8.0
	Gliadin-wheat B	—	0.1	31	N/50 NaOH	>8.0
2-22-23	Hordein	—	0.2	30	N/500 NaOH	>8.0
	Hordein	—	0.5	40	N/500 NaOH	>8.0
2-15-23	Zein	—	0.5	36	N/500 NaOH	>8.0
	Zein	0.6	—	24	N/500 NaOH	>8.0
3-16-23	Zein	—	0.4	36	N/500 NaOH	>8.0
	Zein	—	0.6	38	N/500 NaOH	>8.0
	Zein	—	0.6	39	N/500 NaOH	>8.0
2- 2-23	Millet seed	—	0.3	24	N/500 NaOH	>8.0
	Millet seed	0.7	—	28	N/500 NaOH	>8.0
2-22-23	Gliadin-rye	—	0.4	36	N/500 NaOH	>8.0
	Gliadin-rye	0.9	—	39	N/500 NaOH	>8.0
3-16-23	Gliadin-rye	—	0.5	40	N/500 NaOH	>8.0
	Gliadin-rye	—	0.6	28	N/500 NaOH	>8.0
	Gliadin-rye	—	0.3	22	N/500 NaOH	>8.0
2-22-23	Milk-alcohol-soluble	0.9	—	32	N/500 NaOH	>8.0
	Milk-alcohol-soluble	1.3	—	38	N/500 NaOH	>8.0

TABLE 2

DATE OF EXPERIMENT	LOT OF CASEIN	AMOUNT OF REACTION +	AMOUNT OF REACTION −	NUMBER OF ANIMAL USED	SOLVENT	pH
		°C.	°C.			
5- 9-22	Casein A—50 mgm.	1.45	—	9	N/50 $NaHCO_3$	—
11	Casein A— 5 mgm.	1.95	—	8	N/500 $NaHCO_3$	7.3
16	Casein A— 1 mgm.	1.15	—	12	N/2500 $NaHCO_3$	6.7
20	Casein A— 1 mgm.	1.25	—	12	N/2500 $NaHCO_3$	6.6
9-27-22	Casein A— 1 mgm.	1.70	—	15	N/2500 $NaHCO_3$	7.1
12- 5-22	Casein B—5 mgm.	0.80	—	18	N/500 $NaHCO_3$	6.9
	Casein B	1.00	—	27	N/500 $NaHCO_3$	6.9
	Casein B	1.40	—	34	N/500 $NaHCO_3$	6.9
12-12-22	Casein B	1.40	—	23	N/500 $NaHCO_3$	7.2
	Casein B	1.60	—	24	N/500 $NaHCO_3$	7.2
	Casein B	1.90	—	34	N/500 $NaHCO_3$	7.2
1- 5-23	Casein C—5 mgm.	—	0.60	26	N/500 $NaHCO_3$	7.7
	Casein C	—	0.30	33	N/500 $NaHCO_3$	7.7
	Casein C	—	0.40	35	N/500 $NaHCO_3$	7.7
	Casein C	1.50	—	28	N/500 NaOH	>8.0
	Casein C	1.20	—	32	N/500 NaOH	>8.0
	Casein C	1.60	—	36	N/500 NaOH	>8.0
1-30-23	Casein C	—	0.50	30	N/500 $NaHCO_3$	7.2
	Casein C	0.90	—	38	N/500 $NaHCO_3$	7.2
2- 3-23	Casein C	1.30	—	32	N/500 $NaHCO_3$	7.1
	Casein C	1.00	—	25	N/500 $NaHCO_3$	7.1
	Casein C	1.20	—	36	N/500 $NaHCO_3$	7.1
	Casein C	2.00	—	39	N/500 $NaHCO_3$	7.1
1-17-23	Casein D—5 mgm.	1.20	—	22	N/500 $NaHCO_3$	7.1
	Casein D	0.50?	—	26	N/500 $NaHCO_3$	7.1
	Casein D	1.00	—	24	N/500 NaOH	>8.0
	Casein D	1.10	—	31	N/500 NaOH	>8.0
1-30-23	Casein D	—	0.60	24	N/500 $NaHCO_3$	7.1
	Casein D	—	0.10	31	N/500 $NaHCO_3$	7.1
1-30-23	Casein E—5 mgm.	—	0.60	28	N/500 $NaHCO_3$	7.1
	Casein E	1.80	—	23	N/500 $NaHCO_3$	7.1
1-30-23	Casein F—5 mgm.	1.30	—	22	N/500 $NaHCO_3$	7.0
	Casein F	—	0.20	27	N/500 $NaHCO_3$	7.0
2-19-23	Casein F	0.50?	—	36	N/500 $NaHCO_3$	7.1
	Casein F	0.90	—	27	N/500 $NaHCO_3$	7.1
2-19-23	Casein G—5 mgm.	0.70	—	38	N/500 $NaHCO_3$	7.1
	Casein G	1.10	—	39	N/500 $NaHCO_3$	7.1

TABLE 3—*Concluded*

DATE OF EXPERIMENT	LOT OF CASEIN	AMOUNT OF REACTION		NUMBER OF ANIMALS USED	SOLVENT	pH
		+	−			
		°C.	°C.			
5-30-23	Casein G	1.10	−	30	N/500 $NaHCO_3$	7.2
	Casein G	0.90	−	25	N/500 $NaHCO_3$	7.2
	Casein G	−	0.50?	33	N/500 $NaHCO_3$	7.2
	Casein G	−	0.20	32	N/500 $NaHCO_3$	7.2
3-23-23	Casein H	−	0.20	41	N/500 $NaHCO_3$	6.5
	Casein H	−	0.20	38	N/500 $NaHCO_3$	6.5
	Casein H	−	0.20	39	N/500 $NaHCO_3$	6.5
	Casein H	−	0.40	22	N/500 $NaHCO_3$	6.5
	Casein H	−	0.20	36	N/500 $NaHCO_3$	6.5
	Casein H	−	0.20	40	N/500 $NaHCO_3$	6.5
4- 9-23	Casein H	−	0.30	32	N/500 $NaHCO_3$	6.8
	Casein H	−	0.50	39	N/500 $NaHCO_3$	6.8
3-30-23	Casein I	−	0.20	26	N/500 $NaHCO_3$	7.5
	Casein I	−	0.70	28	N/500 $NaHCO_3$	7.5
	Casein I	−	0.30	40	N/500 $NaHCO_3$	7.5
	Casein I	−	0.30	41	N/500 $NaHCO_3$	7.5
	Casein I	−	0.40	33	N/500 $NaHCO_3$	7.5
6- 5-23	Casein I	1.10	−	40	N/500 $NaHCO_3$	7.3
	Casein I	1.50	−	42	N/500 $NaHCO_3$	7.3
	Casein I	1.00	−	26	N/500 $NaHCO_3$	7.3
4- 9-23	Casein J	0.90	−	22	N/500 $NaHCO_3$	7.1
	Casein J	1.50	−	24	N/500 $NaHCO_3$	7.1
	Casein J	1.20	−	41	N/500 $NaHCO_3$	7.1
	Casein J	0.80	−	38	N/500 $NaHCO_3$	7.1
	Casein J	−	0.50	28	N/500 $NaHCO_3$	7.1
6- 5-23	Casein K	1.00	−	30	N/500 $NaHCO_3$	7.1
	Casein K	0.90	−	39	N/500 $NaHCO_3$	7.1
	Casein K	1.10	−	44	N/500 $NaHCO_3$	7.1

out all the experiments with casein there was never any indication of anaphylaxis or immunity due to former injections into the same animal.

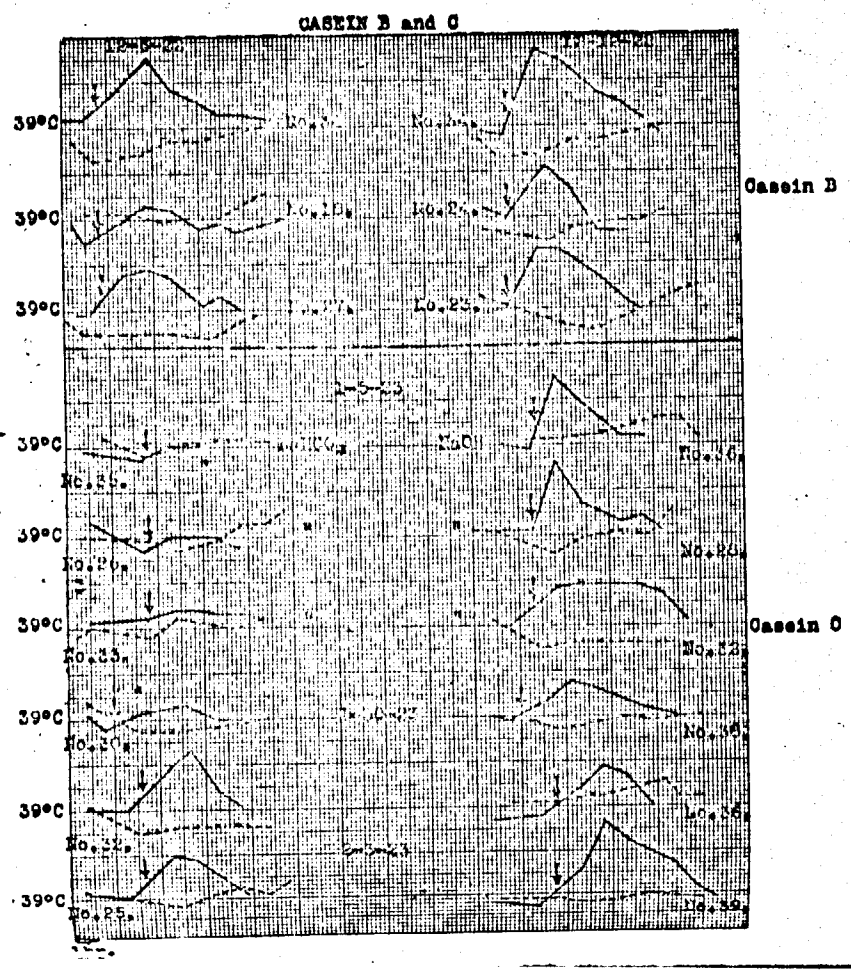
Various types of purification were studied in order to determine whether the febrile reaction was due to the protein itself or to a contamination product of the protein. For example, casein A was redissolved in alkali and reprecipitated with acid five times but still gave a fever when injected. As seen from the analyses—ash = 3.38 per cent, moisture = 4.39 per cent, fat = 0.2 per cent, and nitrogen = 14.7 per cent—casein B was practically fat-free and this would indicate that fat as an impurity is not the cause of fever.

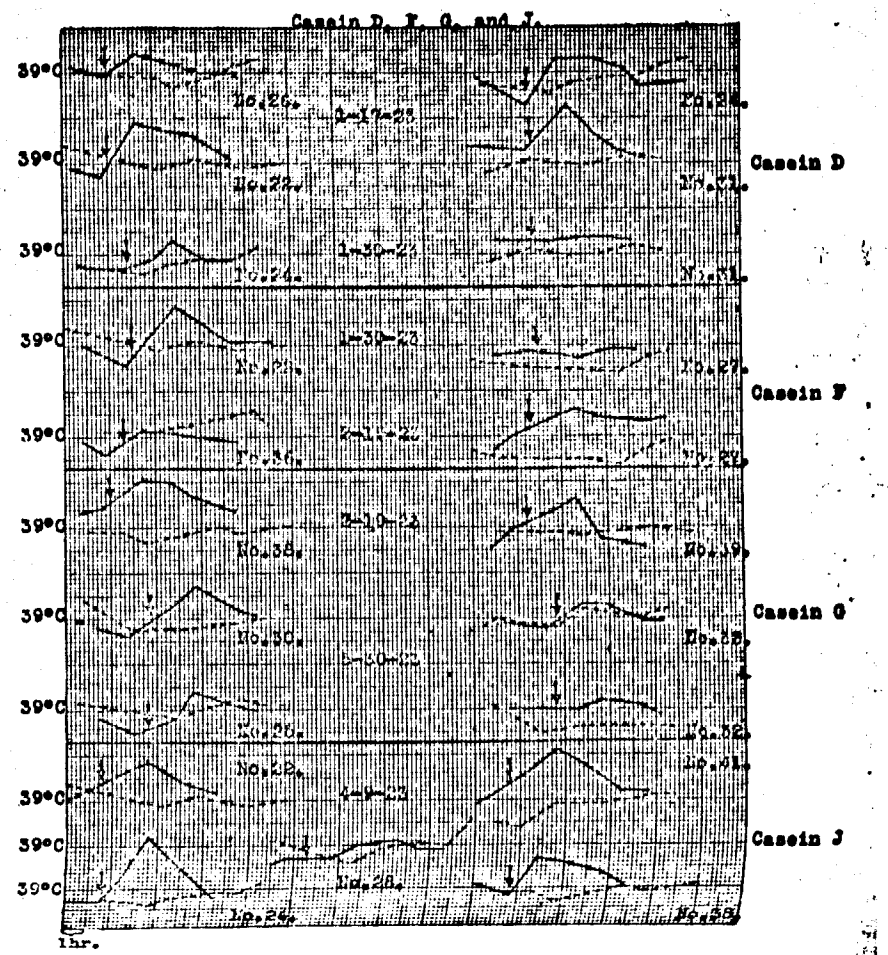
Casein B was then freed of salts by Loeb's (15) method—that is, by washing it six times with water, the pH of which was 4.6 or the same as the isoelectric point of casein. Each time it was thoroughly mixed with the water by grinding in a mortar for five to ten minutes, then filtered and finally dried by means of 70 per cent and 95 per cent alcohol, and ether. The isoelectric water used was made from freshly distilled water and was shown in two tests on rabbits to have no fever-producing power. This lot of casein C was therefore very low in ash—0.4 per cent—and might possibly have contained less pyrogenic contamination by virtue of its being washed with inactive water. The results with this preparation were the most promising so far obtained. For example, when dissolved in $NaHCO_3$, as in all previous experiments, and injected, it gave no temperature rise. However, in order to test the effect of a different solvent the casein was dissolved in NaOH but in this case fever was obtained. Twenty-five days later the same lot of casein, dissolved in $NaHCO_3$, was tested and this time gave a fever in one rabbit but not in another. Four days after this, four rabbits responded with a fever. This apparent development of a fever-producing property in an originally inactive casein is significant and is further evidence against the importance of the casein itself.

An attempt to repeat the experiment by preparing another lot of ash-free casein D with the same technic, however, was unsuccessful. Even greater care was employed in the preparation of this casein and Cohn's (7) method of redissolving at a pH of 7.0 in order to eliminate casein in combination with divalent ions was used. It was precipitated then at a pH of 4.7, washed with water at a pH of 4.7, and this process repeated six times. The ash content was only 0.1 per cent but nevertheless febrile reactions were obtained, indicating that the salt impurities are not the important factors. Forty-four days later this casein dissolved in $NaHCO_3$ gave two negative reactions. The apparent inactivation of the casein with time is unexplainable and, of course, directly opposed to the results obtained with the previous lot of casein.

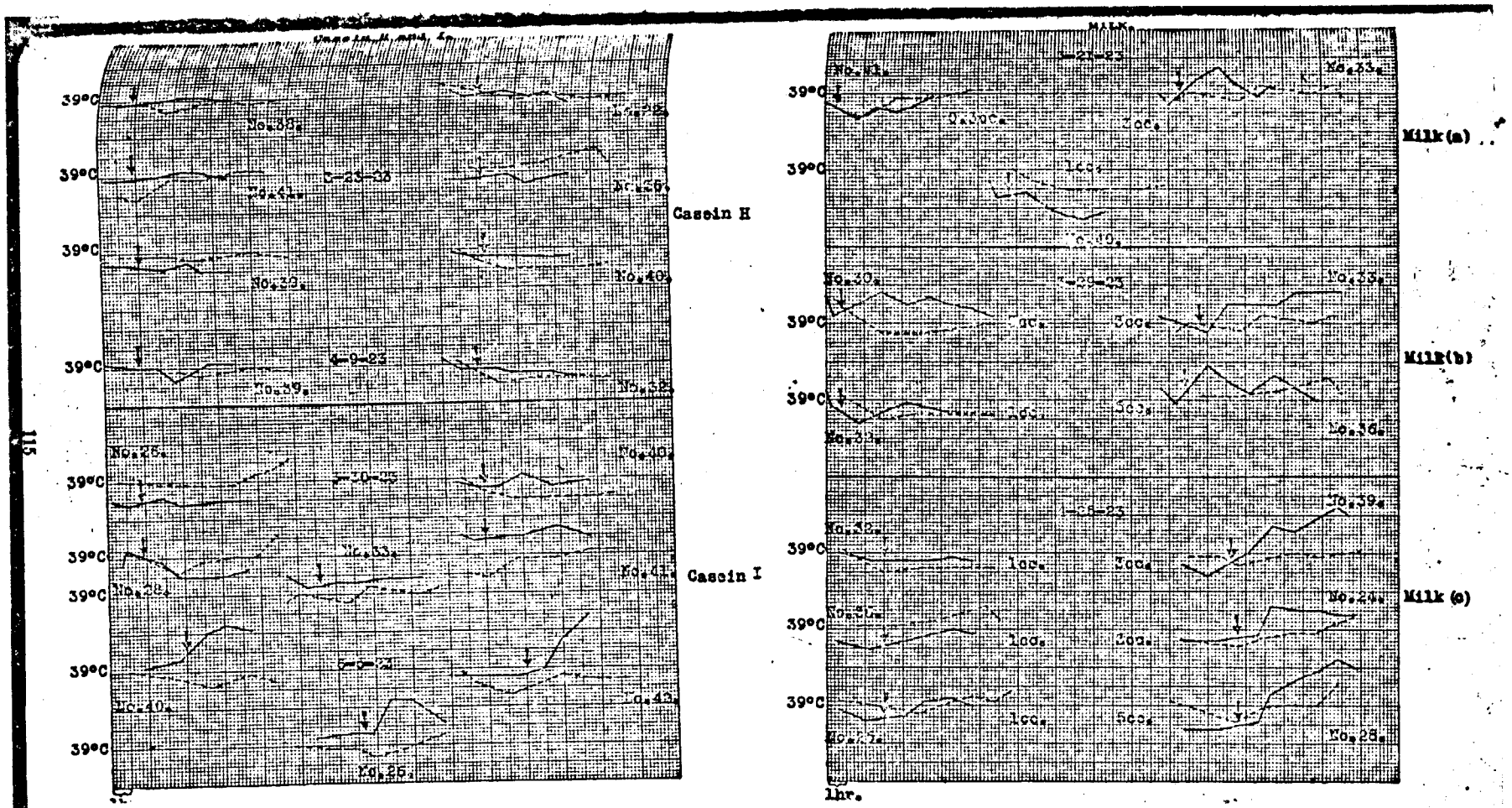
Two other samples of casein D and E, the latter of which was prepared at the Connecticut Experiment Station, gave negative and positive reactions.

Another type of purification suggested by Vaughan's (28) work in which he ascribed the toxic portion of Witte "peptone." to the alcohol-soluble fraction was undertaken. For example, casein C was thoroughly washed by grinding in a mortar with absolute alcohol six times, but this preparation —casein G—still produced a fever. Extraction with hot alcohol did not prove any more efficacious in rendering casein inactive. Casein C after being boiled with 95 per cent alcohol three times for ten-minute periods and then being filtered each time still gave a fever—casein J.


CASEIN B and C
39°C
Casein B
Casein C


Casein D, F, G, and J.
39°C
Casein D
Casein F
Casein G
Casein J
1hr.

1339



Charts A-D. The dotted lines represent normal temperature curves. The number of each animal is found with its curves. The arrows indicate the time at which the injections were made.

Therefore the fever-producing property of casein would not seem to be due to adhering fat, salts or an alcohol-soluble substance. But the fact that several negative reactions were obtained after certain types of purification led one to suspect that the fevers were due to a contaminating substance rather than to the casein itself. Since bacteria were the source of the pyrogen in water, may not this also be the case with casein? Therefore, instead of trying to remove a pyrogenic substance already present in the casein, attempts were made to prepare a casein in which bacterial contamination was prevented from occurring.

TABLE 3

DATE OF EXPERIMENT	LOT OF MILK	AMOUNT OF REACTION		NUMBER OF ANIMAL USED	AMOUNT INJECTED
		+	−		
		°C.	°C.		cc.
3-21-23	Milk *a* fresh	−	0.40	41	0.3
	Milk *a*	−	0.70	40	1.0
	Milk *a*	1.00	−	33	3.0
3-29-23	Milk *b* fresh	−	1.30	32	1.0
	Milk *b*	0.60	−	30	3.0
	Milk *b*	1.00	−	33	3.0
	Milk *b*	1.00	−	36	5.0
4-25-23	Milk *c* fresh	−	0.20	32	1.0
	Milk *c*	−	0.50	36	1.0
	Milk *c*	−	0.60	25	1.0
	Milk *c*	1.80	−	39	3.0
	Milk *c*	0.90	−	24	3.0
	Milk *c*	1.80	−	28	5.0

Milk *a* was obtained under as sterile conditions as possible. A healthy Jersey cow was led out doors, brushed, and the udder sterilized with surgical technic. The milker's hands were similarly sterilized. A pint of milk was discarded and then about a quart was milked into a sterile bottle. The milk was then brought to the laboratory within an hour. The authors wish to express appreciation to Miss Elizabeth Carey for obtaining the milk in the manner described.

A nutrient broth culture showed slight contamination with streptococcus lactis.

Three rabbits were injected with the milk *a*—in 0.3 cc., 1 cc. and 3 cc. doses respectively—and a slight fever was obtained only in the case of the 3 cc. dose. (See table 3.)

From the remainder of the milk, casein H was prepared as quickly as possible.

A dilute solution of hydrochloric acid was made by adding a few drops of concentrated HCl to a water which had just been freshly distilled and shown to be inactive when injected into a rabbit. With this acid the casein was precipitated

at its isoelectric point—pH 4.6—from the milk during constant stirring. The casein was then filtered off with suction, ground in a mortar several times with some of the freshly distilled water, the pH of which was 4.7 and filtered each time. Following this the casein was treated similarly twice with alcohol and then put into ether. The ether mixture was stood on a radiator and allowed to come to a boil, then filtered. This was repeated three more times and then the casein was treated repeatedly with cold ether and finally dried. Although the milk was rich in fat the casein prepared from it was a fine white powder and probably did not contain much fat.

Two days afterward, a 0.1 per cent sodium bicarbonate solution of this casein H was prepared in the usual way and injected. Six rabbits gave absolutely negative results. (See table 2.) Seventeen days later the same casein was tested and still found to be negative.

On another day a sample of fresh sterile milk *b* was obtained with the same technic as described above, and when plated it was found to contain only 3000 to 4000 organisms per cubic centimeter. The injection of 1 cc. of this milk again failed to produce a reaction while 3 and 5 cc. doses did produce a fever. Casein I prepared immediately from this milk in the manner described for casein H and injected on the following day gave no reactions in five rabbits, thus confirming in detail the results obtained in the former experiment. However, when tested again in sixty-six days, positive reactions were obtained. The casein had been kept in a clean, but non-sterile, well-stoppered bottle and it is possible that bacterial contamination may be the cause of the development of a fever-producing property on standing.

However, if casein is prepared from milk in which there are relatively few bacteria and if it is made and tested before the latter have had time to produce a toxic substance in any quantity, no fever is elicited by the injection of the casein. The inactivity of the casein can scarcely be due to a denaturizing effect on the protein, since it was never in contact with acid or alkali beyond the concentration equivalent to its isoelectric point, nor was it subjected to drastic heat or cold at any time. When warmed with the ether the temperature naturally never rose above that of the boiling point of ether. Furthermore, a denaturization cannot be attributed to alcohol treatment since alcohol extraction did not inactivate caseins G and J.

Since non-fever-producing casein can be made from fresh milk and since the milk from which it was made nevertheless gave a fever when injected in doses larger than 1 cc., it was attempted to determine whether or not even this fever could be eliminated if the milk which was taken under sterile conditions was boiled immediately. Such boiling would kill the few bacteria present and would thus prevent a possible formation of the toxic substance before it reached the laboratory to be tested. Milk *c* treated in this way, however, gave the same results as the two previous samples—that is, fever with doses above 1 cc.

Therefore the following confirmed facts can be stated in regard to the fever-producing properties of milk. There is something in milk which produces a fever in rabbits in doses greater than 1 cc. This is not a bacterial product which develops in the milk in vitro. It is possible, however, that such a product may be formed in vivo since it is practically impossible to obtain sterile milk and some organisms live in the ducts of the mammary gland. From previous work it can be seen that the casein, sugar, salts and fat constituents of the milk are not responsible for the fever. But the many other components of the milk, such as proteins, other than casein, and possibly some unknown factors are yet to be studied.

The fact that casein could be successfully prepared from milk in such a manner as to be free from fever-producing properties and still not be denaturized, inspired an attempt to prepare egg proteins, edestin and hemoglobin similarly.

Ovalbumin crystallized in the usual way in nine different cases produced positive reactions, and this includes experiments in which the hydrogen ion concentration of the solution was varied from more acid than pH 5.2 to more alkaline than pH-8.0 If the purified protein made from egg white produces a fever, it might be expected that the egg white itself, if injected in a dose sufficient to contain an equivalent amount of ovalbumin, would also produce a fever. Such an experiment was performed.

A strictly fresh egg was sterilized according to Schottelius' (24) method. It was washed with freshly distilled water, which was also sterile. The shell was then pricked with a sterile needle and some of the egg white drawn into a syringe. One-tenth cubic centimeter of the egg white, equivalent to 12 mgm. of ovalbumin, was injected into the ear vein of a rabbit, and then another syringe was quickly transferred to the needle and 5 cc. physiological saline were injected. Two-tenths cubic centimeter, equivalent to 24 mgm. of ovalbumin, was injected in the same manner into another rabbit. Neither of these rabbits showed the slightest temperature fluctuation. (See table 4.)

This absence of a reaction with fresh, sterile egg white can scarcely be explained by assuming that the salts in the egg white inactivated the protein, since similar salts have been tested previously with and without other proteins and no indication of such a phenomenon has ever been detected. Hydrogen ion concentration cannot explain it on the basis of former experiments. Some change had unquestionably been produced in the protein in the process of isolation that had rendered it capable of disturbing the heat-regulating mechanism of the rabbit. In view of the experience with casein and with the pyrogenic substance in water, are we not justified in asking whether or not this same pyrogenic substance might be the ultimate cause of the fever-producing power of samples of crystallized ovalbumin?

TABLE 4

DATE OF EXPERIMENT	PROTEIN INJECTED	AMOUNT INJECTED	TEMPERATURE VARIATION +	TEMPERATURE VARIATION −	pH	NUMBER OF ANIMAL	SOLVENT
		mgm.	°C.	°C.			
12- 8-22	Crys. Ovalbumin	5	1.35	–	6.6	22	Water
	Crys. Ovalbumin	5	2.50	–	6.6	27	Water
	Crys. Ovalbumin	5	1.80	–	6.6	31	Water
2- 9-23	Crys. Ovalbumin	5	1.90	–	4.6	33	Water
	Crys. Ovalbumin	5	1.80	–	4.6	22	Water
	Crys. Ovalbumin	5	1.10	–	6.5	26	Water
	Crys. Ovalbumin	5	2.00	–	6.5	28	Water
	Crys. Ovalbumin	5	2.40	–	8.0	38	Water
	Crys. Ovalbumin	5	1.60	–	8.0	30	Water
1-17-23	Fresh egg white	24	–	0.20	–	23	0.8 per cent NaCl
	Fresh egg white	12	–	0.70	–	33	0.8 per cent NaCl
4-25-23	Ovoglobulin (a) + ovomucin	5	–	0.20	>8.0	38	N/500 NaOH
	Ovoglobulin (a) + ovomucin	5	–	0.50	>8.0	41	N/500 NaOH
4-24-23	Ovalbumin (a) + conalbumin + ovomucoid	5	–	0.50	>8.0	33	N/500 NaOH
	Ovalbumin (a) + ovomucoid + conalbumin	5	0.80	–	>8.0	40	N/500 NaOH
	Ovalbumin (a) + ovomucoid + conalbumin	5	–	0.20	>8.0	22	N/500 NaOH
5-24-23	Ovoglobulin (b) + ovomucin	5	1.40	–	>8.0	41	N/500 NaOH
	Ovoglobulin (b) + ovomucin	5	0.90?	–	>8.0	26	N/500 NaOH
	Ovoglobulin (b) + ovomucin	5	1.30	–	>8.0	31	N/500 NaOH
	Ovoglobulin (b) + ovomucin	5	0.70	–	>8.0	42	N/500 NaOH
5-24-23	Ovalbumin (b) + conalbumin + ovomucoid	5	1.30	–	>8.0	33	N/500 NaOH
	Ovalbumin (b) + conalbumin + ovomucoid	5	0.70	–	>8.0	40	N/500 NaOH
	Ovalbumin (b) + conalbumin + ovomucoid	5	1.60	–	>8.0	32	N/500 NaOH
	Ovalbumin (b) + conalbumin + ovomucoid	5	1.20	–	>8.0	22	N/500 NaOH
5-30-23	Ovalbumin (b) + conalbumin + ovomucoid	5	1.20	–	>8.0	22	N/500 NaOH
	Ovalbumin (b) + conalbumin + ovomucoid	5	0.80	–	>8.0	26	N/500 NaOH
6- 8-23	Ovoglobulin (c) + ovomucin	5	–	0.50	>8.0	38	N/500 NaOH
	Ovoglobulin (c) + ovomucin	5	–	0.40	>8.0	40	N/500 NaOH
	Ovoglobulin (c) + ovomucin	5	–	0.40	>8.0	26	N/500 NaOH
6- 8-23	Ovalbumin (c) + conalbumin + ovomucoid	5	0.90?	–	>8.0	42	N/500 NaOH
	Ovalbumin (c) + conalbumin + ovomucoid	5	1.30	–	>8.0	39	N/500 NaOH
	Ovalbumin (c) + conalbumin + ovomucoid	5	1.10	–	>8.0	33	N/500 NaOH

With this question in mind an attempt was made to prepare proteins in such a way as to prevent bacterial contamination during the preparation. Merely a gross separation of the proteins was made and there was no effort to isolate them in a highly purified state at first.

Eggs laid within the previous twenty-four hours were used and the whites were diluted with freshly distilled water to seven times their volume. The ovoglobulin and most of the ovomucin were precipitated and filtered off in this way. From the filtrate the remaining proteins, a mixture of ovalbumin, conalbumin and ovomucoid were precipitated with alcohol. These two fractions of egg proteins when dissolved in NaOH and injected gave no reactions.

The experiment was repeated but this time ovalbumin was obtained from fresh egg white by the usual $(NH_4)_2SO_4$ method except that the entire process was carried out in one day and consequently all the ovalbumin was not obtained as crystals and that part which was obtained was not repurified. The ovoglobulin plus ovomucin fraction was precipitated by dilution as before. With these two fractions of egg proteins, fevers were elicited. A 0.5 per cent $(NH_4)_2SO_4$ solution however, did not produce a febrile reaction.

One more attempt was made to repeat this experiment and the two fractions, an ovoglobulin plus ovomucin and an ovalbumin were prepared as in the previous experiment. Negative reactions were obtained with the globulin fraction but positive with the albumin fraction.

It is evident that more work should be carried out with the egg proteins but the incomplete evidence so far offered is promising and suggestive that some, if not all, of the egg proteins in their purified state will not produce fever.

The same suggestion was applied to a study of edestin from the hempseed. One lot of the protein, consistently gave no febrile reaction in eight experiments on four different days, as seen in table 5. A sample of edestin prepared at the Connecticut Experiment Station gave a marked fever. A third preparation, made in the usual way except that freshly distilled water was used throughout and the entire process was completed in one day, gave fever reactions. The inconstancy of the results with different lots of edestin points to the necessity for further experimentation with this protein, as in the case with the egg proteins, before conclusions can be drawn.

A few experiments were performed with whole blood, serum and hemoglobin. The blood from two dogs, *a* and *b*, was used but the results obtained were not consistent. As can be seen from table 6 the whole blood taken from dog *a* and injected immediately produced no reaction while that from dog *b* produced a fever. The serum injections always were followed by fever except in one case where there was a marked fall in temperature.

The hemoglobin was prepared on the same day that the whole blood was injected and it was tested the following day. The protein was obtained by laking the blood with freshly distilled water and then adding absolute

TABLE 5

DATE OF EXPERIMENT	PROTEIN INJECTED	TEMPERATURE VARIATION +	TEMPERATURE VARIATION −	NUMBER OF ANIMAL	SOLVENT USED	pH
		°C.	°C.			
5-23-22	Edestin A	–	0.50	8	10 per cent NaCl	6.6
	Edestin A	–	0.50	9	10 per cent NaCl	6.7
5-30-22	Edestin A	–	0.80	21	N/500 NaOH	>8.0
	Edestin A	–	0.60	24	N/500 NaOH	>8.0
	Edestin A	–	0.80	30	N/500 NaOH	>8.0
	Edestin B	–	0.30	22	8 per cent NaCl	6.8
	Edestin A	–	0.10	31	8 per cent NaCl	6.8
	Edestin B	–	0.70	32	8 per cent NaCl	6.8
1-29-23	Edestin B	1.60	–	25	N/500 NaOH	>8.0
	Edestin B	0.90	–	28	N/500 NaOH	>8.0
6- 8-23	Edestin C	1.10	–	44	10 per cent NaCl	
	Edestin C	0.60	–	41	10 per cent NaCl	
	Edestin C	0.90	–	30	10 per cent NaCl	

TABLE 6

DATE OF EXPERIMENT	PROTEIN INJECTED	TEMPERATURE VARIATION +	TEMPERATURE VARIATION −	NUMBER OF ANIMAL	AMOUNT INJECTED
		°C.	°C.		cc.
5-28-23	Whole dog blood *a*	–	0.40	36	3
	Serum dog blood *a*	0.80	–	22	4
6- 1-23	Hemoglobin *a*	–	0.70	42	5 (diluted)
	Hemoglobin	–	0.20	40	5 (diluted)
	Hemoglobin	–	0.60	41	5 (diluted)
	Hemoglobin	Died		22	5 (strong)
	Hemoglobin	–	0.60	26	5 (strong)
6- 4-23	Whole dog blood *b*	2.10	–	36	5
	Whole dog blood	0.50	–	31	2
	Serum dog blood *b*	0.90	–	26	3
	Serum dog blood	0.70	–	38	5
	Serum dog blood	–	1.10	32	5
6- 5-23	Hemoglobin *b*	–	0.80	33	5 (diluted)
	Hemoglobin	–	1.00	31	5 (strong)

alcohol. After standing in a freezing mixture, then centrifuging, washing, and dissolving in a small amount of water, the per cent of hemoglobin was determined colorimetrically. By dilute solution is meant one containing

5 mgm. hemoglobin in 5 cc. of solution; by strong, 375 mgm. in 5 cc. solution. The reactions with the dilute solutions were mostly negative, while with the strong solutions one animal died as if from shock, another gave a doubtful reaction, and a third showed a fall in temperature. The one which died, however, had been injected previously with serum from the same dog.

The results, therefore, are too scanty and variable to permit of any deductions except that the few negative reactions obtained here again point to a promise for future efforts.

SUMMARY AND CONCLUSIONS

Of twenty-one proteins, made and preserved in the usual manner, all gave febrile reactions when injected, except the alcohol-soluble ones. Of these latter—six in number—two gave negative results constantly, three gave some positive results, and one gave only positive reactions.

Casein when prepared in the usual manner always produced fever. The amount of casein injected did not seem to be an important factor since 50, 5 and 1 mgm. when injected in the same volume of fluid produced equally intense reactions. Furthermore there was never any evidence of anaphylaxis in the rabbits regardless of how many previous injections or at what intervals they were given.

Extensive processes of purification of casein showed that impurities such as fat, salts and alcohol-soluble products were not the cause of fever, but the few negative results obtained suggested the presence of a pyrogenic contamination in the reactive preparations.

With a knowledge of the cause of the pyrogenic property of some distilled waters, casein was prepared in a manner to prevent bacterial contamination. This preparation gave no fever. The experiment was duplicated. Non-pyrogenic casein, however, may become pyrogenic on standing under non-sterile conditions, again pointing to the bacterial nature of contamination.

Fresh, practically sterile milk, containing only 3000 to 4000 organisms per cubic centimeter, gave fever in doses larger than 1 cc. Therefore, either sufficient bacterial toxic products are formed in vivo in the milk or else there is some other constituent of the milk than fat, salts, and casein which is able to elicit fever in rabbits.

Fresh sterile egg white did not produce a fever. Although ordinary crystallized ovalbumin injections were followed by fever, some suggestive negative results were obtained with egg proteins prepared in a manner to eliminate bacterial contamination. Similarly promising but inconclusive negative results were obtained with certain samples of edestin and hemoglobin. While the results with these three proteins have not as yet

been adequately confirmed, they are presented as promising evidence and merely suggest to the authors that completely satisfactory conditions have not yet been obtained for their preparation as in the case with casein.

These results, together with the numerous negative reactions obtained with the alcohol-soluble proteins, where bacterial contamination would necessarily be checked by the method of preparation, and the knowledge of the cause of water fevers, would seem to indicate that protein per se may after all not be the cause of fever in the so-called "protein fevers."

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Effect of Casein, Lactalbumin, and Ovalbumin on 3-Methylcholanthrene-Induced Mammary Carcinoma in Rats [1, 2]

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SUMMARY—Diets containing large proportions (27–64%) of casein promoted the development of mammary adenocarcinomas in female rats of the Wistar strain receiving daily gastric instillations of 3-methylcholanthrene. This effect could be dissociated from effects of body-weight gain on the appearance of the tumors, which are inhibited by caloric restriction.—J Nat Cancer Inst 33: 243–253, 1964.

IN THE systematic study of a laboratory cancer model, the possible effects of dietary constituents are among the natural questions of interest. At the Fels Research Institute, we have been concerned with carcinoma of the breast induced in female Wistar rats by the gastric instillation of 3-methylcholanthrene (*1–3*). The present report summarizes experiments on the effects of casein, lactalbumin, and ovalbumin added as major sources of protein in the diet of the animals. A preliminary abstract of the work has been published (*4*).

MATERIALS AND METHODS

Female rats of the Wistar-derived colony were used. At the age of 4 to 5 weeks, when the animals weighed 60 to 80 g, they were included in a control or an experimental group, individually marked, and placed on one of the diets to be studied. Rats that received 3-methylcholanthrene were gastrically intubated daily, except Sundays; the carcinogen was dissolved in 0.5 cc of olive oil.

All animals were kept in individual metal cages, with an unlimited supply of water and the diet unless otherwise specified. They were weighed once a week and examined for the presence of tumors. The tumors were excised when they reached 1 to 2 cm in diameter, and the animals then observed for the appearance of additional tumors. In some specified groups the rats were killed when a second tumor appeared, but otherwise they were observed for 52 to 56 weeks unless their general physical condition was deteriorating.

All tumors were prepared for microscopy, and all those recorded proved histologically to be mammary adenocarcinomas of the type and with morphologic variations of those previously described (*1–3*).[5]

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[3] Deceased July 30, 1963.

[4] With the technical assistance of Dolores Thatcher, Mary Acuff, and Ronald Wieder.

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The basic laboratory diet was the Rockland complete rat/mouse diet. The ingredients, as provided by the manufacturer (Teklad, Inc., Monmouth, Ill.), are:

> Soybean meal, fish meal, dried skim milk, round oats, wheat standard middlings, ground yellow corn, ground barley, feeding oatmeal, ground whole wheat, dehydrated alfalfa meal, 1 percent animal fat, vitamin A palmitate, 0.5 percent dicalcium phosphate, 1 percent calcium carbonate, 1 percent salt. The average analysis is given as: protein, 24 percent; fat, 4 percent; fiber, 4.9 percent; carbohydrates, 56 percent; nitrogen-free extract, 52 percent; ash, 7.8 percent. The composition of minerals, vitamins, and amino acids are also available. The calculated caloric content is 542 per kilogram.

The special diets were prepared by the Nutritional Biochemical Company, Cleveland, Ohio, and their compositions were as follows:

Diet No. 1:
- 64% Vitamin-free casein
- 22% Sucrose
- 8% Vegetable oil
- 2% Brewer's yeast, USP
- 4% Salt mixture, USP, No. 2, plus Vitamine-diet fortification mixture.

Diet No. 2:
- 27% Vitamin-free casein
- 59% Sucrose
- Oil, yeast, salt, and vitamin mixture as in diet 1.

Diet No. 3:
- 64% Purified lactalbumin
- 22% Sucrose
- Oil, yeast, salt, and vitamin mixture as in diet 1.

Diet No. 4:
- 64% Ovalbumin
- 22% Sucrose
- Oil, yeast, salt, and vitamin mixture as in diet 1.

Diet No. 5:
- 13.5% Vitamin-free casein
- 13.5% Ovalbumin
- 59% Sucrose
- Oil, yeast, salt, and vitamin mixture as in diet 1.

Diet No. 6:
- 7% Vitamin-free casein
- 20% Ovalbumin
- 59% Sucrose
- Oil, yeast, salt, and vitamin mixture as in diet 1.

The acceptance of these diets by the rats was determined by the weekly body-weight increment. In addition, the actual amounts consumed were measured for 9 to 10 days in groups of mature female rats of approximately the same age and weight. The mean values per rat per day were:

Laboratory diet (Rockland)	14.8 g
Diet 1 (64% casein)	13.4 "
Diet 2 (27% casein)	12.8 "
Diet 3 (64% lactalbumin)	10.0 "
Diet 4 (64% ovalbumin)	8.4 "

The specific conditions of the individual experimental groups and the results of each experiment are presented together for convenience.

EXPERIMENT 1

Groups of female rats were placed on the usual laboratory diet, on the 64 percent casein and 27 percent casein diets (*see* diets 1 and 2). No methylcholanthrene or other carcinogen was given to these animals.

The results are presented in table 1. One of 54 rats on the laboratory diet developed 2 breast cancers at 13 months of age. On the 64 percent casein diet, 6 of 66 rats developed breast cancer between 28 and 48 weeks of the experiment, and of these 6 animals, 2 had 2 breast cancers and 1 had 3 breast cancers. Another rat had a mammary fibroadenoma at 40 weeks. On the 27 percent casein diet, 2 of 24 rats had breast cancers, and in 1 of these 2 additional breast cancers appeared. One fibroadenoma was noted at the 44th week of the experiment.

The body weights of the animals in the 3 groups are also given in table 1. The mean body weight at 48 weeks was significantly lower on the laboratory diet (254 g, SD 39.8) than on the 64 percent casein diet (285 g, SD 37.2).

The proportion of rats with breast cancers is higher in the 2 casein-diet groups than in the controls, but the difference fails to reach statistical significance. Furthermore, the greater body weight attained by the rats that received casein would suggest that the observed effect may be attributable to caloric or other food restriction in the rats on the usual laboratory diet.

EXPERIMENT 2

All female rats in both groups of this experiment were given a single dose of 15 mg 3-methylcholanthrene in 1.5 cc olive oil by gastric tube. They were then placed on either the laboratory diet or diet 1, containing 64 percent casein.

The results are given in table 2. There were

TABLE 1.—Mammary carcinoma in female Wistar rats maintained on 3 diets—no methylcholanthrene given (expt. 1)

Group and diet	Weeks:	0	4	8	12	16	20	24	28	32	36	40	44	48	52	Breast tumors Number	Breast tumors Percent	Additional breast tumors Number	Additional breast tumors Tumors/rat	Other tumors
1 Rockland	Rats alive	54	54	54	54	54	54	54	52	52	52	52	51	51	30					
	Rats with breast cancer														1	1	2	1	0.03	0
	Mean body weights	75	153	186	199	210	215	222	230	233	237	241	242	254						
2 64% Casein	Rats alive	66	66	66	66	64	62	62	61	61	61	60	60	59	0					
	Rats with breast cancer								2	2	1			1		6	10	4	0.15	1F*
	Mean body weights	72	144	181	198	210	223	228	241	246	257	265	272	284						
3 27% Casein	Rats alive	24	24	24	24	24	23	23	23	23	23	23	22	22	6					
	Rats with breast cancer									1			1			2	10	4	0.25	1F*
	Mean body weights	71	152	174	190	204	213	223	236	246	250	254	266	275						

*F = fibroadenoma of breast.

TABLE 2.—Mammary carcinoma in female Wistar rats receiving a single gastric instillation of 15 mg methylcholanthrene and maintained on 2 diets (expt. 2)

Group and diet	Weeks:	0	4	8	12	16	20	24	28	32	36	40	44	48	52	Breast tumors Number	Breast tumors Percent	Additional breast tumors Number	Additional breast tumors Tumors/rat	Other tumors
4 Rockland	Rats alive	22	22	22	22	22	22	22	21	21	21	21	21	21	16					
	Rats with breast cancer											1	1			2	9	2	0.2	1L*
	Mean body weights	76	143	178	194	204	214	217	220	225	230	231	238	242						
5 64% casein	Rats alive	22	21	21	21	20	20	20	20	20	20	19	19	19	0					
	Rats with breast cancer									1			1	2		4	19	6	0.3	0
	Mean body weights	77	150	185	197	207	219	226	230	233	247	259	268	264						

*Lymphoma.

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twice as many tumors in the casein-fed group as in the laboratory-diet group, but the difference is not statistically significant. Also, the weight increment of the casein-fed group was again above that of the laboratory-diet group, as presented in table 2, although the difference is not statistically significant at 48 weeks (264, SD 32.4, vs. 242, SD 50.0).

EXPERIMENT 3

The rats in this experiment were given intragastric instillations of 5 mg of 3-methylcholanthrene in 0.5 cc of olive oil daily for 10 days, for a total dose of 50 mg. They were placed on 1 of 3 diets on the day of the first instillation of methylcholanthrene, 20 to 23 animals per group. The response of 2 groups in tumors and in body weight of animals on the laboratory diet was almost identical, and the responses of 2 groups on 64 percent casein (diet 1) were almost the same. For presentation in table 3, the 2 groups on each diet were combined. In the 27 percent casein groups (diet 2), however, 1 group had a significantly greater weight increment (317 g, SD 60.0, vs. 250 g, SD 28.5), and they are summarized separately. We do not know the reason for this difference in weight gain, shown in group 8 of table 3, but this fortuitous occurrence did provide additional information on the role of body weight in mammary carcinogenesis.

The weight increments during the experiment are indicated in table 3. These are identical for the groups on laboratory diet and on the 64 percent casein diet and for 1 of the 2 groups on 27 percent casein. Comparison of groups 8 and 9, on the 27 percent casein, shows a significant difference in weight, but the proportions of rats that develop mammary cancer are similar, although the small difference favors the group with the greater weight increment. The mammary tumor responses in the 3 casein-diet groups are not statistically different and were combined, giving a 42 percent cancer occurrence in 85 rats. This is significantly higher than the 15 percent response in 46 rats maintained on the laboratory diet.

Under the conditions of the carcinogenic stimulus of 50 mg of 3-methylcholanthrene, therefore, rats on diets containing 27 or 64 percent casein manifested a significantly greater carcinogenic response than animals fed a satisfactory laboratory diet. This difference was not related to differences in body-weight gain, and thus, presumably, was not attributable to dietary restriction.

EXPERIMENT 4

The conditions of this experiment were the same as in experiment 3. Rats received 5 mg 3-methylcholanthrene for 10 days for a total of 50 mg. The same control group as for experiment 3 was used in this series. At the initiation of methylcholanthrene instillations, the animals were placed on diets 3, 4, 5, or 6, containing lactalbumin, ovalbumin, and casein in the proportions indicated previously.

In this series, the purpose was to determine the effects of protein sources other than casein, and to study further the effect of weight-gain restriction on the carcinogenic reaction. After it was noted that the rats on ovalbumin gained less weight than on the laboratory diet, 25 rats were placed on 64 percent casein diet restricted to the amount that allowed these animals to gain weight at approximately the same rate as the rats on ovalbumin (groups 11 and 12). The animals of group 12 were weighed daily, and the amount of food for daily consumption was restricted to a level indicated by the weekly weight gain of group 11.

The results are given in table 4. The weight increments were almost identical for all the groups except group 11, on the 64 percent ovalbumin, and group 12, on the restricted 64 percent casein diet.

The number and the proportion of animals that developed breast cancers are not sufficient to yield statistical significance of the differences, yet the results are consistent with the observations of total investigations. In contrast with the significantly higher cancer occurrence recorded on the 64 and 27 percent casein diets of experiment 3, 64 percent lactalbumin diet produced a minor or no effect.

On the 64 percent ovalbumin diet there was, if anything, an inhibition of carcinogenic effect. This was in part or in whole related to the decrease in weight gain, which is significantly lower in this group 11 than in group 6 (at 48 weeks, 205 g, SD 21.7, vs. 249 g, SD 21.6).

An important aspect of the investigation is

TABLE 3.—Mammary carcinoma in female Wistar rats receiving 5 mg 3-methylcholanthrene for 10 days (total dose 50 mg) and maintained on Rockland rat diet and casein diets

Group and diet	Weeks:	0	4	8	12	16	20	24	28	32	36	40	44	48	52	Breast tumors: Number	Breast tumors: Percent	Second tumors	Other tumors
6 Rockland	Rats alive	46	46	46	46	46	46	44	44	44	43	41	41	39	6				
	Rats with breast cancer				2		1		1			1	1		1	7	15	2	1F* 1L†
	Mean body weights	70	139	168	185	202	207	215	221	227	230	235	242	249					
7 64% Casein	Rats alive	42	42	41	41	40	39	38	38	37	35	35	35	35	8				
	Rats with breast cancer				2	2	3	2	1	1	1	1		2		15	36	6	2F*
	Mean body weights	70	141	173	194	204	206	213	221	226	231	241	246	245					
8 27% Casein, high weight	Rats alive	22	22	22	22	22	22	22	21	21	21	20	18	18	5				
	Rats with breast cancer				1	3		2	2		2			2		12	55	4	1L†
	Mean body weights	70	156	192	212	227	232	241	254	258	271	289	301	321	317				
9 27% Casein, low weight	Rats alive	21	21	20	18	18	18	17	17	17	16	15	15	15	6				
	Rats with breast cancer				2				1	1	2	1		1	1	9	43	3	1F*
	Mean body weights	73	142	167	182	193	199	211	217	224	226	233	237	250					

*Fibroadenoma of breast.
†Lymphoma.

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TABLE 4.—Mammary carcinoma in female Wistar rats receiving 5 mg 3-methylcholanthrene for 10 days and maintained on diets with lactalbumin, ovalbumin, and restricted casein (expt. 4)

Group and diet	Weeks:	0	4	8	12	16	20	24	28	32	36	40	44	48	52	Breast tumors: Number	Breast tumors: Percent	Second tumors	Other tumors
6 Rockland	Rats alive	46	46	46	46	46	46	44	44	44	43	41	41	39	6				
	Rats with breast cancer				2		1		1			1	1		1	7	15	2	1F* 1L†
	Mean body weights	70	139	168	185	202	207	215	221	227	230	235	242	249					
10 64% Lactalbumin	Rats alive	21	21	20	20	19	19	19	19	19	19	19	18	18	0				
	Rats with breast cancer						1			1			1	1	1	5	24	0	2F* 1L†
	Mean body weights	75	147	173	185	196	202	220	212	214	225	236	235	252					
11 64% Ovalbumin	Rats alive	21	20	19	19	18	17	17	17	17	17	17	17	17	0				
	Rats with breast cancer								1							1	5	0	0
	Mean body weights	68	111	129	143	156	166	172	174	182	190	197	200	203	213				
12 64% Casein, restricted	Rats alive	25	24	24	22	22	22	22	21	21	21	21	21	21	8				
	Rats with breast cancer												1	1		2	8	1	0
	Mean body weights	75	120	128	139	155	166	177	182	179	187	196	204	210					
13 13.5% Casein, 13.5% ovalbumin	Rats alive	20	20	20	19	19	19	19	19	19	17	17	17	17	4				
	Rats with breast cancer				1	1	2		1		1		1			7	35	2	0
	Mean body weights	70	137	169	190	200	209	216	226	236	236	243	247	255					
14 7% Casein, 20% ovalbumin	Rats alive	23	23	23	23	23	23	22	22	20	20	20	19	18	6				
	Rats with breast cancer						1			3						4	17	0	2F* 2L†
	Mean body weights	69	135	165	186	199	204	208	220	226	233	247	247	242					

*Fibroadenoma of breast.
†Lymphoma.

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shown by the response of group 12, on the restricted 64 percent casein diet. The proportion of animals with cancer is the same as in the ovalbumin group. It is evident that the carcinogenic response is inhibited by restriction in food consumption at a level producing 20 percent less weight gain than the 64-casein or the laboratory diets fed *ad libitum*.

Groups 13 and 14, in which 13.5 and 7 percent casein were combined with ovalbumin, were intended to provide further information to one of two possibilities: 1) that casein contains some factors promoting carcinogenesis or 2) that ovalbumin contains some factors inhibiting carcinogenesis. The responses recorded do not deviate significantly from the control group on the laboratory diet, but there is a suggestion that on the 13.5 percent casein and 13.5 percent ovalbumin diet the proportion of rats with cancer lies intermediate between that of the laboratory-diet group 6 and the 27 and 64 percent casein groups 7, 8, and 9 of experiment 3. The proportion of cancers in animals on the diet of 7 percent casein and 20 percent egg albumin is identical as in the laboratory-diet group 6. These observations further support the impression that diets with 13.5 to 64 percent casein promote the carcinogenic reaction induced by methylcholanthrene, and that this effect is not attributable to general nutritional conditions reflected by body weight.

EXPERIMENT 5

In this experiment, a comparison was made of the carcinogenic response after a higher dose of 3-methylcholanthrene, given in 5 mg doses daily (except Sunday) gastric instillations for 9 weeks, for a total dose of 270 mg.

At the first dose of methylcholanthrene the rats were divided into 2 groups, one maintained on Rockland laboratory diet *ad libitum* and the other on diet No. 1, containing 64 percent casein, *ad libitum*. The animals were killed when a second cancer had appeared, unless general health deterioration was observed.

The results are presented in table 5. The differences in weights and in the proportion of breast cancers that developed are not significant. Nevertheless, even at this level of methylcholanthrene, all but 1 of 22 rats on the casein diet developed breast cancer, whereas on the laboratory diet, 5 of 24 animals remained free from cancer during the experiment.

In this experiment there also appeared 1 fibroadenoma of the breast and 2 lymphomas (1 lymphocytic and 1 myelocytic leukemia) in the casein-diet groups, and no other neoplasms in the group on the laboratory diet. The occurrences of tumors other than breast cancers in the previous 4 experiments were therefore summarized. Among 146 rats on the laboratory diet, 1 fibroadenoma of the breast and 2 lymphomas were observed. Among 239 rats on diets containing 13.5 to 64 percent casein, there were 6 fibroadenomas of the breast and 3 lymphomas. The differences are obviously not significant and fail to indicate that dietary variations in the experiments produced any effects on the occurrence of these neoplasms.

Among the rats that received no methylcholanthrene, in experiment 1, 2 of 144 developed fibroadenoma of the breast and none developed lymphomas. Of the 241 rats that received methylcholanthrene, for a total dose of 15 to 270 mg, 6 developed mammary fibroadenomas and 5 developed lymphomas. Again, the small number of these tumors preclude statistical manipulation, but the impression is that fibroadenomas were not affected, whereas a minimal increase in lymphomas may have occurred.

EXPERIMENT 6

In this experiment an attempt was made to gain insight into whether the enhancement of mammary carcinogenesis in rats maintained on high casein diets could be localized to the stage of carcinogenesis or to a later stage of development and growth of the neoplasms. For this purpose, 3 groups of rats were given 2 mg 3-methylcholanthrene daily (except Sunday) for 17 weeks, for a total dose of 204 mg methylcholanthrene. All were maintained on the Rockland diet until the 14th week of the experiment. At that time, group 18 was placed on diet 1, containing 64 percent casein, and group 19 on diet 2, with 27 percent casein. These diets were then continued for the course of the experiment, and the animals killed when they developed 2 mammary carcinomas.

TABLE 7.—Summary of results on casein-diet effect on breast cancer induction with 3-methylcholanthrene in female Wistar rats

	Rockland laboratory diet			64 Percent casein diet		
Methylcholanthrene (mg)	Number of rats	Mean body weight (g)	Breast cancer (%)	Number of rats	Mean body weight (g)	Breast cancer (%)
0	54	254	2	66	284	4
15	22	242	9	22	264	19
50	46	249	15	42	245	36
270	24	226	75	22	—	90

received a diet with 64 percent casein and were exposed to the carcinogenic stimulus of a range of doses of 3-methylcholanthrene administered through gastric tube. The data are quite consistent and include a greater number of breast cancers in rats that presumably received no methylcholanthrene as well as at all doses of the carcinogen. Of special significance, it seems to us, is that the clearest difference was obtained at the higher doses of methylcholanthrene, in which the body-weight increment of the casein-fed rats was identical with that in the laboratory-diet controls.

We conclude that casein contains some factors accelerating carcinogenesis induced in the breast with a polycyclic hydrocarbon. Perhaps this effect is also present in the presumably "spontaneous" cancers that appeared in rats not receiving methylcholanthrene. Of course, this Institute has used methylcholanthrene in large amounts since 1948, and it is possible that some undetected contamination of the diet or other environment occurred. Samples of the diet were sent to Dr. Hans Falk, of the National Cancer Institute, Bethesda, Maryland, for the detection of the presence of methylcholanthrene, and the results were negative. Nevertheless, we may have here an example of contamination of food by known or unknown carcinogens. A designed experimental model of this situation was reported by Huggins and Grand (*10*), who elicited subcutaneous sarcoma in rats fed methylcholanthrene when such rats were given injections of presumably inert materials, which thus localized the carcinogen at the site of injury.

Tannenbaum and Silverstone (*11*) in 1949 reported that the addition of 9 to 45 percent of casein to food did not affect the occurrence of "spontaneous" mammary carcinoma in mice of the C3H strain. This is not a contradiction of our findings, but an indication of a different effect under different circumstances. We wonder whether breast cancers in mice induced by different doses, or at different times of life, by the Bittner virus may provide results consistent with our findings in rats where a different carcinogenic stimulus was used.

The results of this investigation also provide ample demonstration of the clear effects of restricted diet on the inhibition of tumor development. Ovalbumin at levels of 64 percent was not as dietarily acceptable to rats as the casein or the laboratory diet, and resulted in a 20 percent reduction in weight increment of the animals. The number of breast cancers was reduced to below that seen with the laboratory diet. An identical effect was observed when the 64 percent casein diet was restricted so that the gain in weight was held down to that seen with ovalbumin.

An attempt was made to determine at what phase of the neoplastic process casein might be considered to have its effect. The incomplete evidence suggests that this more likely occurs at a phase beyond the actual carcinogenic transformation of the breast epithelium and thus in the subsequent development and growth of the neoplasm. It would be useful, in the further pursuit of this problem, to determine whether high casein diet influences the absorption of methylcholanthrene through the gastrointestinal tract and its known deposition in the fat pads surrounding the mammary epithelium (*12*).

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DIETARY REGULATION OF GLYCOLYTIC ENZYMES

I. ADAPTIVE CHANGES IN RAT JEJUNUM

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SUMMARY

1. The effects of dietary fructose, sucrose, glucose, casein and fasting upon the activity of several glycolytic enzymes [fructokinase (ketohexokinase, EC 2.7.1.3), hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1), glucokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.2), Fru-1-*P* aldolase (ketose-1-phosphate aldehyde-lyase, EC 4.1.2.7), Fru-1,6-P_2 aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) and aldose reductase (alditol:NADP oxidoreductase, EC 1.1.1.21)] were studied in the jejunum of rats.

2. Individual sugars (glucose, sucrose and fructose) have specific carbohydrate effects on certain jejunal glycolytic enzymes. Fructose had a specific adaptive effect upon fructokinase and Fru-1-*P* aldolase, while glucose exerted its adaptive effect upon hexokinase and glucokinase. The addition of calories in the form of casein to fasted rats caused a non-specific increase in the activity of all jejunal glycolytic enzymes studied.

3. Changes in rat jejunal glycolytic enzymes due to diet reflect those in rat liver.

4. The ratio of Fru-1,6-P_2/Fru-1-*P* aldolase activities in the liver was close to 1.0 for all diets whereas in the jejunum the ratio of activities is altered by diet. The fact that the jejunal activity ratio was lower than 1 in fructose-fed rats suggests that the intestine may contain an aldolase with different properties from those in other tissues.

INTRODUCTION

Dietary sucrose increases the activity of sucrase and maltase in the jejunum of rat and man[1-3]. In man fructose seemed to be the specific sugar responsible for this increased jejunal sucrase activity[3]. The feeding of a high-sucrose diet to rats enhanced fructose absorption[2]. Based upon the relative phosphorylation and absorption rates of several sugars in rat small intestine, it was suggested that intestinal hexokinase plays an integral part in the absorption mechanism of glucose[4]. These data suggested that fructose might affect specific glycolytic enzymes (fructokinase (ketohexokinase, EC 2.7.1.3), Fru-1-*P* aldolase (ketose-1-phosphate aldehyde-lyase, EC 4.1.2.7)). Similarly glucose might exert an adaptive effect upon its specific enzymes

(hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1), glucokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.2) and Fru-1,6-P_2 aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13)). To test this hypothesis, we studied the effects of sucrose, fructose, glucose, casein and fasting on the activity of fructokinase, Fru-1-P aldolase, Fru-1,6-P_2 aldolase, glucokinase, hexokinase and aldose reductase in the rat jejunum. Simultaneous assays were made in the liver.

MATERIALS AND METHODS

Assay animals. Male Holtzman rats weighing between 320 and 400 g were housed in individual cages with water *ad libitum*. 5 groups of 8 rats each were fasted for 3 days. One group was killed after fasting. Each of the remaining 4 groups were fed one of the following diets for 3 days: 68% by wt. of fructose, sucrose, glucose or casein. The composition of the diet is shown in Table I. No significant differences were noted in the weight gains and food consumption of the 4 groups refed after fasting.

Collection, storage and preparation of tissues. The rats were killed by cervical fracture and a portion of the jejunum just beyond the ligament of Treitz and liver was collected. The jejunum was slit longitudinally and the mucosa scraped from the intestine with a glass slide. The fresh jejunal mucosa was homogenized immediately. The livers were stored at −85° until the enzymes were assayed. The enzyme activities from tissue frozen for 2 weeks were similar to those in fresh tissue.

TABLE I

COMPOSITION OF THE DIETS

Ingredients	*Diets (% by wt.)*				
	1	*2*	*3*	*4*	*5*
Sucrose	68				Fasted only
Fructose		68			
Glucose			68		
Casein	17	17	17	85	
Crisco	10	10	10	10	
Salt mixture	4	4	4	4	
NBC vitamins	1	1	1	1	

The jejunal mucosa was weighed and homogenized in a Kontes–Duall grinder with 20 vol. of a Tris–EDTA buffer (pH 7.5) containing 20 mM Tris, 120 mM KCl, 5 mM $MgSO_4$ and 0.1 mM EDTA (disodium salt). The liver was weighed, crushed in a Harvard tissue press and then homogenized in a Potter–Elvehjem tissue grinder with a 20-fold vol. of the same buffer. The hepatic and jejunal homogenates were centrifuged at 104000 × *g* for 60 min in a Spinco model L-2 ultracentrifuge at 4°. Following aspiration of the surface lipid layer, the supernatant fluid was decanted and the resulting extract used for the enzyme assays.

Enzyme assays. Enzyme activities were assayed as follows: fructokinase[5], hexokinase and glucokinase[6], aldose reductase[7], Fru-1-P and Fru-1,6-P_2 aldolase[8]. Protein was determined by the method of LOWRY *et al.*[9].

RESULTS

The protein concentrations in the jejunum and liver are shown in Table II. The individual enzyme activities in the jejunum are shown in Tables III and IV. The activities of fructokinase and Fru-1-*P* aldolase were greatest in the fructose-fed group. The activities decreased in order with sucrose, glucose, casein and fasting, respectively. Hexokinase activity was highest in the glucose-fed rats and decreased in order with sucrose, fructose and casein, respectively. The jejunal glucokinase activity was highest with glucose and sucrose, less with fructose and least with casein and fasting. The Fru-1,6-P_2 aldolase activity was similar in the fructose-, glucose- and sucrose-fed rats. Aldose reductase was highest on glucose and lowest on fasting.

The relationships of the enzyme activities in the liver to the various diets (Tables IV and V) were similar to those in the jejunum except that the glucokinase activity in the livers of the sucrose-fed rats was intermediate between the glucose- and fructose-fed rats.

In both tissues, the enzyme activities were uniformly lowest in the fasted group. The enzyme activity in the casein-fed rats was slightly higher than that in the fasted group, but lower than that of the carbohydrate-fed rats. In both tissues, the aldose reductase activity was greatest in the glucose-fed rats and lower in the fructose- and sucrose-fed rats.

Comparison of the data for jejunal (Tables III and IV) and hepatic (Tables IV and V) enzyme activities reveals differences in the fasting activities of fructokinase and Fru-1-*P* aldolase which were higher in the liver. The maximal fructokinase activi-

TABLE II

PROTEIN CONCENTRATIONS OF THE HIGH-SPEED SUPERNATANTS OF LIVER AND JEJUNUM HOMOGENATES OF RATS FED DIFFERENT DIETS

Protein concentrations are expressed as mg protein per g tissue (mean ± S.E. for 8 rats).

Tissue	*Diet*				
	Fructose	*Sucrose*	*Glucose*	*Casein*	*Fasting*
Liver	116.6 ± 12.3	132.7 ± 10.3	110.9 ± 20.1	130.3 ± 10.4	108.7 ± 17.3
Jejunum	80.1 ± 8.9	84.6 ± 7.4	70.3 ± 8.4	92.0 ± 0.2	95.6 ± 0.8

TABLE III

EFFECT OF DIET UPON GLYCOLYTIC ENZYMES IN RAT JEJUNUM

Activities are expressed as nmoles of substrate metabolized per min per mg protein (mean ± S.E. for 8 rats).

Enzyme	*Diet*				
	Fructose	*Sucrose*	*Glucose*	*Casein*	*Fasting*
Fructokinase	41.51 ± 2.38	27.12 ± 1.25	15.39 ± 0.36	7.28 ± 2.48	4.50 ± 1.45
Hexokinase	1.69 ± 0.18	2.03 ± 0.26	4.18 ± 0.14	1.33 ± 0.10	0.91 ± 0.05
Glucokinase	3.68 ± 0.56	5.50 ± 0.36	5.13 ± 0.51	2.99 ± 0.26	2.29 ± 0.34
Aldose reductase	5.20 ± 0.42	5.69 ± 0.40	7.00 ± 1.77	4.15 ± 0.33	3.44 ± 0.23

ties (fructose diet) were the same for both tissues but the maximal Fru-1-*P* aldolase was greater in the intestine (fructose diet). The maximal glucokinase activity was greater in the liver than in the intestine (glucose diet) and in all dietary groups greater per cent of the glucose phosphorylating activity was accounted for by glucokinase in the liver than in the intestine.

TABLE IV

COMPARISON OF Fru-1,6-P_2 AND Fru-1-*P* ALDOLASE RATIOS IN RAT JEJUNUM AND LIVER

Activities are expressed as nmoles of substrate per min per mg protein (mean ± S.E. for 8 r.

Assay	*Diet*				
	Fructose	*Sucrose*	*Glucose*	*Casein*	*Fasting*
Jejunum					
Fru-1,6-P_2 aldolase	45.84 ± 4.48	40.79 ± 2.73	45.31 ± 3.44	22.47 ± 1.82	10.01 ± [illegible]
Fru-1-*P* aldolase	69.08 ± 3.22	45.15 ± 1.84	16.41 ± 1.60	9.20 ± 0.60	6.02 ± [illegible]
Fru-1,6-P_2/Fru-1-*P* aldolase ratio	0.66	0.90	2.76	2.44	1.66
Liver					
Fru-1,6-P_2 aldolase	30.00 ± 1.48	28.69 ± 1.97	30.90 ± 1.50	16.26 ± 2.00	14.31 ± 0.81
Fru-1-*P* aldolase	42.06 ± 1.65	29.26 ± 1.40	30.32 ± 2.00	16.46 ± 2.36	12.94 ± 0.86
Fru-1,6-P_2/Fru-1-*P* aldolase ratio	0.86	0.98	1.02	0.99	1.10

TABLE V

EFFECT OF DIET UPON GLYCOLYTIC ENZYMES IN RAT LIVER

Activities are expressed as nmoles of substrate metabolized per min per mg protein (mean ± S.E. for 8 rats).

Enzyme	*Diet*				
	Fructose	*Sucrose*	*Glucose*	*Casein*	*Fasting*
Fructokinase	43.81 ± 1.87	30.05 ± 1.26	23.09 ± 1.85	10.71 ± 1.98	10.12 ± 0.80
Hexokinase	2.40 ± 0.29	2.43 ± 0.20	5.29 ± 0.72	1.32 ± 0.22	0.61 ± 0.10
Glucokinase	6.65 ± 0.99	8.76 ± 0.98	13.98 ± 1.22	3.83 ± 0.95	2.28 ± 0.53
Aldose reductase	4.80 ± 0.48	3.71 ± 0.38	7.08 ± 0.81	2.06 ± 0.23	1.32 ± 0.09

The effects of the various dietary alterations on intestinal and hepatic aldolase activity are shown in Table IV. In comparison to the fasted group of rats the feeding of casein or any of the high carbohydrate diets increased the activity of both the Fru-1-*P* and Fru-1,6-P_2 aldolases. The activity of Fru-1-*P* aldolase was highest on the fructose diet whereas the Fru-1,6-P_2 aldolase activity was essentially the same on the glucose, sucrose or fructose diets. The ratio of activity of Fru-1,6-P_2 to Fru-1-*P* aldolase was different for different diets being 2.76 for the glucose diet in comparison to 0.66 for the fructose diet. This variation in the ratio of intestinal aldolase activities with different diets is not seen in the liver. In this tissue, the aldolase activity ratios are close to 1 for all diets, though the ratio falls to 0.86 on the fructose diet.

DISCUSSION

These results show clearly that jejunal glycolytic enzymes metabolizing a substrate are highest when that substrate is fed in the diet. In addition to the specific response of enzymes to their specific substrate there is a lesser increase when non-substrate carbohydrate is fed, *e.g.* increase in fructokinase when glucose is fed. These changes in jejunal glycolytic enzymes as a result of diet are parallel, in general, to the changes in the hepatic glycolytic enzymes on the same diet.

In jejunum, when fructose is fed, the fructose-metabolizing enzymes, fructokinase and Fru-1-*P* aldolase, reached their maximal activity. These are the enzymes initially concerned with the metabolism of fructose. Fru-1,6-P_2 aldolase is also increased by fructose, sucrose and glucose. It is interesting that Fru-1,6-P_2 aldolase may be in a pivotal position in the transformation of fructose to glucose. On the other hand, fructose had relatively little effect on hexokinase, glucokinase and aldose reductase. In contrast, glucose caused a maximal change in hexokinase, glucokinase, aldose reductase and Fru-1,6-P_2 aldolase and lesser changes in fructokinase and Fru-1-*P* aldolase.

In addition to the specific enzyme changes by their dietary substrates, changes were seen in enzymes by non-substrate dietary components. Glucose increased fructokinase and Fru-1-*P* aldolase and casein gave a greater overall response in all the enzymes as compared to the fasting state. Sucrose, except for Fru-1,6-P_2 aldolase changes, gave results intermediate between fructose and glucose.

In general, similar results occurred in hepatic enzymes. If dietary components affect hepatic and jejunal glycolytic enzymes the same it would be possible to obtain an index of hepatic enzyme changes by measuring jejunal enzymes. This may be of importance in the study of man where jejunal tissue is accessible, but hepatic tissue is not practical to obtain in sufficient quantity in serial fashion.

Mammalian tissues contain three different types of Fru-1-*P* and Fru-1,6-P_2 aldolases[10-12]. These three, referred to as Type A (muscle), B (adult liver) and C (brain), can be differentiated on the basis of differing electrophoretic and immunologic characteristics[11], by differing peptide patterns after tryptic digestion[10] and by different substrate specificities[13]. The enzyme activity ratio of Fru-1,6-P_2 to Fru-1-*P* is 50 for Type A, 1 for Type B and 10 for Type C[10]. The hybrid enzymes formed by mixing associated chains of Types A and B, A and C or B and C give activity ratios which intermediate between those for the parent aldolase. It has not been possible to arate Fru-1-*P* aldolase activity from Fru-1,6-P_2 aldolase[14-16]. Inhibition of aldolase ivity by specific antibodies to Types A and B has shown that adult rabbit intestine tains a mixture of Types A and B[17]. The data in Table IV indicate that rat intestine must contain a mixture of aldolases.

Aldolase A has little activity with Fru-1-*P* in comparison to Fru-1,6-P_2, so at an increase in Fru-1-*P* aldolase activity due to a change in aldolase A content uld be associated with a 50-fold greater increase in activity with Fru-1,6-P_2 as bstrate. The increases in the aldolase activity with either Fru-1-*P* or Fru-1,6-P_2 as bstrate in casein and glucose-fed rats (in comparison to fasting) could have been ue to an increase in both aldolases A and B. On the other hand, the activity ratios the aldolases in the fasted rats or the casein and glucose-fed rats could be an dication that the adult gut contains an aldolase similar to that in fetal liver and

hepatomas[16]. Most important is the fact that if fructose feeding in comparison to either the casein diet or fasting led to an inductive increase in intestinal aldolase B, the increase in activity with Fru-1-*P* as substrate should be associated with a similar increase in activity with Fru-1,6-P_2 as substrate. The data indicate that Fru-1-*P* aldolase increased in the rat eating sucrose or fructose in comparison to glucose without a change in Fru-1,6-P_2 aldolase activity.

The simplest explanation for these data is that the adaptive response of intestinal aldolases is specifically related to the type of sugar in the diet. Casein or glucose feeding increases the activity of an aldolase with predominant activity with Fru-1,6-P_2 (Type A or hybrid type). In contrast, sucrose and fructose feeding increase the activity of an aldolase with predominant activity with Fru-1-*P*. Further, sucrose and fructose feeding must repress the activity of the predominantly Fru-1,6-P_2 aldolase type. The alternative to this conclusion is that an aldolase with activity with Fru-1-*P* only is present in the intestine. As yet there is no evidence to support the existence of this type of aldolase. What might be called the "fructose type of aldolase" has a relatively large activity with Fru-1,6-P_2 as substrate in comparison to the Fru-1-*P* activity of the "glucose type". Thus, the sucrose induced repression of the "glucose type of aldolase" does not impair the glycolytic metabolism of glucose.

In view of the fact that the activity ratio of Fru-1,6-P_2 to Fru-1-*P* was lower than 1 in fructose-fed rats (as low as 0.37 in one rat), it is possible that the intestine contains an aldolase with properties different from those in other tissues. This remains to be determined.

Dietary sucrose increased jejunal sucrase and the ability of the intestine to absorb fructose[1-3]. It was suggested that increased cell permeability might allow a more rapid access of the disaccharide to the active site within the cellular compartment[2]. Although altered cellular permeability might be instrumental in explaining these data, we propose another possibility. Since fructose is non-actively transported[19], absorption depends upon the diffusion gradient from lumen to cell. Increased rates of fructose metabolism might lead to an increased diffusion gradient across the epithelial cell membrane. Our data shows that the activities of the two initial glycolytic enzymes in the metabolism of fructose (fructokinase and Fru-1-*P* aldolase) are markedly increased in rats fed sucrose and fructose diets. This suggests that the rapid metabolism of fructose in the intestinal epithelial cell may aid in accelerating the absorption of fructose in rats fed sucrose diets. However, it is necessary to correlate enzyme activities with precise absorption measurements to confirm this hypothesis.

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Dietary Regulation of Galactose-Metabolizing Enzymes: Adaptive Changes in Rat Jejunum

Abstract. *The effects of dietary galactose, sucrose, fructose, glucose, casein, and fasting upon the activity of four galactose-metabolizing enzymes (galactokinase, galactose-1-phosphate uridyltransferase, uridine diphosphate galactose 4-epimerase, and galactose dehydrogenase) were studied in the jejunum of rats. Galactose produced the greatest increase in enzyme activity, fructose and sucrose produced effects intermediate between galactose and glucose, and casein produced a greater activity increase than fasting, but less than the sugars.*

Dietary sucrose increases jejunal sucrase and maltase (*1*) and specific dietary sugars increase specific jejunal glycolytic enzymes in man and rat (*2*). We now report the effect of dietary galactose upon galactose-metabolizing enzymes.

Dietary galactose alters the hepatic galactokinase activity of young rats (*3*). The prolonged administration of galactose produced a slight but significant rise in liver galactokinase activity in adult rats (*3*). We have studied the effects of dietary galactose, sucrose, fructose, glucose, casein, and fasting upon the activity of galactose-metabolizing enzymes galactokinase (E.C. 2.7.1.6), galactose-1-phosphate uridyltransferase (E.C. 2.7.7.12), uridine diphosphate galactose 4-epimerase (E.C. 5.1.3.2), and galactose dehydrogenase (E.C. 1.1.1.48) in adult rat duodenum, jejunum, and ileum.

Twenty-four male Holtzman rats (320 to 400 g) were housed in individual cages with free access to water. Six groups of four rats each were fasted for 3 days. One group was killed after fasting. Each of the remaining five groups were fed as follows. The basic diet for the 3 following days consisted of (by weight) casein, 17 percent; Crisco, 10 percent; salt mixture, 4 percent; and NBC vitamin diet fortification mixture, 1 percent. To this diet we added 68 percent of sucrose for group 1; 68 percent of fructose for group 2; 68 percent of glucose for group 3; 68 percent of galactose for group 4; 68 percent of casein for group 5 (the total casein for this group being 85 percent). No significant differences were noted in the weight gains and food consumption of the five groups fed after fasting. The average total food consumption and standard error for each group during refeeding were: group 1, 65.5 ± 3.8 g; group 2, 62.5 ± 3.1 g; group 3, 64.0 ± 2.3 g; group 4, 59.9 ± 5.3 g; group 5, 57.0 ± 6.3 g.

The rats were stunned by cervical fracture; and portions of the duodenum, jejunum, and ileum were collected. The intestinal segments were slit longitudinally, and the mucosa was scraped with a glass slide. After storage at − 85°C, the mucosa was homogenized in a Kontes-Duall grinder with 20 volumes of a tris-ethylenediaminetetraacetic acid buffer, *p*H 7.5, containing 120 m*M* KCl, 20 m*M* tris, 5 m*M* $MgSO_4$, and 0.1 m*M* ethylenediaminetetraacetic acid (Na_2). The homogenates were centrifuged at 104,000*g* for 60 minutes in a Spinco model L2 ultracentrifuge at 4°C. The resulting supernatant fraction was used for the enzyme assays.

Activities of galactokinase (*3*), galactose-1-phosphate uridyltransferase (*4*), uridine diphosphate galactose 4-epimerase (*4*, p. 185), and galactose dehydrogenase (*5*) were determined for each sample, and protein was determined by the method of Lowry *et al.* (*6*).

Activities of the individual enzymes in the jejunum are shown in Table 1. These data show that galactose produces the greatest increase in enzymes with decreasing effects from fructose, sucrose, glucose, and casein, respectively.

In *Escherichia coli*, a single operon (the gal operon) contains the linked structural genes for the three enzymes specific for galactose utilization: galactokinase, galactose-1-phosphate uridyltransferase, and uridine diphosphate galactose 4-epimerase (*7*). These three enzymes, which are collectively called the gal enzymes, are coordinately induced by D-galactose (*8*). Our data show that the same three enzymes are also adaptively increased in jejunum of galactose-fed rats. The activities of galactokinase and uridyltransferase were increased eightfold, and the epimerase activity was increased fourfold, as compared to the activities in fasted rats. These observations are consistent with the idea of coordinate control of the three enzymes in the jejunum of rats fed galactose. Though consistent, this neither proves nor disproves the existence of a gal operon in rat jejunum.

In rat liver, the activities of galactose dehydrogenase and galactokinase are of similar magnitude (*9*). Based upon the relative activities of these two enzymes, our data suggest that galactose is metabolized chiefly by galactokinase in the rat jejunum. The ratios of the activities of galactokinase to galactose dehydrogenase was 1.8 : 1 in fasted rats and 4.5 : 1 in galactose-fed rats.

Comparisons of enzyme activities in different sites of the small intestine (Table 2) showed that the jejunum was the most active metabolic site in the galactose-fed rats. The finding of highest enzyme activity in the jejunum coincides with the fact that the jejunum

Table 1. Effect of diet upon galactose-metabolizing enzymes in rat jejunum. Enzyme activity was measured in nanomoles per minute per milligram of protein.

Diet	Enzyme activity (mean ± S.E.)			
	Galactokinase	Uridyl-transferase	Epimerase	Galactose dehydrogenase
Galactose	20.77 ± 1.14*	75.94 ± 3.08	41.10 ± 2.99	4.66 ± 0.07
Fructose	11.61 ± 0.72	39.81 ± 2.88	31.08 ± 1.60	2.96 ± 0.08
Sucrose	9.98 ± 0.42	34.60 ± 0.42	29.95 ± 0.51	3.05 ± 0.17
Glucose	6.90 ± 0.48	26.80 ± 0.87	24.19 ± 0.95	2.38 ± 0.08
Casein	4.69 ± 0.18	17.66 ± 0.54	18.22 ± 0.83	1.84 ± 0.09
Fasting	2.68 ± 0.29	8.99 ± 0.29	11.09 ± 0.96	1.53 ± 0.10

* Data for 4 rats.

Table 2. Effect of diet upon galactose-metabolizing enzymes in different sites of the small intestine of rats fed galactose.

Small intestine site	Enzyme activity (mean ± S.E.) (nmole min^{-1} mg^{-1})			
	Galactokinase	Uridyl-transferase	Epimerase	Galactose dehydrogenase
Duodenum	12.76 ± 0.18*	73.39 ± 0.53	30.98 ± 0.44	4.14 ± 0.09
Jejunum †	20.77 ± 1.14	75.94 ± 3.08	41.10 ± 2.99	4.66 ± 0.07
Ileum	10.38 ± 0.44	64.68 ± 1.14	22.26 ± 0.35	3.96 ± 0.09

* Data for four rats. † The data for the jejunal enzymes are taken from Table 1.

is the most active site of absorption of monosaccharides in the small intestine (10).

Our data demonstrate the adaptive nature of several galactose-metabolizing enzymes (galactokinase, galactose dehydrogenase, uridyltransferase, and uridine diphosphate galactose 4-epimerase) in the jejunum of rats. These results, coupled with previous observations on certain jejunal glycolytic enzymes (2), provide a convenient model for studying the regulation of intestinal enzymes in vivo.

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Experimental Liver Cancer in Rats and its Inhibition by Rice-Bran Extract, Yeast, and Yeast Extract*

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It is a well established fact that the incidence of primary cancer of the liver among Orientals is much higher than the incidence among Europeans and Americans.

Nagayo (9) made a statistical study of liver cancer among Japanese, Chinese, and Koreans. In Japan, primary liver cancer ranks third in frequency of occurrence in males (6.7 per cent), and sixth in females (4.6 per cent). In China, hepatic cancer ranks fifth in males (8.1 per cent), and eighth in females (0.56 per cent), while in Korea, it ranks third in males (9.8 per cent), and fourth in females (6.6 per cent).

De Leon's study on malignancy among the Filipinos (2) shows that tumor incidence among Filipinos is not very different from that of occidental countries except in the high percentage of primary carcinomas of the liver. Of the 1,502 cases of malignant tumors admitted to the Philippine General Hospital in 1910-14 and 1922-26, 95 or 6.32 per cent were primary liver cancer.

Pack and Le Fevre (16) analyzed 19,129 patients with neoplastic diseases admitted for diagnosis and treatment at the Memorial Hospital, New York City, during 1917-29. They found that primary hepatic cancers form 0.17 per cent of the admissions to this institution.

Not only the racial characteristics, but also the dietary habits of the East and West differ greatly. Many attempts have been made to prove that diet has some effect on the incidence of cancer, but only in recent years has scientific evidence succeeded in showing that the nature of the food consumed has some definite influence in carcinogenesis.

In 1935 Sasaki and Yoshida (17) experimentally produced liver cancer in rats by feeding o-amino-azotoluene, and in 1937 Kinosita (6) using dimethyl-amino-azobenzene, commercially known as butter-yellow,[1] produced liver cancer in a much shorter time. In both cases, rice was used as the basal food for the animals, to correspond exactly to the staple food of the rice-eating countries of the Orient. The use of rice in their experiment is very important because when wheat bread was given the production of liver cancer was markedly reduced (4, 14).

Several inhibitory substances were tested by Japanese workers in their research. Okada (13) in 1938 reported that daily ingestion of a small amount of rice-bran oil prevented the production of liver cancer in rats fed butter-yellow. In the same year, Kinosita (5) and Ando (1) found that the addition of yeast to the butter-yellow rice diet decreased the production of liver cancer. Nakahara, Mori, and Fujiwara (11) used dried beef liver as a supplementary food with butter-yellow rice and demonstrated inhibition of liver cancer in rats.

At present it is important not only to verify the findings of the Japanese workers, but also to isolate the substance present in rice-bran oil, yeast, and beef liver that inhibits the production of liver cancer.

In view of these facts it seems desirable to give an account of experiments in which primary tumors of the liver, hepatoma and cholangioma, were produced in a high percentage of rice-fed rats which received butter-yellow. The formation of these tumors was prevented by feeding rice-bran extract, yeast, or yeast extract.

Experimental

In the present series of experiments we used p-dimethyl-amino-azobenzene (No. 338, Eastman Kodak Co.), discovered by Kinosita to be highly active in producing liver cancer in rats. This substance was dissolved in olive oil in the proportion of 3 per cent.[2] Twenty cc. of this solution was evenly mixed with 1,000 gm. of coarsely ground, unpolished Texas rice (fresh crop); *i.e.*, 0.6 gm. butter-yellow per kg. The rats were allowed to take as much of the mixture as they desired. The basal diet was supplemented with a small slice, about 1 gm., of fresh carrot per rat daily. Unlimited water was allowed.

As a preliminary study, the susceptibility of 3 different strains of rats to the carcinogenic action of butter-yellow was determined. Previous work had not taken into consideration the possible variability of the experimental animals.

Kinosita and other Japanese workers used albino rats of a mixed stock. In the present study we used young adult male and female rats of 3 pure strains, Wistar, Sherman, and Evans stock. They were 2 to 4 months

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[1] Butter-yellow was synthesized by Griess in 1875. It has been used to color oils, oleomargarine, and other vegetable fat substitutes for butter. In recent years the use of it in foods has been prohibited. However, Yellow A. B. (1-phenylazo-2-naphthylamine), and Yellow O. B. (1-o-tolylazo-2-naphthylamine), oil-soluble coal tar dyes are permitted in this country. These substances are somewhat related in structure to butter-yellow and the possibility of their acting in an analogous manner to butter-yellow cannot be overlooked.

[2] The 3 per cent butter-yellow solution in olive oil is a saturated solution. The butter-yellow readily dissolves in olive oil in this per cent when warmed on a water bath.

old, weighing 120 to 190 gm. at the beginning of experiments.

The amount of food consumed by each rat was carefully noted in order to calculate the approximate amount of butter-yellow ingested. A record of each animal was made, including the general physical condition, its weight at frequent intervals, and the duration of life, until death or until the animal was sacrificed.

Animals were killed at various stages from the beginning of butter-yellow feeding up to the development of advanced hepatic cancer, and the livers of these animals were examined grossly and microscopically.

The results obtained from this study are summarized in Table I and presented in graphic form in Fig. 7. This study included 8 groups of experiments, involving a total of 144 rats of the Wistar stock, 146 rats of the Sherman stock, and 132 rats of the Evans stock.

At the end of 2 months of butter-yellow feeding, the livers had irregular surfaces with more or less well defined nodules (Fig. 1.B). Histologically there was extensive proliferation of the fibrous connective tissue with lymphocytic infiltration, forming islands of hyperplastic liver cells (annular cirrhosis) (Fig. 2.B). Some nodules at this stage showed adenomatous hyperplasia of bile ducts with beginning malignant transformation into a cholangioma (Fig. 3).

At the end of 3 months of butter-yellow feeding, the livers contained nodules reaching 8 mm. in diameter. Some of the nodules were strikingly grayish white or yellowish in color on the reddish brown background of the whole organ (Fig. 1.C). Microscopic sections of the liver showed proliferation of the liver cords into papillomatous projections in acinar spaces giving the appearance of bile ducts in some areas (cholangioma type) (Fig. 4.A,B). Other sections of the liver showed liver cords proliferating into trabeculae with irregular

TABLE I: COMPARISON OF LIVER CHANGES IN RATS OF VARIOUS STRAINS MAINTAINED ON BUTTER-YELLOW RICE DIET *

	Days																	
	1-24		25-40		41-60		61-75		76-90		91-120		121-150		151-180		181-220	
Strain	Liv. cancer	No liv. cancer	Liv. cancer	No liv. cancer	Liv. cancer	No liv. cancer	Liv. cancer	No liv. cancer	Liv. cancer	No liv. cancer	Liv. cancer	No liv. cancer	Liv. cancer	No liv. cancer	Liv. cancer	No liv. cancer	Liv. cancer	No liv. cancer
Sherman	0	8	0	12	3	13	16	14	25	4	24	0	22	0	5	0	..	..
Wistar	0	7	0	14	6	16	10	10	12	5	21	4	14	0	18	0	7	0
Evans	0	5	0	14	0	22	0	10	2	9	11	11	12	4	11	2	19	0

* The sum of the numbers in the two columns under each period of days is the total number of animals of each strain killed for examination in that period.

All diagnoses were confirmed by microscopic examination.

The present results confirm the findings of Kinosita in every respect. The liver cancers were found to be various histological types of hepatomas as follows: trabecular or solid type; glandular or cholangioma type; and mixed type. Cirrhosis with adenomatous hyperplasia of bile ducts preceded these malignant tumors. Since the histology of these experimental liver cancers has been well described by Kinosita (6) and later by Nakahara, Mori, and Fujiwara (12), it seems unnecessary to enter into details. However, in view of the interest which has developed recently as to the possibility of a dietary factor as a causation of certain types of cancer in man, we felt it would be of interest to summarize our findings.

During the first 30 days' feeding on butter-yellow rice diet, the livers of rats showed no great changes in size, color, or shape. (Fig. 1.A). However, microscopic sections showed swelling of cells, much narrowing of sinusoids, and hyperchromatic appearance of nuclei. The surface of the capsule was smooth (Fig. 2.A).

and hyperchromatic nuclei (hepatoma type) (Fig. 5. A,B).

At the end of 5 months of butter-yellow feeding, the livers showed large, isolated, massive nodules measuring about 3 cm. in diameter. There were usually 1 or 2 such nodules in each lobe. Some of the nodules were strikingly whitish in color and soft; others were hemorrhagic and cystic (Fig. 1.D). Microscopic sections showed solid hepatomas in which the liver cells were enlarged; mitotic figures were present; and giant hyperchromatic nuclei were frequent (Fig. 6).

As may be seen from the data in Table I and Figure 7, the susceptibility of rats of different strains to the carcinogenic action of butter-yellow is not the same. Among rats of the Evans strain, the first liver cancer was noted on the 81st day after the beginning of the experiment. In the case of the Wistar strain the first liver cancer occurred in a rat dying 52 days after the beginning of the experiment, and in the case of the Sherman strain, the first tumor appeared after 49 days.

In the case of the Evans strain 2 out of 13 rats, or 15 per cent, living between 151 to 180 days did not

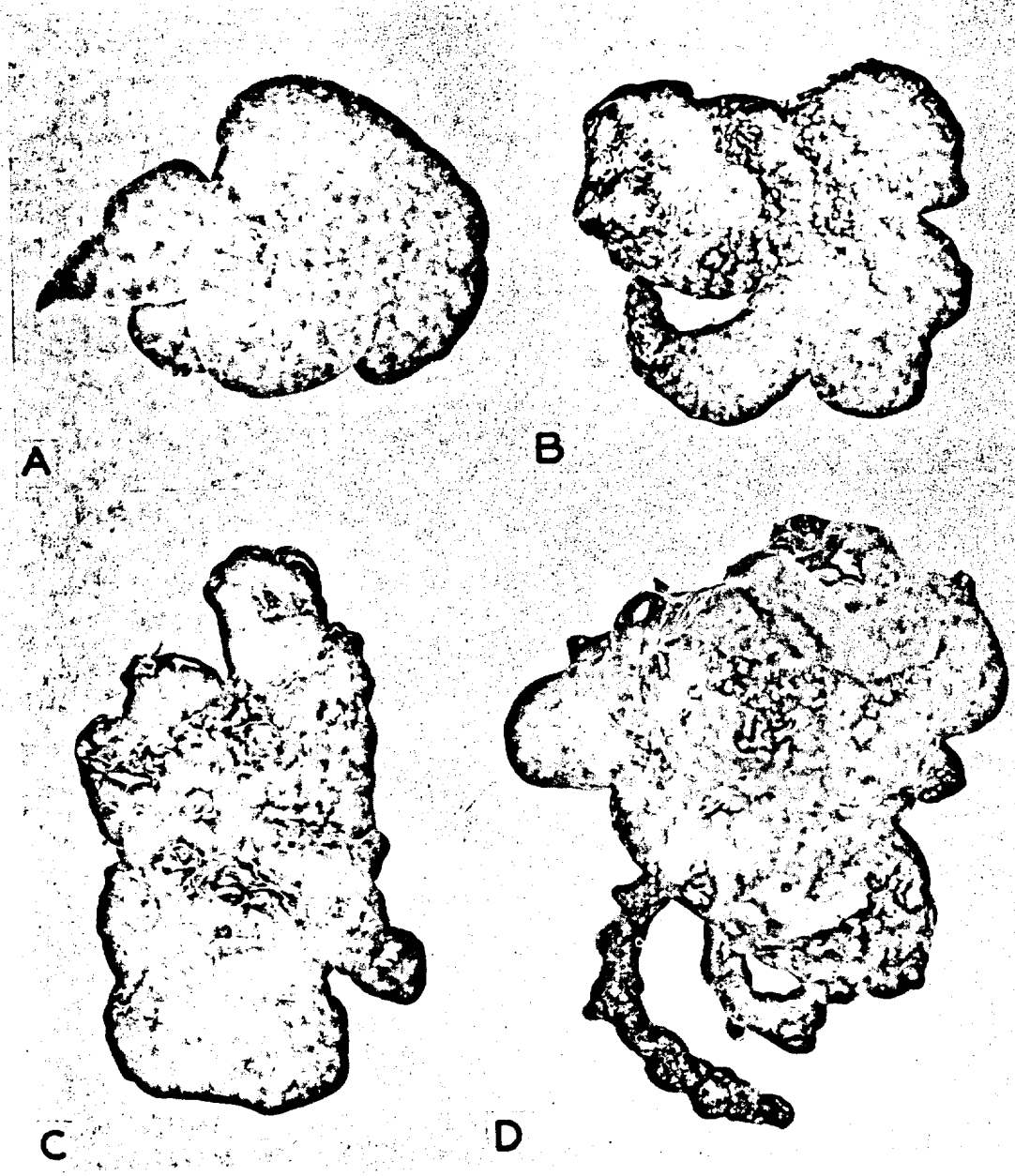

FIG. 1.

Gross appearance of livers of rats fed on butter-yellow rice diet for various lengths of time. Photographs of the livers were made at the same magnification, × 0.9.

A. Liver of a rat (30 days), showing practically normal gross appearance.
B. Liver of a rat (60 days), showing nodular cirrhosis.
C. Liver of a rat (90 days), showing hepatoma-nodules.
D. Extremely large liver of a rat (150 days), showing hepatoma-nodules and mesenteric metastasis.

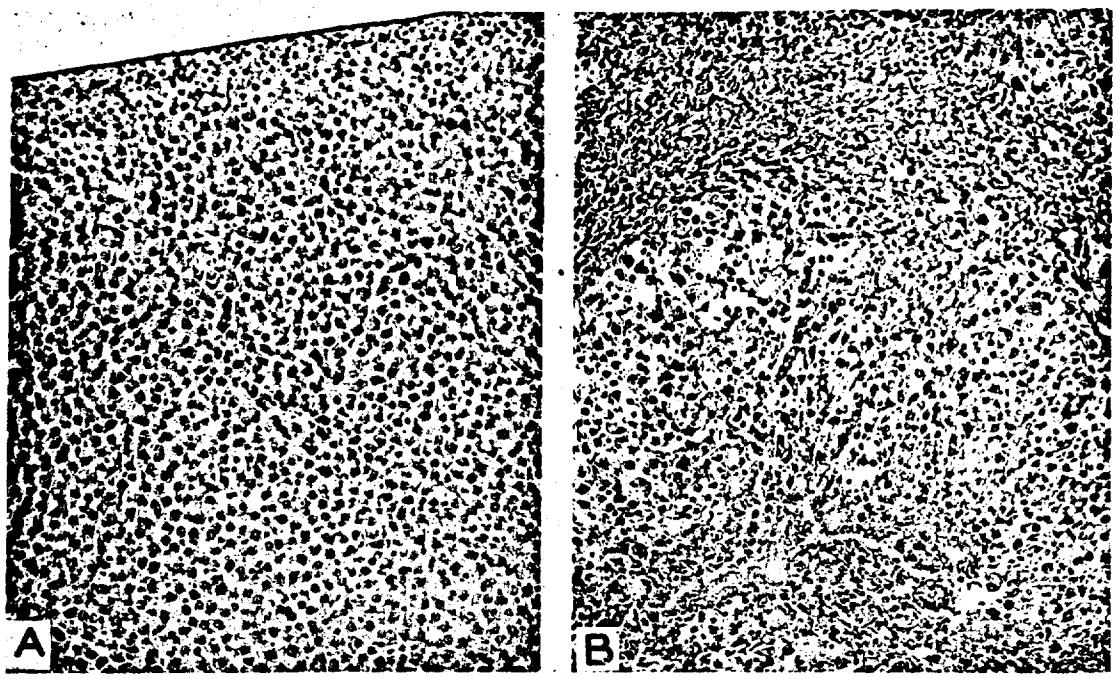

FIG. 2.

A. Section of a practically normal liver (30 days on butter-yellow rice diet), showing swelling of cells, much narrowing of sinusoids, and smooth surface of the capsule. (For gross liver see Fig. 1.A.)

B. Section of a cirrhotic liver (60 days), showing annular cirrhosis with marked lymphocytic infiltration. (For gross liver see Fig. 1.B.)

develop liver cancer, but showed liver cirrhosis. All of the 19 rats surviving 181 to 220 days of butter-yellow feeding showed liver cancer.

In the case of the Wistar and Sherman strains, all the rats that were maintained upon the butter-yellow rice diet for 124 days and 92 days, respectively, developed typical liver cancer.

Of the 185 rats from the 3 strains which died or were sacrificed between 91 and 220 days after the beginning of the experiment 164, or 89 per cent, had liver cancer and the other 21, or 11 per cent, showed liver cirrhosis with or without bile duct adenomatosis. Of the 101 rats of the Wistar and Sherman strains which died or were sacrificed between 100 and 200 days after the beginning of the experiment 98, or 97 per cent, showed liver cancer; and the other 2, or 3 per cent, showed liver cirrhosis with or without adenomatous hyperplasia.

Under the conditions of our experiments only about 3 per cent of the rats died during the first month without noteworthy liver change, and about 15 per cent died during the first 60 days of feeding. The low mortality rate in our rats is surprising because Harada, Mizuta, and Maruya (3) at Kinosita's laboratory found 45 per cent mortality in their rats during the first month. Similarly, Nakahara and co-workers (12) reported that many of their butter-yellow fed rats died

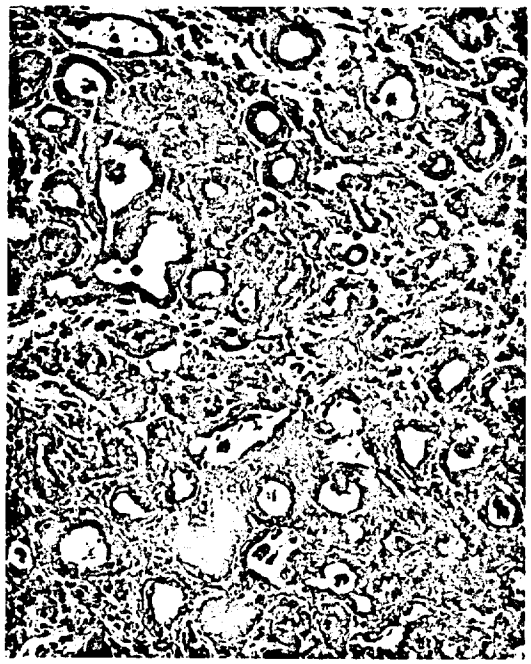

FIG. 3.

Section of a liver (60 days), showing bile duct adenomatosis with beginning malignant transformation into a cholangioma.

FIG. 4.

A. Section of a liver cancer (90 days), showing cholangiomatous type of tumor. (For gross liver see Fig. 1.C.)
B. Same section at higher magnification.

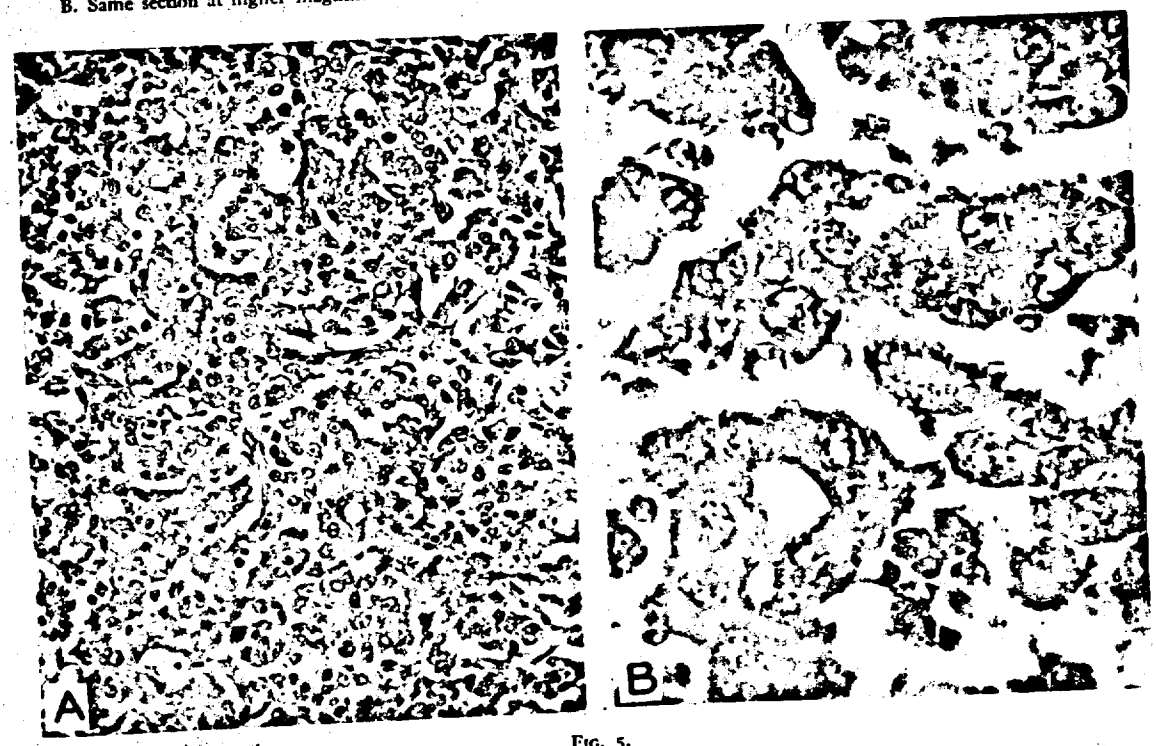

FIG. 5.

A. Section of a liver cancer (hepatoma) (90 days), showing trabecular type. (For gross liver see Fig. 1.C.)
B. Same section at higher magnification.

early without showing any significant change in the liver.

Although the number of days of feeding was the same, individual rats consumed different amounts of butter-yellow. Therefore, we calculated approximate amounts of butter-yellow consumed by each animal from a large number of rats and found about 162 mgm. of butter-yellow for the first 50 days and 120 mgm. for the second 50 days, or a total of 282 mgm. for 100 days.

Nutrition of young adult rats, of the Wistar and Sherman strains, during ingestion of the butter-yellow rice diet with supplement of fresh carrots, may be briefly summarized as follows: During the first 10 days individual animals consumed about 8 gm. of the basal diet with butter-yellow daily, during the next 20 days about 5 to 6 gm., and during the second and third months about 4 gm. The majority lost body weight from the beginning of the experiment, at an average of about 0.5 gm. daily. Some gained weight slightly during the first 2 weeks, and maintained their weight until the onset of liver cancer. At the end of 2 months, when the livers of most of the rats were cirrhotic, the general appearance of the animals was good, but thereafter, when malignant changes began to appear in the liver, the condition of the animals was poor.

Fig. 6.

Section of a liver cancer (150 days), showing solid type of hepatoma. (For gross liver see Fig. 1.D.)

After the rats had been fed butter-yellow for 90 days, 2 types of liver with cancer were observed. One was very small, weighing about 2.1 gm., and the other of normal size, weighing about 8.9 gm. Livers of normal rats weighing about 150 gm. have an average weight of 8 gm. In some of the well-nourished rats living 150 days or more, the liver reached a huge size, weighing from 24 to 75 gm. The cancers occupied about 90 per cent of the organ (Fig. 1.D). Small livers showed more fibrosis or cirrhotic changes with comparatively few cancer nodules, while the normal or large livers contained more numerous multicentric patches of carcinoma and much less fibrosis or cirrhotic change. From these observations we are inclined to believe that the state of nutrition of the animal has much to do with the type of tumor which develops. In undernourished rats the livers were abnormally small and more cirrhotic, while in the well-nourished rats the livers were normal or very large with more cancerous nodules.

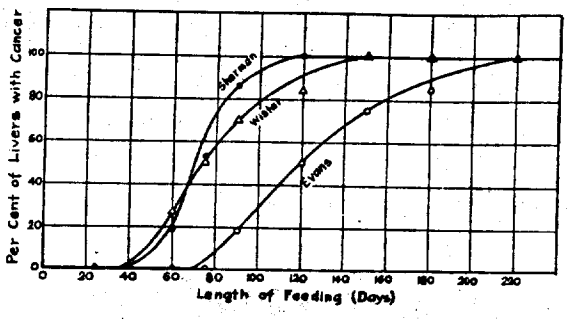

Fig. 7.

Curves showing percentage of liver cancer in rats of the Sherman, Wistar, and Evans strains maintained on a butter-yellow rice diet.

Among rats of the Sherman, Wistar, and Evans strains examined between 150 and 220 days of butter-yellow feeding, 6 out of 12 livers of Sherman rats, 16 out of 35 livers of Wistar rats, and 6 out of 37 livers of Evans rats were very large. Only among rats surviving for a longer period and exhibiting large livers were metastases found. Sixteen out of 84 rats had widespread metastases in the mesentery and omentum, and 4 of them also had lung metastases. Most of the metastases were of cholangioma type.

Ascites was found only among rats surviving 150 days or longer and exhibiting large livers (Fig. 1.D). Among 84 such rats 11 had ascites.

The spleens of rats fed butter-yellow rice diet for about 65 days were greatly enlarged in size. This splenomegaly coincided with the development of liver cirrhosis. Many of the spleens weighed 2 to 3 times the normal weight. However, with the development

of the liver cancers the enlarged spleens gradually returned to normal size.

None of the 135 young adult female rats kept with male rats became pregnant while on the diet. On the other hand, 10 mated female rats maintained upon a ration of unpolished rice and carrots produced 1 to 3 litters of young during an experimental period of 200 days. This is in accord with the observation of Kusumoto (8) who noted ovarian atrophy in these animals.

Effect of Feeding Carrot

In the preceding experiments we supplemented the basal diet of butter-yellow and rice with a small slice of fresh carrot per rat every day in order to maintain the normal metabolism and maintain the normal physical condition of the animal. The carrot is rich in vitamin A.

Table II: Effect of Absence of Carrots on the Production of Liver Cancer by Butter-Yellow

Rat no.	No. of days survived	Liver findings
1	12	Normal
2	60 *	Cirrhosis
3	61	Cirrhosis
4	65	Cirrhosis
5	69	Cholangioma
6	75	Cholangioma
7	77	Cholangioma
8	77	Cholangioma
9	83	Cholangioma
10	85	Cholangioma
11	86	Cholangioma
12	90 *	Cholangioma
13	90	Hepatoma
14	97	Hepatoma
15	107 *	Cholangioma
16	112	Cholangioma, Hepatoma

* Indicates the animal was sacrificed and examined.

The following experiment records the results obtained with 16 rats of the Sherman strain fed on the butter-yellow rice diet without the supplement of carrots.

The data in Table II show that young adult rats maintained upon the butter-yellow rice diet alone died definitely earlier than those maintained upon a similar diet with the supplement of carrots (Table I). The animals without carrots continued to lose weight until death.

In the livers of these animals there were numerous small nodules with a few large ones. Histological examination revealed that about 75 per cent of these liver cancers were cholangiomatous in type. When the basal diet was supplemented with carrots the animals lived longer. The livers grew larger and showed many large cancer nodules, mostly of the hepatoma type. It is clear from this finding that daily ingestion of a small slice of fresh carrot has no noticeable effect on the production of liver cancer by butter-yellow, but it influences the survival of the animal and consequently the type of liver cancer.

Effect of Feeding Rice-Bran Extract

Before the Third International Cancer Congress at Atlantic City, September 1939, Kinosita (7) stated that the production of liver cancer by butter-yellow is stimulated by feeding with polished rice instead of unpolished rice, but is slightly inhibited by feeding with bread. When 1 cc. of rice-bran oil (ether soluble substance) is given daily to the rat the production of butter-yellow cancer is also definitely hindered.

Kinosita obtained rice-bran oil by pressing the rice-bran from fresh crop rice (Fall harvest rice) at a temperature of 60 to 70° C. In our study the same kind of rice-bran oil was prepared. Rice-bran from a fresh crop of Texas rice was continuously extracted for 24 to 48 hours with ether free from H_2O_2. From 1,800 gm. of rice-bran, about 400 cc. of a greenish brown oily emulsion was obtained. The extract is supposed to contain vitamins A, B_1, and E. Vitamin B_2 was present in a very small amount.

The vitamin A content of the rice-bran extract was tested by feeding 10 young albino rats upon a synthetic diet (19) consisting of casein, 18 per cent; cane sugar, 10 per cent; starch, 36 per cent; salt mixture, 3 per cent; yeast, 3 per cent; lard, 20 per cent; and rice-bran extract, 10 per cent. After 3 months, xerophthalmia failed to appear in these animals and their physical appearance was normal.

Tests for vitamin B_1 were made on 4 pigeons. When these animals developed symptoms of polyneuritis upon feeding with polished rice, the administration of 1 to 2 gm. of the rice-bran extract to each bird caused a disappearance of the beriberi symptoms within 10 hours. The pigeons remained normal for several days upon continued feeding with polished rice alone.

Vitamin E content was assayed by feeding young albino rats, 4 males and 6 females, upon a synthetic diet consisting of casein, 18 per cent; cane sugar, 10 per cent; starch, 26 per cent; salt mixture, 3 per cent; yeast, 3 per cent; butter fat, 30 per cent; and rice bran extract, 10 per cent. Three of the 6 female rats gave birth to 1 to 2 litters of young during the experimental period of 100 days. This shows the presence of vitamin E.

The content of riboflavin, vitamin B_2, in the rice-bran extract was estimated by the fluorometric method. Spectroscopic analysis showed the presence of unsaturated hydrocarbons related to ergosterol, pro-vitamin D. The rice-bran extracts were kept in a refrigerator until used.

In this experiment we used 90 rats of the Sherman and Wistar strains. One group of 60 rats was maintained on the mixture composed of 96 per cent unpolished rice, and 4 per cent rice-bran extract, supplemented with a small slice of fresh carrot per rat daily. Thirty rats of the second group were kept on unpolished rice and fresh carrots, but without rice-bran extract.

TABLE III: EFFECT OF FEEDING RICE-BRAN EXTRACT AND UNPOLISHED RICE ON THE PRODUCTION OF LIVER CANCER BY BUTTER-YELLOW

Rat no.	Sex	No. of days fed	Body weight (gm.) Initial	Maximum	Final	Liver findings *
1	♂	100	134	157	157	—
2	♂	100	121	136	120	—
3	♀	100	125	145	142	—
4	♂	106	150	155	154	—
5	♂	110	147	177	170	—
6	♂	110	150	151	142	±
7	♂	112	115	122	122	±
8	♀	114	117	122	100	—
9	♂	116	119	136	133	—
10	♀	120	142	142	127	—
11	♀	120	132	132	118	—
12	♂	120	137	137	70	±
13	♀	125	124	151	149	—
14	♀	128	121	121	100	—
15	♂	129	136	155	155	—
16	♂	130	126	126	108	—
17	♀	130	129	139	144	±
18	♂	138	122	144	144	±
19	♂	139	120	151	151	—
20	♂	139	132	132	122	—
21	♂	139	116	127	120	±
22	♂	149	148	148	130	—
23	♀	150	127	160	147	—
24	♀	150	117	128	120	±
25	♂	150	122	122	117	±
26	♀	153	145	145	109	—
27	♀	161	115	133	100	—
28	♂	161	159	159	120	—
29	♂	161	150	150	124	—
30	♂	161	167	167	165	—
31	♀	161	135	138	117	±
32	♂	161	151	151	110	+
33	♂	161	179	179	168	+
34	♂	170	158	165	161	—
35	♀	172	113	118	118	—
36	♂	185	140	140	120	±
37	♀	200	121	135	130	±
38	♀	200	122	129	140	+
39	♂	218	174	160	160	—
40	♂	218	133	133	95	±
41	♂	218	162	173	144	±
42	♂	218	144	150	138	++
43	♂	228	108	128	112	+
44	♂	250	140	140	135	—
45	♂	250	148	148	123	±
46	♀	250	135	149	149	±
47	♂	250	135	135	111	+
48	♀	250	141	173	162	++

* — indicates smooth, practically normal liver; ± indicates nodular cirrhosis with adenomatous hyperplasia; + indicates distinct areas of cholangioma or hepatoma, or both; ++ indicates extensive liver cancer with or without metastasis. In all cases the diagnoses were confirmed by microscopic examination.

Twenty cc. of 3 per cent butter-yellow solution in olive oil were mixed well with 1,000 gm. of unpolished rice, with or without rice-bran extract. In order to prevent possible deterioration, the food mixtures were kept in a cool place.

The results of the above experiments are recorded in Tables III and IV. Since we observed that the susceptibility of rats of the Sherman and Wistar strains to the carcinogenic action of butter-yellow is practically the same, the findings in both groups were combined.

Rats that died or were sacrificed before the period of 100 to 250 days are not included in Table III, experimental group. None of them, however, showed any malignant change. Similarly, animals of the control group that died or were sacrificed before 100 days are not included in Table IV, although many of them showed liver cancer.

TABLE IV: EFFECT OF UNPOLISHED RICE ALONE ON THE PRODUCTION OF LIVER CANCER BY BUTTER-YELLOW

Rat no.	Sex	No. of days fed	Body weight (gm.) Initial	Maximum	Final	Liver findings *
1	♀	100	145	145	130	+
2	♀	100	136	136	83	+
3	♂	105	153	154	117	+
4	♂	128	135	136	130	—
5	♀	128	118	118	75	+
6	♀	130	134	145	124	+
7	♂	134	179	197	136	+
8	♀	136	119	119	74	+
9	♂	141	139	139	125	++
10	♂	150	148	156	146	+
11	♀	176	135	136	92	++
12	♂	182	136	140	119	+
13	♂	182	141	141	110	+
14	♂	182	111	125	122	+
15	♀	182	114	114	64	+
16	♂	182	121	130	130	++
17	♀	182	134	138	115	++
18	♂	184	120	120	80	++
19	♂	188	167	167	165	+
20	♂	200	160	160	105	++

* See Table III for explanation of symbols.

From the data in Tables III and IV, it will be seen that rice-bran extract exerted a definite inhibiting effect upon the production of liver cancer in rats by the oral administration of butter-yellow. This inhibiting action was already demonstrated at a period between 100 and 150 days after the beginning of the experiment when 25 of the 60 rats fed with rice-bran extract were killed for examination. The livers of 17 rats had the normal smooth surface, while in the remaining 8 the surface was slightly granular. Microscopic sections of the smooth livers revealed only beginning connective tissue increase with slight regeneration of the bile ducts

(Fig. 8.A). Histologic examination of the granular livers, observed in rats kept on this diet for 4-5 months, showed proliferation of fibrous connective tissue inclosing islands of liver cords, resembling the cirrhotic condition produced in the liver of rats fed with butter-yellow rice diet without supplementary rice-bran extract for 2 months (Fig. 8.B). There was no evidence of malignant change in any of these granular livers. On the other hand, 9 out of 10 rats on butter-yellow rice diet without the supplementary rice-bran extract produced liver cancer in the same period of 100 to 150 days.

It was observed that rats fed on butter-yellow rice supplemented with rice-bran extract generally gained weight and presented a healthy appearance. Each rat consumed approximately 6 to 8 gm. of the food per day, an amount greater than that consumed by rats fed on butter-yellow rice alone. This observation is important because we are sure that the amount of butter-yellow ingested by each experimental rat during the first 100 days was much greater than 282 mgm., which is the average amount for a rat of the cancer control group.

The inhibiting effect of the rice-bran extract upon experimental liver cancer appears to be a delaying rather than a preventing action because some of the rats finally developed liver cancer. Thus, among 23 rats in this group surviving a period of 153 to 250 days 7, or 30 per cent, were found with typical liver cancer, and 7 others showed liver cirrhosis with adenomatous hyperplasia, a forerunner of malignancy. All control rats had liver cancer. The present result is in accord with the findings of Okada (15), who observed that daily ingestion of rice-bran oil delayed the development of liver cancer in rats fed with butter-yellow bread diet.

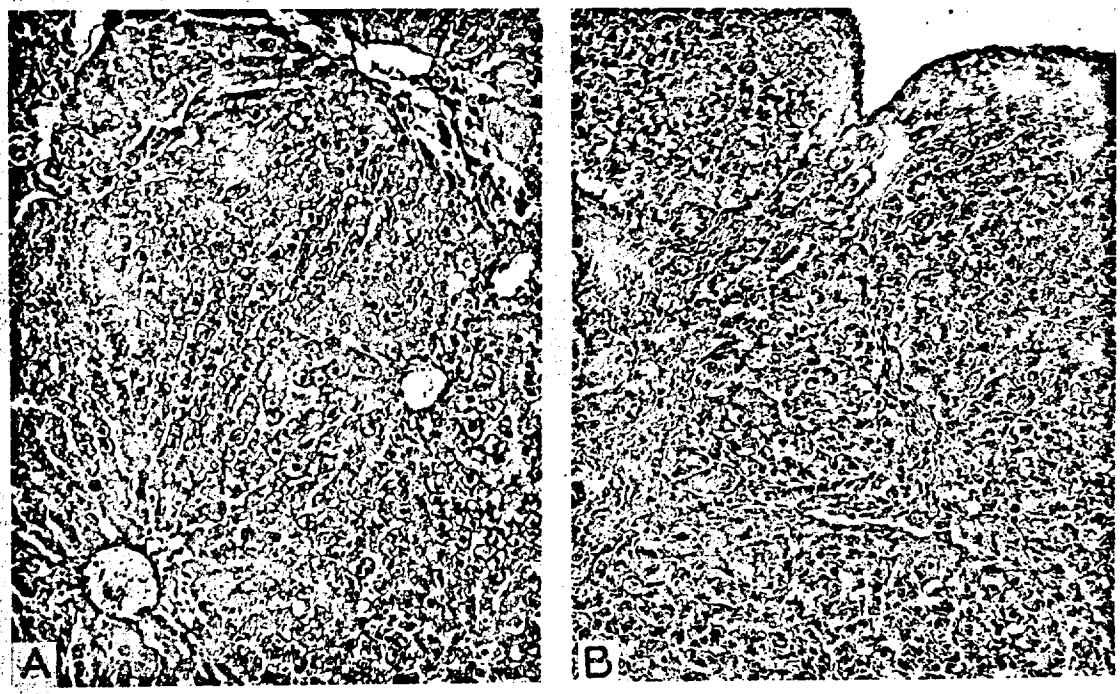

Fig. 8.

A. Section of the liver of a rat fed on butter-yellow rice diet supplemented with rice-bran extract for 150 days. The liver lobules are distinct with slight proliferation of connective tissue.

B. Section of the liver of a similarly fed rat (150 days), showing marked proliferation of fibrous connective tissue inclosing islands of liver cells (cirrhosis). No evidence of malignant change.

Effect of Feeding Yeast Extract

It was shown in the above experiments that the ether extract of rice-bran contains a substance which is inhibitory to the development of liver cancer by butter-yellow feeding. The question then arose: Is the cancer-inhibiting substance a fat soluble substance or a water soluble substance, or both?

In an attempt to answer this question, the effect of an ether extract of yeast upon butter-yellow liver cancer was investigated. Recent studies of Kinosita (5), Ando (1), and Nakahara, Fujiwara, and Mori (10) showed that yeast feeding tends to inhibit the experimental production of liver cancer.

Following the same technic as that of rice-bran ex-

traction, dried brewer's yeast (No. 2040, Fleischmann Laboratories) was continuously extracted with ether for 24 to 48 hours. From 10,000 gm. of yeast 165 gm. of a thick brown-colored extract were obtained. It contained 14.5 γ of vitamin B_2, riboflavin, per gram of the material. It also contained ergosterol.

The experimental group of 30 rats was kept on the mixture containing 99.75 per cent of butter-yellow rice and 0.25 per cent of yeast extract, supplemented with a small slice of fresh carrot per rat daily.

TABLE V: COMPARATIVE EFFECTS OF FEEDING YEAST EXTRACT AND OF OMISSION OF YEAST EXTRACT ON THE PRODUCTION OF LIVER CANCER BY BUTTER-YELLOW

Group fed yeast extract			Group without yeast extract		
Rat no.	No. of days fed	Liver findings *	Rat no.	No. of days fed	Liver findings *
1	100	—	1	100	+
2	100	—	2	100	+
3	100	—	3	100	+
4	100	±	4	105	+
5	104	±	5	110	+
6	106	—	6	127	+
7	106	—	7	134	+
8	130	—	8	141	+
9	130	+	9	151	++
10	145	++	10	164	+
11	149	—	11	176	++
12	178	++	12	179	++
13	183	+	13	184	+
14	188	+	14	188	++
15	189	—	15	197	++
16	193	++			
17	200	—			
18	203	++			
19	208	—			
20	208	±			
21	208	±			
22	208	+			
23	208	+			
24	208	+			
25	208	+			
26	208	++			
27	219	+			
28	223	++			

* See Table III for explanation of symbols.

The control group of 20 rats was maintained on butter-yellow rice without yeast extract, but with fresh carrot.

The results are presented in Table V. Rats used in this study were of the Wistar and Sherman strains.

Table V shows clearly that the development of liver cancer in rats following oral administration of butter-yellow is inhibited by daily ingestion of a small amount, 18 mgm., of yeast extract. Among 28 rats of the experimental group examined after 100 to 223 days, 10 rats, or 36 per cent, showed a smooth normal liver, 4 rats, or 14 per cent, developed liver cirrhosis with adenomatous hyperplasia, and 14 rats, or 50 per cent, had cholangioma or hepatoma, or both. On the other hand, all of the 15 rats, or 100 per cent, in the control group examined between 100 and 197 days showed typical liver cancer.

The yeast extract protected 33 per cent of the animals surviving more than 200 days from the carcinogenic action of butter-yellow.

Animals fed on the butter-yellow rice diet with the supplement of yeast extract presented a much healthier appearance and lived much longer than the control animals. In the experimental group, rats Nos. 4, 8, 13, 14, 16, 18, 27, and 28 became weak and emaciated. Some of these animals died and others were sacrificed when it became apparent that they would not survive. The remaining 20 rats were sacrificed for examination. In the control group, 12 rats died or were killed because of their terminal condition. Rats Nos. 6, 13, and 15 were sacrificed for examination.

EFFECT OF FEEDING 15 PER CENT WHOLE YEAST

Recently Nakahara, Fujiwara, and Mori (10) reported that the addition of yeast to a rice diet decreased the rate of liver cancer production. The experiment on which this conclusion was based follows:

> One group of 57 albino rats was maintained on unpolished rice with the occasional addition of fresh carrots, and another group of 56 rats on the mixture of 85 per cent unpolished rice and 15 per cent dried baker's yeast, also supplemented with carrots. From 0.5 to 1.0 cc. of 2.5 per cent olive oil solution of dimethyl-amino-azobenzene, butter-yellow, was injected intraperitoneally once weekly during the entire experimental period. The results showed that among 6 rats in the group without yeast surviving 255 to 279 days, 3 were found with typical liver cancer, while for the corresponding period of survival none of the 10 rats in the yeast-fed group showed liver cancer. Rats in the first group were maintained upon an inadequate diet, especially lacking in vitamins. Rats in the second group were maintained upon a better balanced diet, although it was not an adequate diet.

From these experiments it seems that a certain factor or factors of diet may control the experimental development of liver cancer. Confirmation of the Japanese workers' findings and further studies on the role of diet in the development of cancer are imperative.

In the present study we used brewer's yeast (No. 2040, Fleischmann Laboratories) instead of baker's yeast, and fed dimethyl-amino-azobenzene instead of giving it by intraperitoneal injection.

The experimental group of 40 rats, Wistar and Sherman strains, was kept on the mixture of unpolished rice, 85 per cent, and dried yeast, 15 per cent, supplemented with a small slice of fresh carrot per rat daily.

The control group of 20 rats was maintained on unpolished rice without yeast, but supplied with fresh carrot.

Twenty cc. of 3 per cent butter-yellow solution in

olive oil were mixed well with 1,000 gm. of unpolished rice with or without yeast. Rats were allowed these mixtures *ad libitum*.

The results are presented in Tables VI and VII.

The data in Table VI show clearly that yeast feeding produced a very striking inhibition of liver cancer development. All of the 34 rats, with the exception of 2,

TABLE VI: EFFECT OF FEEDING WHOLE YEAST, 15 PER CENT, AND UNPOLISHED RICE ON THE PRODUCTION OF LIVER CANCER BY BUTTER-YELLOW

Rat no.	Sex	No. of days fed	Body weight (gm.) Initial	Maximum	Final	Liver findings *
1	♂	104	147	160	160	—
2	♂	104	134	174	174	—
3 †	♂	142	150	214	214	—
4	♀	150	153	190	177	—
5	♀	150	171	197	181	—
6	♂	150	154	163	150	—
7	♂	150	165	165	145	—
8	♀	177	163	165	165	—
9	♂	189	125	167	147	—
10	♂	189	120	164	138	—
11	♂	189	115	179	158	—
12	♂	200	145	168	158	—
13	♂	200	154	155	145	—
14	♂	200	120	148	118	—
15	♂	200	122	128	124	—
16	♂	200	121	150	140	—
17 †	♂	210	140	160	114	—
18	♂	221	131	176	176	—
19	♂	223	167	177	168	—
20	♀	223	164	180	166	—
21	♀	223	154	154	128	—
22	♀	223	133	133	132	±
23	♀	233	189	198	191	—
24	♂	233	204	242	204	—
25	♀	233	180	182	170	—
26	♂	233	190	190	178	—
27	♂	250	120	150	120	—
28	♂	250	138	154	135	—
29	♂	250	141	155	139	—
30	♂	250	130	159	145	—
31	♂	284	123	160	152	—
32	♂	284	121	169	163	—
33	♀	284	142	153	153	—
34	♂	284	139	175	145	±

* See Table III for explanation of symbols.
† Died.

examined between 104 and 284 days after the beginning of the experiment had smooth and practically normal livers. Rats Nos. 22 and 34, sacrificed on 223 and 284 days, respectively, had livers with slight granulation of the surface. Histologically they exhibited cirrhosis with adenomatous hyperplasia.

In contrast, the animals fed the butter-yellow rice diet without yeast developed typical liver cancer in all cases in a comparatively shorter period of time. Rats Nos. 9, 11, and 12 of this group had large livers with large cancer nodules, and 2 of them also had metastases in the mesentery and omentum.

As may be seen in Table VI, about 90 per cent of the animals fed the butter-yellow rice diet with yeast gained weight and presented a healthy appearance throughout the experimental period. On the other hand, only 25 per cent of the rats of the control group receiving no yeast gained during the first 60 days, and thereafter their physical appearance was poor. All of the 12 rats of this group living 101 to 192 days died or were killed because of their terminal condition (Table VII).

Animals on the butter-yellow rice diet containing 15 per cent of yeast consumed from 7 to 9 gm. of the food daily. This amount is decidedly greater than the amount consumed by those fed on the butter-yellow rice diet without a supplement of yeast.

The inhibiting effect of yeast feeding upon the production of liver cancers is distinctly superior to that

TABLE VII: EFFECT OF UNPOLISHED RICE ALONE ON THE PRODUCTION OF LIVER CANCER BY BUTTER-YELLOW

Rat no.	Sex	No. of days fed	Body weight (gm.) Initial	Maximum	Final	Liver findings *
1	♂	101	135	135	122	+
2	♂	101	153	153	115	+
3	♂	104	134	134	70	+
4	♂	107	168	168	86	+
5	♀	125	140	140	93	+
6	♂	139	180	196	136	+
7	♂	156	145	145	138	+
8	♂	163	145	156	146	+
9	♂	167	155	157	134	++
10	♂	170	154	154	140	+
11	♂	188	169	169	168	++
12	♀	192	135	167	160	++

* See Table III for explanation of symbols.

of the ether extract of yeast (Table V). In the latter case, the butter-yellow rice diet contained 0.25 per cent of yeast extract. From 10,000 gm. of dried yeast 165 gm. of ether extract were obtained. Therefore, 0.25 gm. of the ether extract is equivalent to 15 gm. of dried yeast. This suggests that the anticarcinogenic substance present in yeast is either not readily soluble in ether, or it is partially inactivated during the ether extraction.

EFFECT OF FEEDING PROTEIN

In other series of experiments, purified protein was added to the butter-yellow rice diet instead of yeast in order to determine whether or not an increased quantity of protein of known composition would inhibit the development of liver cancer.

Purified casein (18) was used in this series because it is known to contain all the amino acids essential to animal nutrition. It is especially rich in sulfur, containing amino acids such as cystine.

Twenty cc. of 3 per cent butter-yellow solution in olive oil were mixed well with 1,000 gm. of the mixture consisting of unpolished rice, 82 per cent, and purified casein, 18 per cent. As before, a small quantity of fresh carrot was given daily.

In this experiment we used 20 rats of the Wistar and Sherman strains. The results are presented in Table VIII.

The data in Table VIII show that the addition of milk protein had no inhibiting effect upon the production of liver cancer. A similar result was obtained by Nakahara, Mori, and Fujiwara (12), who used purified fish protein in their experiment.

TABLE VIII: EFFECT OF PROTEIN ON THE PRODUCTION OF LIVER CANCER BY BUTTER-YELLOW.

Rat no.	Sex	No. of days fed	Body weight (gm.) Initial	Maximum	Final	Liver findings *
1	♂	100	174	184	117	—
2	♂	100	165	165	152	±
3	♀	100	147	147	126	±
4	♀	125	132	134	82	+
5	♀	134	77	108	108	—
6	♀	134	75	96	75	+
7	♀	159	65	125	80	+
8	♂	164	65	150	145	+
9	♂	164	160	183	130	++
10	♂	186	144	199	190	++
11	♂	198	133	165	155	++
12	♂	203	191	219	201	+
13	♂	210	149	174	125	++
14	♂	223	91	128	105	++
15	♂	234	149	156	156	++
16	♂	251	152	163	160	++
17	♂	284	173	204	180	+
18	♂	284	185	185	141	++
19	♂	284	125	167	158	++

* See Table III for explanation of symbols.

Animals on the protein-sufficient diet lived much longer than those maintained on a similar diet without an extra supply of protein. Many of these animals gained weight and appeared healthy throughout the experiment.

The livers of these animals grew larger and showed many large cancer nodules, mostly of the hepatoma type. Rats Nos. 9, 10, 11, 13, 14, 15, 16, 18, and 19 had very large livers and rats Nos. 9, 10, and 12 also had metastases in the mesentery and omentum.

EFFECT OF FEEDING 3 AND 6 PER CENT WHOLE YEAST

In the preceding experiments the failure of butter-yellow to produce liver cancers in rats fed on a diet of unpolished rice containing 15 per cent of yeast was clearly demonstrated.

In the following experiments an attempt was made to find out whether or not a smaller amount of yeast would have the same effect. In the present experiment 3 per cent and 6 per cent of yeast was added to the basal diets. Sugiura and Benedict (19) reported in an earlier paper that 3 per cent of yeast in a synthetic diet deficient only in vitamin B complex was sufficient to promote normal growth in young rats.

Fifty rats of the Sherman strain were used. One group of 10 rats was maintained on the mixture of unpolished rice, 94 per cent, and dried yeast, 6 per cent, and another group of 20 rats on the mixture of unpolished rice, 97 per cent, and dried yeast, 3 per cent. Twenty rats of the third group were kept on unpolished rice without yeast. All animals received butter-yellow in the proportion of 20 cc. of a 3 per cent solution per kilogram of food. In all 3 groups the rats received a small amount of fresh carrot daily.

After 150 days the experiment was discontinued; all rats then living were sacrificed and examined. The results obtained from the yeast feeding are presented in Tables IX and X. In every case, the diagnoses were confirmed by microscopic examination.

TABLE IX: EFFECT OF FEEDING WHOLE YEAST, 6 PER CENT, ON THE PRODUCTION OF LIVER CANCER BY BUTTER-YELLOW FOR 150 DAYS

Rat no.	Sex	Body weight (gm.) Initial	Maximum	Final	Liver findings *
1	♂	141	164	134	—
2	♂	136	171	146	—
3	♀	120	163	130	—
4	♀	118	145	103	—
5	♂	142	180	170	±
6	♂	132	132	100	±
7	♀	121	164	122	±
8	♂	130	160	143	+
9	♂	130	130	107	+
10	♀	125	135	116	+

* See Table III for explanation of symbols.

As may be seen in Table IX, the addition of 6 per cent yeast to unpolished rice had a distinct, although not complete, inhibiting effect on the progression of pathological changes leading to the development of liver cancer. Thus, among 10 rats, 4, or 40 per cent, had grossly and histologically normal livers; 3, or 30 per cent, cirrhotic livers; and 3, or 30 per cent, livers with a few small tumor nodules. The nodular livers contained cholangiomas. On the other hand, all of the 11 rats fed with butter-yellow rice diet without addition of yeast and examined on the 150th day, showed typical liver cancer.

The addition of 3 per cent yeast to the butter-yellow rice diet had a slight inhibiting effect upon the production of liver cancer. Among 20 rats, 6, or 30 per cent, showed no malignant change in the livers; 14, or 70 per cent, livers with numerous large and small tumor nodules. On histological examination these livers were

found to have cholangioma or hepatoma, or both. Rat No. 19 had metastases in the mesentery, and rat No. 20 had metastases in the lung, mesentery, and peritoneum.

The above results suggested the possibility that a vitamin-like substance or substances played a role in the inhibition of development of the liver cancers. Therefore, it is of interest to examine the riboflavin, vitamin B_2, content of the various food materials used in this study. Riboflavin estimations were made by the fluorometric method. The riboflavin content of the brewer's yeast employed was 70 γ/gm., yeast extract, 14.5 γ/gm., rice-bran extract, 0.5 γ/gm., brown rice, 0.5 γ/gm., and fresh carrot, 0.4 γ/gm.

TABLE X: EFFECT OF FEEDING WHOLE YEAST, 3 PER CENT, ON THE PRODUCTION OF LIVER CANCER BY BUTTER-YELLOW DURING 150 DAYS

Rat no.	Sex	Body weight (gm.) Initial	Maximum	Final	Liver findings *
1	♀	141	160	160	—
2	♂	121	180	164	—
3	♀	122	144	119	—
4	♂	144	153	140	±
5	♂	165	192	184	±
6	♂	122	167	125	±
7	♂	144	166	162	+
8	♀	136	150	137	+
9	♀	139	139	123	+
10	♂	118	118	118	+
11	♂	130	136	124	+
12	♂	118	119	117	+
13	♀	111	114	108	+
14	♀	127	136	118	+
15	♀	126	138	112	+
16	♂	120	165	155	++
17	♂	136	138	122	++
18	♂	146	160	131	++
19	♂	122	160	146	++
20	♂	140	164	124	++

* See Table III for explanation of symbols.

The brewer's yeast (No. 2040, Fleischmann Laboratories) contained vitamins B_1, 50-55 units/gm., B_2, 70 γ/gm., B_6, 60 γ/gm., H, 0.4 γ/gm., nicotinic acid, 90-340 γ/gm., pantothenic acid, 150-250 γ/gm., and ergosterol, 8.3 mgm./gm., but vitamins A, C, and E were absent.

SUMMARY AND CONCLUSIONS

1. Various factors which influence the production of liver cancer in rats by feeding p-dimethyl-amino-azobenzene, butter-yellow, have been investigated.

2. Under the conditions of the experiments only about 3 per cent of the rats died during the first month against 45 per cent reported by the Japanese workers.

3. The albino rats of the Sherman and Wistar strains were more susceptible to the carcinogenic action of butter-yellow than those of the Evans strain.

Of the 185 rats from the 3 strains which died or were sacrificed between 91 and 220 days after the beginning of the experiment 164, or 89 per cent, had liver cancer and the other 21, or 11 per cent, liver cirrhosis with or without bile duct adenomatosis. Of the 101 rats of the Wistar and Sherman strains which died or were sacrificed between 100 and 200 days 98, or 97 per cent, showed liver cancer and the other 3, or 3 per cent, had liver cirrhosis with or without adenomatous hyperplasia.

In the case of the Evans strain 19 out of 33 rats, or 58 per cent, living between 100 and 150 days, and 11 out of 13 rats, or 85 per cent, living between 151 and 180 days respectively had liver cancer, and all others, had liver cirrhosis with or without adenomatous hyperplasia. All of the 19 Evans rats surviving 181 to 220 days of butter-yellow feeding showed liver cancer.

4. The liver cancers were found to be of various histological types of hepatomas: trabecular or solid type; glandular or cholangioma type; and mixed type. Cirrhosis with adenomatous hyperplasia of bile ducts preceded these malignant tumors.

5. Daily ingestion of a small slice of fresh carrot has no noticeable effect on the production of liver cancer by butter-yellow, but it has an influence on the survival of the animal and consequently upon the type of liver cancer.

6. Rats fed butter-yellow and rice-bran extract (ether soluble) did not develop liver cancer during a period of 150 days. The inhibiting effect of the rice-bran extract upon experimental liver cancer appears to be transient because some of the rats finally developed the disease.

7. The development of liver cancer in rats fed butter-yellow is inhibited by the daily ingestion of a small amount of ether extract of yeast. The inhibition is less marked than that exerted by the rice-bran extract.

8. Butter-yellow did not produce liver cancer in rats fed on a diet of unpolished rice containing 15 per cent of brewer's yeast. All of the 34 rats examined between 104 and 284 days after the beginning of the experiment showed smooth and practically normal livers.

9. The inhibiting effect of yeast feeding upon the production of liver cancer was distinctly diminished when the basal diet of butter-yellow rice contained less than 15 per cent yeast. With the addition of 6 per cent yeast to the butter-yellow rice diet, 40 per cent of animals had normal livers; 30 per cent, cirrhotic livers; and 30 per cent, livers with a few tumor nodules when examined on the 150th day. With the addition of 3 per cent yeast to the basal diet, 30 per cent showed no malignant change in the livers; and 70 per cent,

livers with many large and small cancer nodules. On the other hand, all of the rats, 100 per cent, fed with butter-yellow rice diet without addition of yeast and examined on the 150th day, showed typical liver cancer.

10. The addition of purified protein, casein, to the butter-yellow rice diet had no inhibiting effect upon the production of liver cancer. Animals on the protein-sufficient diet lived much longer than those maintained on a similar diet without extra supply of protein. The livers of these animals grew larger and showed many large cancer nodules.

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STUDIES ON ARTIFICIAL FEEDING OF INFANTS.

IV FURTHER INVESTIGATIONS IN VARIOUS ADJUNCTS TO ARTIFICIAL FOODS SPECIALLY IN THE MATTER OF KASEIN AND VITAMINE B.

By

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In the previous papers I proved that the dextri-maltosed foods such as Borden's Horlicks and Kinomeal were not perfectly satisfactory as food in their present composition. To study the cause of this defect I made experiments by adding various kinds of sugar to these foods to see what result could be obtained which are explained fully in series III. Not being satisfied or certain about the results obtained I have made further research on the subject which I will proceed to explain in detail.

I took for my experiments 6 albino-rats born of the same mother at the same time. I divided them into 3 pairs. The first pair I fed on ordinary mixed diet for 32 days from birth during which time its gain in weight was very satisfactory as can be seen by the attached diagram. On the 33rd day I fed one of the pair on Horlick's malted milk and the second one I fed on Horlick,s to which I added 0.5 gram of Paranutrin (Vitamine B in commerce). This I continued 21 days with both animals. The first one on the 33rd day weighed 59 grams and during the 3 weeks of special food it only gained 4.5 grams.

The brother at the beginning weighed 59.7 grams it gained during the same period of time only 1.4 grams. This proved that the addition of paranutrin was of no benefit as a nutritive adjunct to the Horlick's malted milk.

In the second stage I increased the paranutrin to 1.0 gram daily in the diet of the second rat, with the first rat I continued the same diet.

The first rat gained 13 grams in 21 days, the second rat only gained 8.5 in the same time. This shows again the addition of paranutrin in a double quantity had no effect as a nutritive adjunct to Horlick. In the third period I again doubled the quantity of paranutrin in the special food for the second rat, the first one still remaining on the same diet. The result was

that the second rat during the time lost weight 0.4 gram, while the first rat gained 6.5 grams. This again goes to prove that paranutrin in even such a large percent has no power as an adjunct to the Horlick's malted milk although Paranutrin has been proved to be a very powerful stimulant for growth as it contains a great amount of vitamine B.

In the fourth period to No. 2 rat I added 13% of casein to 100% Horlicks as the special diet for 21 days during this time the increase in weight was very sudden and tremendous for it gained 37.7 grams in that time, while No. 1 rat to which was fed on the diet from the beginning only gained 16 grams during the 21 days. This proves conclusively that the small gain in weight of No. 1 rat was not caused by the lack of vitamine B in the food.

This deficiency can be corrected very satisfactorily by the addition a certain amount of casein. The want of a proper amount of casein in these dextrimaltosed foods may be the cause of the defective power of nutrition.

Then I took the second pair from the same mother, which I fed on the same ordinary mixed diet as the first pair for 32 days from birth. They gained very satisfactorily during this period.

On the 33rd day I began experiments and I fed No.3 rat on Borden's malted milk, and No.4 rat I fed on Borden's malted milk to which I added 0.5 gram of Paranutrin as before. No. 3 rat died after 8 days, loosing 5.5 grams in weight.

No. 4 rat at the beginning weighed 56.5 grams, at the end of 21 days experiment it weighed 55.5 grams. Thus it had not gained during this period rather it had lost, the weight 1.0 gram.

In the second period I increased the Paranutrin to 1.0 gram daily. it gained 9.5 grams during 21 days. In the 3rd period I again increased Paranutrine to 2.0 per diem and continued the same food as before during the next 21 days. In this period it gained 10.0 grams. Thus during these 3 periods the rat gained very little, which proves again that Paranutrin in even such a large per cent has no power as an adjunct to the Borden's malted milk to correct the deficiency of the nutritive value. In the 4th period I added 11 grams casein to 100 grams Borden's malted milk. Thus making the content of nitrogen nearly to 25% same as for No. 2 rat. In this period the rat suddenly increased its weight gaining 17.5 grams during 21 days.

This again proves that the small gain in weight of No. 3 rat was not caused by the lack of vitamine B in the food and can be corrected very satisfactorily by the addition of casein to the amount of 25%. Comparing the result of the first and 2nd pairs I can suggest that. Horlick can be better corrected than Borden's in a manner like this. To ascertain what is the result with Kinomeal I took the 3rd pair of the same mother. The 3rd pair Nos. 5 and 6 rat were fed on ordinary mixed diet as before during 32 days from

birth. On 33rd I gave No. 5 rat Kinomeal, during 21 days experiment it did not gain at all and on the other hand it lost 4.0 grams.

No. 5 rat, which I fed on Kinomeal adding 0.5 gram of paranutrin daily, after 21 days this rat also lost weight of 1.5 grams.

On the 2nd period, I increased the Paranutrin to 1.0 gram per diem with the first rat I continued the same diet (Kinomeal)

In this period No. 5 rat gained 4.0 grams while No.6 rat gained 5.7 grams. In the third period I again increased paranutrin to 2.0 grams daily while with No. 6 rat continued the same Kinomeal as before.

In this period No. 6 rat lost 1.0 gram of its weight while No. 5 rat gained 1.5 grams. In the 4th period. I added 14 gram casein to 100 gram Kinomeal and fed No. 6 rat during 21 days while No. 5 rat continued on the same Kinomeal. In this period No. 6 rat gained 4 grams while No. 5 rat gained 4.8 grams during the same period.

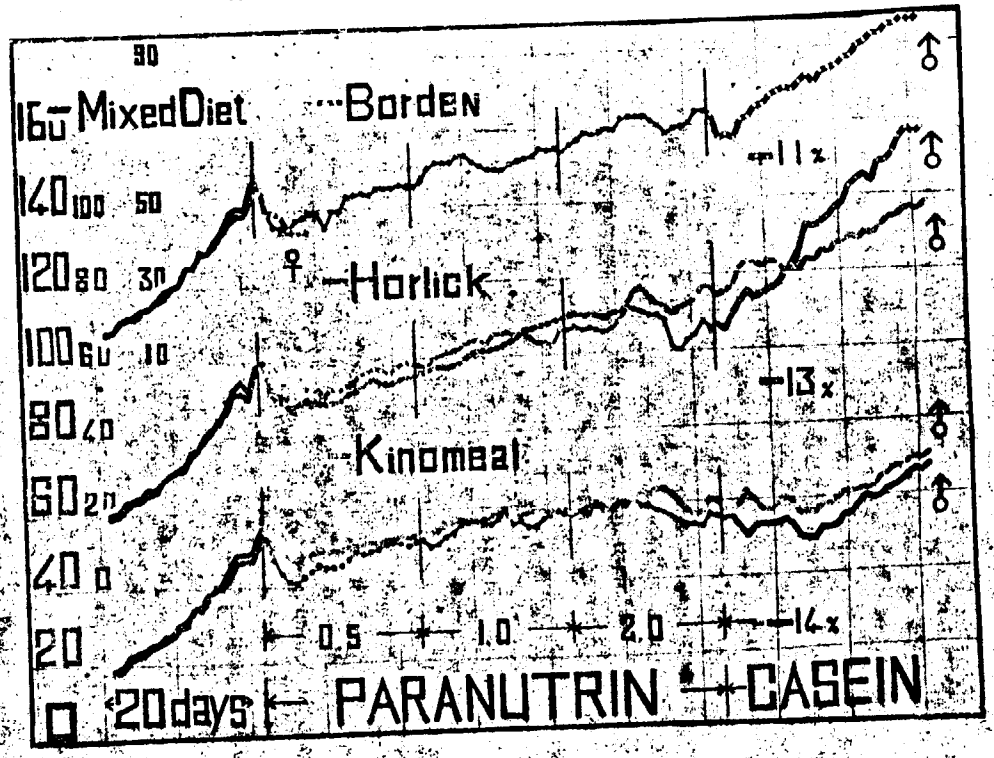
From this pair I learned also the addition of vitamine B in a sufficient amount had no influence on the growth of the animal. The addition of casein as an adjunct has also not much influence in this respect probably owing to the weakness of the animals. but it has tendency to produce a more after adding casein as can be seen by the attached Diagram.

GENERAL CONCLUSIONS.

From the above experiments I can prove conclusively that the snalll gain in weight of dextri-maltose i milk preparations as Horlick, Borden's and Kinomeal were not caused by the lack of vitamine B in the food.

Whis deficiency it seems can be corrected very satisfactorily by the addition of a certain amount of purified casein, thus suggesting that the want of a proper amount of casein in these dextri-maltosed foods may be the cause of the defective power of nutrition, which will be proved by further experiments.

Chart 1. Influence of Vitamine B and Casein.



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AN IMMUNOLOGICAL STUDY OF COELIAC DISEASE AND IDIOPATHIC STEATORRHOEA

SEROLOGICAL REACTIONS TO GLUTEN AND MILK PROTEINS

BY

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The mechanism of the harmful action of wheat and rye gluten in coeliac disease and idiopathic steatorrhoea remains uncertain. That these proteins are responsible for the continued disability in the great majority of cases of coeliac disease was first shown by Dicke (1950). This observation was extended by Weijers and van de Kamer (1950) and first confirmed in this country by Anderson *et al.* (1952). The present theories of pathogenesis have been reviewed recently by Weijers and van de Kamer (1960) and by Frazer (1960). The more popular theory postulates that there is a congenital enzyme deficiency, with resulting incomplete digestion of gluten. Consequently a glutamine-containing polypeptide accumulates sufficiently to damage the intestinal mucosa and to cause harmful systemic effects after absorption. The second theory holds that gluten itself, or a breakdown product, acts through an immunological mechanism. The two theories are not mutually exclusive, because the postulated injurious polypeptide might behave antigenically.

French *et al.* (1957) reviewed earlier work and showed that adults with idiopathic steatorrhoea also responded to a gluten-free diet. Many patients with idiopathic steatorrhoea have histories suggesting that the disease started in childhood, and the term "adult coeliac disease" has been proposed as a better one than idiopathic steatorrhoea or non-tropical sprue (Cooke, 1958).

An obstacle to the acceptance of the immunological theory of causation has been the lack of satisfactory demonstration of antibodies to the protein concerned. We have therefore made a systematic search for antibodies to a breakdown product of gluten in the sera of patients with coeliac disease, idiopathic steatorrhoea, and other types of steatorrhoea.

As well as testing for the presence of antibodies to gluten, we have also studied the antibodies to purified milk proteins, for two reasons. First, there have been reports of clinical remissions in patients with coeliac disease after the withdrawal of milk from the diet (Kunstadter and Shultz, 1953; Breton *et al.*, 1959). Secondly, we have recently found that, compared with healthy subjects, patients with ulcerative colitis are much more likely to have antibodies at high titre to milk proteins (Taylor and Truelove, 1961).

Materials and Methods

Sera.—These were obtained from children with coeliac disease and adults suffering from various types of steatorrhoea. Control sera were taken from children at operation for tonsillectomy or circumcision, under general anaesthesia, and from healthy adults, either the staff of the Nuffield Department of Clinical Medicine or visitors to patients in a general medical ward. All sera were stored at −20° C. until use.

Antigens.—The antigen used to test for antibodies to gluten was a peptic-tryptic digest of wheat gluten (fraction III) as described by Frazer *et al.* (1959). Gluten itself is insoluble in water and the usual salt solutions used in immunological procedures. Fraction III has been shown to cause clinical and biochemical relapse when fed to children with coeliac disease who have been treated with a gluten-free diet (Frazer *et al.*, 1959). Freshly prepared fraction III was used in a 1/5 dilution in phosphate-saline buffer at *p*H 6.4. It was not previously freeze-dried. The chief proteins of milk, casein, α-lactalbumin and β-lactoglobulin were used at a concentration of 0.5% in phosphate-saline buffer at *p*H 6.4. These proteins were supplied to us in a highly purified form by Dr. R. Aschaffenburg, of the National Institute for Research in Dairying, who has described the methods of preparation (Gunther *et al.*, 1960).

Tests Performed.—Sera were examined for precipitating antibodies by the gel-diffusion method of Ouchterlony (1948), using undiluted sera against fraction III. A standard complement-fixation test (Mayer *et al.*, 1948; Price, 1950) for complement-fixing antibodies to fraction III was performed. The complement-fixation test as described by Berger (1958), using alcoholic extracts of gluten and gliadin, was also attempted. The coated tanned-red-cell test (Boyden, 1951) modified by Gunther *et al.* (1960) was used with fraction III and each of the three milk proteins. The tests were read at 2 and 24 hours and the results were scored as described previously (Taylor and Truelove, 1961). The sera were tested in batches, each batch being composed of experimental and control sera. Finally all the sera from both groups were tested on a single day against the preparation of fraction III, and the results showed close agreement with the earlier batch results.

Results

Gel-diffusion Tests.—Repeated tests have been made with the sera of all the classes of patients and control subjects studied against fraction III, and the results have been uniformly negative.

Complement-fixation Tests.—No evidence of complement-fixing antibodies to fraction III has been found.

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Since positive complement fixation with alcoholic extracts of wheat gluten and gliadin by the sera of patients with coeliac disease and normal children have been reported by Berger (1958), we have repeated these tests, using a constant serum dilution of 1/4 and diluting the antigen. The results have been uniformly negative.

Coated Tanned Red Cells.—Positive results were obtained with many of the sera, which were tested in dilutions of 1/20, 1/200, 1/2,000, and 1/20,000.

Results in Children

The reactions in 24 children with coeliac disease have been compared with those in 50 children not suffering from this disease. The subjects with coeliac disease were all under the age of 15 years and consisted of children who, with one exception, had developed the symptoms of coeliac disease during the first three years of life. All suffered, or had suffered, from frank steatorrhoea as judged by biochemical tests. None had evidence of other diseases which cause steatorrhoea, such as fibrocystic disease (mucoviscidosis). In addition, all the subjects either had biopsy evidence of partial or subtotal villous atrophy of the small-intestinal mucosa (Doniach and Shiner, 1957) or had shown a favourable response to a gluten-free diet.

Reactions to Gluten Fraction III

Table I and Fig. 1 show that the spectrum of results obtained with fraction III is different in coeliac disease from that found in health, with fewer coeliac sera giving negative reactions and more giving positive reactions at higher titres (1/2,000 and 1/20,000). Although there is considerable overlap between the results in the normals and those with coeliac disease, the distributions are sharply different, with more than twice as many positive results at the higher titres in coeliac disease. In essence, therefore, patients with coeliac disease are much more likely than normal children to have circulating antibodies at high titre to fraction III.

In view of the fact that most patients diagnosed as suffering from coeliac disease are to-day treated with a gluten-free diet, which in itself might alter the serum reactions, we have analysed our material with this point in mind (Table II). Unfortunately our numbers are small, but none of the three patients on normal diet had negative or low-titre reactions (1/20) to fraction III. This is an issue which deserves further study.

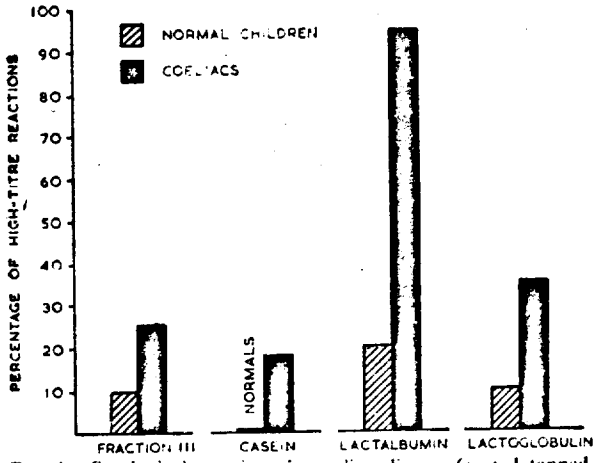

FIG. 1.—Serological reactions in coeliac disease (coated tanned-red-cell test).

TABLE II.—*Serological Reactions to Gluten Fraction III in Coeliac Disease in Relation to Gluten-free Diet*

Diet	Neg. or 1/20	1/200 and Over
Normal	0	3
Gluten-free for <1 year	2	3
" " >1 "	6	7

Likewise, our present small numbers make it impossible to draw any firm conclusions when the serological reactions are related to the biopsy findings (Table III).

TABLE III.—*Serological Reactions to Gluten Fraction III in Coeliac Disease in Relation to Biopsy Findings*

Histological Changes in Biopsy Specimen	Neg. or 1/20	1/200 or Higher
Normal	2	0
Villous atrophy	3	9

Among the 14 patients who had biopsy performed, the two with negative biopsy findings were both on a gluten-free diet which may permit reversion of the mucosa towards normality (Anderson, 1960). Those with positive biopsy findings but negative or low-titre serological reactions to fraction III had all been on a gluten-free diet for several years.

Reactions to Milk Proteins

Table IV and Fig. 1 show the results obtained with the purified individual cow's milk proteins. Once again the sera from patients with coeliac disease show a relative scarcity of negative reactions and a relative excess of positive reactions at high titre. For each milk protein, the differences between coeliac sera and normal sera are significant statistically, although the differences are least pronounced in the case of lactoglobulin.

Independence of Reactions to Gluten Fraction III and to Milk Proteins

In view of the fact that the sera of patients with coeliac disease show more strongly positive reactions to both fraction III and milk proteins, it is of interest to know whether these reactions are related or independent of one another. Table V sets out the association between the results for fraction III and the individual milk proteins in the form of simple contingency tables. It can be seen that there is no obvious correlation, either positive or negative.

Results in Adults

The reactions in 60 cases of steatorrhoea in adults have been compared with those in 64 healthy adult subjects. The steatorrhoea patients comprised 45 examples of idiopathic steatorrhoea, seven with pancreatic steatorrhoea, and eight with post-gastrectomy steatorrhoea.

TABLE I. *Serological Reactions to Gluten Fraction III in Coeliac Disease*

	Detailed Results						Compressed Version				Significance of Difference
	Neg.	1/20	1/200	1/2,000	1/20,000	Total	Neg. or 1/20	1/200	1/2,000 or 1/20,000	Total	
Control children	25	15	5	4	1	50	40 (80%)	5 (10%)	5 (10%)	50 (100%)	$\chi^2 = 13{\cdot}4$, n = 2, P<0·01
Coeliac disease	5	4	9	4	2	24	9 (37·5%)	9 (37·5%)	6 (25%)	24 (100%)	

Reactions to Gluten Fraction III

Table VI shows the results obtained with fraction III. It can be seen that the patients with idiopathic steatorrhoea have a much higher incidence of positive reactions than the normal subjects. For example, the reactions at high titre (1/2,000 or 1/20,000) are more than 10 times as frequent in idiopathic steatorrhoea as in health. The adult patients with idiopathic steatorrhoea (Fig. 2) therefore show essentially the same difference from their normal controls as do children with coeliac disease. By contrast, the patients with pancreatic steatorrhoea and post-gastrectomy steatorrhoea give results which appear to conform with those of healthy adults, although a more extensive study will be necessary to settle this point.

Reactions to Milk Proteins

When the results obtained with milk proteins are examined (Table VII) it can be seen that, as with fraction III, the patients with idiopathic steatorrhoea frequently have strongly positive reactions to these proteins and that the differences from the normal subjects are considerable and reach very high levels of statistical significance. In effect, the adult patients with idiopathic steatorrhoea resemble the children with coeliac disease in showing an excessive number of strongly positive reactions to milk proteins as well as to fraction III. By contrast, the patients with pancreatic or post-gastrectomy steatorrhoea resemble normal subjects in their reactions to milk proteins as well as to fraction III.

It should be mentioned here that the number of high-titre reactions to casein with normal adult sera is lower than that reported in a previous communication (Taylor and Truelove, 1961). This difference appears to be due to a change in technique, the use of rabbit-serum-saline as the serum diluent making the system with this antigen more stable and reducing the number of 1/2,000 and 1/20,000 reactions. The overall difference between normal and ulcerative colitis sera remains unchanged. It is interesting that this technical modification does not affect the results whether using lactalbumin or lactoglobulin.

Independence of the Reactions to Gluten Fraction III and to Milk Proteins

As in the children, the reactions to fraction III and to the milk proteins appear to be essentially independent, as can be seen from Table VIII.

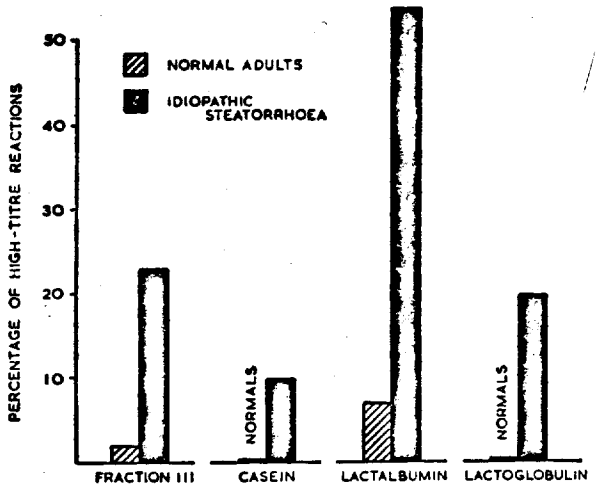

FIG. 2.—Serological reactions in idiopathic steatorrhoea in adults (coated tanned-red-cell test).

TABLE IV.—*Serological Reactions to Cow's Milk Proteins in Coeliac Disease*

Antigen	Subjects	Detailed Results						Compressed Version				Significance of Difference
		Neg.	1/20	1/200	1/2,000	1/20,000	Total	Neg. or 1/20	1/200	1/2,000 or 1/20,000	Total	
Casein	Control children	6	37	7	0	0	50	43 (86%)	7 (14%)	0 (0%)	50 (100%)	$\chi^2 = 29.6$, n = 2, P<0.001
	Coeliac disease	0	3	11	3	0	17	3 (17.6%)	11 (64.7%)	3 (17.6%)	17 (100%)	
α-Lactalbumin	Control children	8	13	19	8	2	50	21 (42%)	19 (38%)	10 (20%)	50 (100%)	$\chi^2 = 29.5$, n = 2, P<0.001
	Coeliac disease	0	0	1	8	8	17	0 (0%)	1 (5.9%)	16 (94.1%)	17 (100%)	
β-Lactoglobulin	Control children	26	11	8	4	1	50	37 (74%)	8 (16%)	5 (10%)	50 (100%)	$\chi^2 = 7.4$, n = 2, P<0.05
	Coeliac disease	2	5	4	6	0	17	7 (41.2%)	4 (23.6%)	6 (35.2%)	17 (100%)	

TABLE V.—*Showing Lack of any Definite Association Between Serological Reactions in Coeliac Disease to Gluten Fraction III and to Individual Milk Proteins*

Gluten Fraction III	Casein				α-Lactalbumin				β-Lactoglobulin			
	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total
Neg. and 1/20	2	3	1	6	0	1	5	6	2	2	2	6
1/200	1	3	2	6	0	0	6	6	4	1	1	6
1/2,000 and 1/20,000	0	5	0	5	0	0	5	5	1	1	3	5
Totals	3	11	3	17	0	1	16	17	7	4	6	17

TABLE VI.—*Serological Reactions to Gluten Fraction III in Steatorrhoea*

	Detailed Results						Compressed Version				Significance of Difference
	Neg.	1/20	1/200	1/2,000	1/20,000	Total	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total	
Adult controls	45	14	4	1	0	64	59 (92.2%)	4 (6.2%)	1 (1.6%)	64 (100%)	$\chi^2 = 21.0$, n = 2, P<0.001
Idiopathic steatorrhoea	14	11	10	9	1	45	25 (55.6%)	10 (22.2%)	10 (22.2%)	45 (100%)	
Pancreatic "	3	3	1	0	0	7	6	1	0	7	
Post-gastrectomy "	4	2	1	1	0	8	6	1	1	8	

Reactions to Gluten Fraction III in Relation to the Clinical Picture in Idiopathic Steatorrhoea

Age at Onset of Disease.—The patients with idiopathic steatorrhoea included a number in whom the symptoms began during childhood, a group which conforms to the conception of adult coeliac disease (Cooke, 1958). It is of interest to compare the immunological reactions of this group of cases with those of the remainder in whom idiopathic steatorrhoea appeared to develop during mature life. Table IX shows that there is no significant difference between these two groups of cases that comprise the total group of idiopathic steatorrhoea.

Small-intestinal Biopsy Findings.—Of the 45 patients with idiopathic steatorrhoea, the results of small-intestinal biopsy were available for 25. Among these 25, all except one showed villous atrophy of the mucosa. The immunological reactions in this group of patients were similar to those in the remainder in whom biopsy had not been employed as one of the diagnostic criteria.

Anaemia.—Among the 44 patients with idiopathic steatorrhoea, 34 had had anaemia as a prominent feature of their illness. These 34 subjects were more liable to show high-titre reactions to fraction III than the remainder; but the difference was not statistically significant, and more extensive observations will be necessary to decide the issue.

Gluten-free Diet.—Among the patients with idiopathic steatorrhoea there appeared to be little difference in the serological reactions of those being treated on a gluten-free diet and those on an unrestricted diet, although precise details of the diet were sometimes uncertain, so that further study is needed.

Discussion

The present study shows beyond doubt that circulating antibodies to fraction III are much more often present in high titre in patients with coeliac disease and idiopathic steatorrhoea than in healthy subjects. In addition, patients with these diseases frequently possess a high titre of antibodies to the proteins of cow's milk. Do these findings imply that gluten exerts its harmful action in these diseases by an immunological mechanism? If so, do milk proteins similarly have an aetiological role in these diseases, at any rate in some patients?

It has long been known from animal experiments that wheat gluten and gliadin can behave as antigens (Wells and Osborne, 1913). The mechanism by which, in man, ingested protein results in the formation of circulating antibodies is unknown. Anderson *et al.* (1926) have produced some evidence that infants absorb protein from cow's milk and eggs, but concluded that this absorption ceased after a few months of life. The fact that some of our control subjects had high titres of circulating antibodies to fraction III is sufficient evidence

TABLE VII.—*Serological Reactions to Cow's Milk Proteins in Steatorrhoea*

		Detailed Results						Compressed Version				Significance of Difference
		Neg.	1/20	1/200	1/2,000	1/20,000	Total	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total	
Casein	Adult controls	13	29	5	0	0	47	42 (89·4%)	5 (10·6%)	0 (0%)	47 (100%)	$\chi^2=14·3$, n=2, P<0·001
	Idiopathic steatorrhoea	4	19	15	4	0	42	23 (54·8%)	15 (35·7%)	4 (9·5%)	42 (100%)	
	Pancreatic steatorrhoea	6	0	1	0	0	7	6	1	0	7	
	Post-gastrectomy steatorrhoea	4	2	1	0	0	7	6	1	0	7	
α-Lactalbumin	Adult controls	10	13	21	3	0	47	23 (48·9%)	21 (44·7%)	3 (6·4%)	47 (100%)	$\chi^2=28·3$, n=2, P>0·001
	Idiopathic steatorrhoea	1	3	16	13	9	42	4 (9·5%)	16 (38·1%)	22 (52·4%)	42 (100%)	
	Pancreatic steatorrhoea	5	0	2	0	0	7	5	2	0	7	
	Post-gastrectomy steatorrhoea	1	2	4	0	0	7	3	4	0	7	
β-Lactoglobulin	Adult controls	26	18	3	0	0	47	44 (93·6%)	3 (6·4%)	0 (0%)	47 (100%)	$\chi^2=23·9$, n=2, P<0·001
	Idiopathic steatorrhoea	10	10	14	7	1	42	20 (47·6%)	14 (33·3%)	8 (19·1%)	42 (100%)	
	Pancreatic steatorrhoea	6	0	1	0	0	7	6	1	0	7	
	Post-gastrectomy steatorrhoea	6	0	1	0	0	7	6	1	0	7	

TABLE VIII. *Showing Lack of any Definite Association Between Serological Reactions in Idiopathic Steatorrhoea to Gluten Fraction III and to Individual Milk Proteins*

Gluten Fraction III	Casein				α-Lactalbumin				β-Lactoglobulin			
	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total
Neg. and 1/20	16	6	1	23	3	11	9	23	15	3	5	23
1/200	2	6	2	10	0	2	8	10	2	6	2	10
1/2,000 and 1/20,000	5	3	1	9	1	3	5	9	3	5	1	9
Totals	23	15	4	42	4	16	22	42	20	14	8	42

TABLE IX. *Similarity of Serological Reactions in Idiopathic Steatorrhoea Beginning in Childhood (Adult Coeliac Disease) and in Idiopathic Steatorrhoea Beginning in Mature Life*

	Fraction III				Casein				α-Lactalbumin				β-Lactoglobulin			
	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total
Adult coeliac disease	9	5	3	17	9	5	2	16	1	6	9	16	10	4	2	16
Other idiopathic steatorrhoea	16	5	7	28	14	10	2	26	4	9	13	26	10	10	6	26

TABLE X.—*Serological Reactions to Gluten Fraction III in Ulcerative Colitis*

	Detailed Results						Compressed Version			
	Neg.	1/20	1/200	1/2,000	1/20,000	Total	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total
Adult controls ..	45	14	4	1	0	64	59 (92·2%)	4 (6·2%)	1 (1·6%)	[illegible]
Ulcerative colitis ..	25	22	3	0	0	50	47 (94%)	3 (6%)	0 (0%)	[illegible]

that dietary gluten can act as an antigen in apparently healthy subjects. However, we do not know whether these control subjects with high titres did not have some abnormality of the small intestinal mucosa either at the time of collection of serum or at some time in the past.

The greatly increased titres to fraction III exhibited by steatorrhoea sera might be merely an indication of enhanced absorption of unchanged, antigenic protein when the small-intestinal mucosa is diseased. However, the factor which most militates against this is that serum reactions to fraction III and milk proteins are not related in individual patients.

A further observation is that in ulcerative colitis the serological reactions to fraction III resemble those in healthy subjects (Table X), although this disease resembles idiopathic steatorrhoea in showing abnormally high numbers of high-titre reactions to milk proteins (Taylor and Truelove, 1961). The discrepancy between the behaviour in the two diseases can be best illustrated by summarizing the data as follows, in which a significant surplus of high-titre reactions compared with normal proportions is indicated by a + sign.

	Idiopathic Steatorrhoea	Ulcerative Colitis
Fraction III	+	−
Casein	+	+
Lactalbumin	+	−
Lactoglobulin	+	+

The one proviso that should be made about regarding this discrepancy as ruling out abnormal absorption as the explanation for a surplus of high-titre reactions is that proteins may vary in the ease with which they are digested, and therefore the site of mucosal damage within the gastro-intestinal tract may have an important bearing.

At least the possibility exists that the high-titre reactions in patients with steatorrhoea represent an abnormal degree of specific response to one or more dietary proteins associated with hypersensitivity of the small-intestinal mucosa to these same proteins. Testing this hypothesis requires quantitative measurement of hypersensitivity of the small-intestinal mucosa itself. Rubin *et al.* (1960) have introduced gluten into the small intestine of patients with idiopathic steatorrhoea maintained in remission on a gluten-free diet and have shown that lesions typical of the disease appeared in the previously histologically normal mucosa of the proximal ileum. This elegant study may provide a valuable technique for future investigations of this problem.

Studies of circulating antibodies to dietary proteins may also prove to be of importance in the diagnosis and treatment of various enteropathies. For example, it is well known that some patients with idiopathic steatorrhoea fail to show any beneficial response when gluten is excluded from the diet. We have recently seen one such patient whose jejunal biopsy showed subtotal villous atrophy. Reaction of her serum to fraction III was negative. In such subjects it would be of interest to determine levels of circulating antibodies to other food proteins, such as those of milk, and then to determine by exclusion diets whether any favourable result obtained was related to the level of circulating antibodies.

ADDENDUM.—The serum of the patient just mentioned was found to give high-titre reactions to cow's milk proteins. She was treated with a milk-free diet (but one containing gluten) and has shown an excellent clinical response during the two months that have elapsed. More prolonged observation of this patient, as well as studies of other patients, will be necessary before it is possible to conclude with certainty that milk can play a decisive part in some patients with idiopathic steatorrhoea or coeliac disease.

Summary

Sera of patients with coeliac disease and idiopathic steatorrhoea contain antibodies to a proteolysed fraction of wheat gluten in significantly higher incidence and titre than the sera of control subjects. This is also true for purified cow's milk, casein, α-lactalbumin, and β-lactoglobulin. These findings are discussed in relation to aetiology and treatment of coeliac disease and idiopathic steatorrhoea.

We are indebted to Dr. R. Aschaffenberg, of the National Institute for Research in Dairying, for supplying us with the purified milk proteins. We are most grateful to the following physicians who assisted us by collecting sera from their patients with coeliac disease and idiopathic steatorrhoea and supplying us with clinical data: Dr. Elizabeth Pringle, Dr. Margot Shiner, Dr. Winifred Young, Dr. W. T. Cooke, Dr. Clifford Hawkins, and Dr. John Badenoch. Our anaesthetist colleague, Dr. J. V. Mitchell, kindly collected blood under anaesthesia from children undergoing minor surgical operations. We thank Miss Marjory Alcock, Miss Ruby Gearing, and Mrs. Jean Jeffs for technical assistance. The expenses of the study were partly met by a grant to one of us (S. C. Truelove) from the Medical Research Council, and it is a pleasure to acknowledge this.

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Fate in Rats of Heterologous Protein Labeled "Internally" by S^{35} and "Externally" by I^{131}

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The widespread use of radioactive labels to study protein metabolism prompted us to compare the fate of I^{131} attached as an "external" label to serum albumin with that of S^{35}-amino acids incorporated into the same protein molecule as an "internal" label. The doubly labeled protein was prepared by injecting guinea pigs with a yeast hydrolyzate containing S^{35}-amino acids (*1*), sacrificing the animals after 6 to 12 hr, isolating the serum albumin, and labeling it with traces of I^{131} *in vitro* (*2*). Nine days after the intravenous administration of the doubly labeled guinea-pig serum albumin to rats, the animals were sacrificed. Dry protein powders (*3*) were prepared from the liver homogenates, and the protein-bound radioactivity was determined repeatedly during a period of several weeks. An aliquot of the injected protein was treated similarly.

The activities resulting from I^{131} and S^{35} were calculated from the decay rate of 30-mg samples using the equations $R_0 = S_0 + I_0$ and $R_t = S_0 \times 0.5^{t/87} + I_0 \times 0.5^{t/8}$, where R_0 is the total activity (in counts per minute) and S_0 and I_0 the activities of S^{35} and I^{131} at zero time, respectively; R_t is the total activity at the time t. The results of two such experiments (*4*) are shown in Table 1.

We express our results by the ratio "percentage of persisting S^{35}/percentage of persisting I^{131}" in the proteins of the rat liver. Obviously, this ratio remains 1.0 when both isotopes are metabolized at the same rate. According to Table 1, the percentage of persisting S^{35} is, however, 72 or 58 times higher than that of persisting I^{131}. This may be caused by (i) partial deiodination of the injected protein and persistence of the deiodinated product in the liver; (ii) incorporation into the rat liver proteins of S^{35}-cystine, S^{35}-methionine, or peptides containing these amino acids; or (iii) a combination of these processes. In each of these cases, the percentage of guinea-pig serum albumin persisting in the rat liver will be quite different from the percentages of persisting I^{131} and/or S^{35}. If deiodination or loss of iodinated amino acids, as we believe, is the major factor responsible for our results, the amount of guinea-pig protein deposited in the rat liver would be many times higher than that indicated by the low I^{131} content.

Table 1. Radioactivity of I^{131} and S^{35} in doubly labeled guinea-pig serum albumin and in the liver homogenate of injected rats. (All activities are expressed as counts per minute on the 6th day after the rats were sacrificed.)

	Rat 4-WF	Rat 7-WF
Guinea pig serum albumin		
Injection (mg)	10.4	8.0
S^{35} activity	40×10^3	67×10^3
I^{131} activity	11.2×10^6	13.3×10^6
Rat liver proteins		
Dry weight (g)	1.02	1.20
S^{35} (activity)	0.85×10^3	1.87×10^3
S^{35} (% of injection)	2.1	2.8
I^{131} (activity)	3.2×10^3	6.45×10^3
I^{131} (% of injection)	0.029	0.048
Ratio		
% S^{35}/% I^{131}	72	58

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Goiter Production and Prevention in Rats

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Laboratory studies of the causes of dietary goiter have been handicapped by the lack of an experimental diet that would rapidly and reproducibly produce goiter in the absence of known goitrogenic chemicals. A low iodide diet is available commercially (*1*), prepared according to the recommendation of Remington (*2*). In rats this diet produces goiter very slowly. We have prepared low-iodide diets (*3*) with casein and sucrose as the basic components. These mixtures were not goitrogenic.

In June 1953 we received a communication from Eartly and Leblond (*4*) stating that a commercial cereal (*5*) was low in iodine content and could be used as a diet for rats. For the past 20 mo we have studied iodide metabolism of rats fed this cereal. In this report (*6*) the cereal is referred to as Cereal-G. Repeated analyses have shown that Cereal-G contained 0.015 to 0.035 μg of iodide per gram (*7–11*), and it was consistently goitrogenic.

The commercial Remington diet contained 0.08 μg (*7*) of iodide per gram according to our analyses, although Remington reported that his original mixtures were as low as 0.015 μg of iodide per gram. Our diets of sucrose with 13 and 26 percent casein (*5*) contained, respectively, 0.03 and 0.05 μg of total iodide per gram (*7*).

Groups of Long-Evans male rats, 60 to 100 g initial body weight, were fed one of the afore-mentioned diets for 6 wk to 2 yr. All diets were supplemented with distilled water, and the rats were main-

tained in wire-bottomed cages in an isolated, air-conditioned room ventilated with filtered air. At indicated intervals, groups of the animals were sacrificed. Their fresh thyroid glands were removed and weighed.

Table 1 shows that Cereal-G produced thyroids 3 times the normal size within 6 wk. Some of the Remington-fed rats developed slight goiters after 8 mo; however, after 18 to 26 mo, thyroids of the Remington rats were as large as the glands in the rats fed Cereal-G for 12 mo. The final age and body weight of the Remington-fed rats exceeded the age and weight of our other groups. The thyroid weights of the rats fed Cereal-G increased progressively, but their body growth was retarded. Four of the animals, fed Cereal-G for 12 mo, had thyroids that weighed more than 100 mg/100 g of body weight; control values never exceeded 14 mg/100 g.

Large thyroids were obtained by alternating Cereal-G and Cereal-G with casein. The glands in this group averaged more than 11 times normal weight (largest thyroid weighed 492 mg). When these rats were sacrificed, their health, appetite, and fur were in good condition. The mean thyroid size was large in this group; however, the standard deviations were also large. This resulted in no significant difference ($P = > 0.1$) between the mean thyroid weight of this group and the mean values of the groups fed Remington or Cereal-G for the longest duration.

Cereal-G with 25 percent casein for 8 wk resulted in significant enlargement ($P = 0.01$) of the thyroid. After 20 wk of this diet the thyroid size was normal. The casein-sucrose diets did not produce goiter.

The addition of 1.5 μg of NaI to the daily Cereal-G intake practically prevented the goiter, and 3 μg of NaI per day resulted in thyroid weights similar to those of the controls.

These data may be summarized as follows. Cereal-G produced goiters in rats more rapidly than a somewhat similar mixture of grains (Remington). A casein-sucrose diet was less goitrogenic than casein plus Cereal-G, even though both diets had the same total iodine content. Addition of casein to Cereal-G partially prevented goiter and stimulated body growth; withdrawal of casein from this diet produced the largest goiters that we have observed. The goitrogenic effect of Cereal-G was prevented by 3 μg of NaI per day.

These observations suggest the following possible explanations: (i) The fact that Cereal-G is more quickly goitrogenic than Remington diet indicates that there is either a goitrogen among the original ingredients of Cereal-G or an antigoitrogen in the Remington diet; or else the processing of Cereal-G may have increased its goitrogenic properties. (ii) The iodide requirements of rats may be decreased by casein or increased by Cereal-G. (iii) The goitrogenic property of Cereal-G may be due to an ingredient that interferes with production or utilization of the thyroid hormone or to the lack of a substance required to produce thyroid hormone. (iv) The goiter

Table 1. Thyroid weights of rats fed different diets for progressively increased durations.

Diet	Weeks on diet	No. of rats	Body wt. (g) ± S.D.*	Thyroid wt. (mg) ± S.D.*	Thyroid mg/100gm body wt. ± S.D.*
Fed diet less than 4 mo					
Control (Purina lab. chow)	8	8	153 ± 14	15 ± 1.7	9.8 ± 1.1
Cereal-G	6	9	175 ± 33	54 ± 18	31 ± 10
Cereal-G	12	9	202 ± 41	60 ± 12	29.7 ± 12
Cereal-G + 25% casein	8	14	208 ± 43	31.4 ± 14	15.1 ± 4.1
Cereal-G + NaI (1.5 μg NaI per day)	8	6	152 ± 17	16 ± 3.6	10.5 ± 2.4
Cereal-G + NaI (3.0 μg NaI per day)	8	4	155 ± 17	15 ± 3.4	9.7 ± 2.8
Fed diet 4 to 8 mo					
Control (Rockland Farms)	24	9	197 ± 44	22 ± 4.8	11 ± 1.9
26% casein (with sucrose)	16	5	315 ± 38	23 ± 4.3	7.3 ± 1.3
13% casein (with sucrose)	16	11	260 ± 43	18 ± 5.6	7.0 ± 1.8
Cereal-G + 25% casein	20	6	336 ± 14	32 ± 8.8	10.7 ± 4.1
Remington	32	15	275 ± 18	39 ± 19	14 ± 7.1
Cereal-G	32	11	266 ± 27	192 ± 58	72 ± 20
Fed diet 10 to 26 mo					
Control (Purina lab. chow)	10–50	13	362 ± 38	27 ± 3.6	7.5 ± 0.98
Cereal-G	50	10	249 ± 57	135 ± 55	54.4 ± 22
Cereal-G + 10% casein	50	2	370 ± 71	61 ± 9	17 ± 6
26% casein (with sucrose)	100	5	294 ± 36	26 ± 5.2	8.7 ± 1.7
Remington	80–110	6	438 ± 118	231 ± 146	52.8 ± 33
Cereal-G 3 mo, then Cereal-G + 25% casein 2 mo, then Cereal-G 6 mo		7	329 ± 53	296 ± 104	90.0 ± 53

* S.D. means standard deviation calculated from the range [R. B. Dean and W. J. Dixon, *Anal. Chem.* **23**, 636 (1951)].

prevention effect of NaI may demonstrate that Cereal-G is deficient in iodide; however, these data could result if NaI reduced the effectiveness of a goitrogenic mechanism (*12*) other than iodine deficiency.

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1. Obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.
2. R. E. Remington, *J. Nutrition* **13**, 223 (1937). Remington diet No. 347 contained wheat gluten 18 percent, brewer's yeast 2 percent, corn meal (obtained from "iodide-deficient" areas) 78 percent, calcium carbonate 1 percent, sodium chloride 1 percent.
3. Composition: casein (0.11 μg of iodide per gram, vitamin free) (*1*) 13 or 26 percent, sucrose (Domino) 68 or 55 percent, vegetable fat (Crisco) 10 percent, purified salts, and crystalline vitamins.
4. H. Eartly and C. P. Leblond, private communication.
5. Produced by Mead Johnson and Company, Evansville, Ind., and marketed under the trade name Pablum Mixed Cereal. This cereal is stated to contain "wheat meal (farina), oatmeal, yellow corn, wheat germ, tribasic calcium phosphate, powdered alfalfa leaf, dried yeast, sodium chloride, thiamine hydrochloride, riboflavin, and reduced iron." The mixture is "precooked" and dried to a moisture content of 7 percent. This cereal was purchased on the open market, 1-mo supply at a time, from July 1953 through January 1955.

6. This investigation was supported by grants from the U.S. Atomic Energy Commission and U.S. Public Health Service. Such support should not be construed as endorsement of, or concurrence in, the findings and opinions expressed herein, which are solely my own.

7. Analysis for total iodine in dietary materials are subject to many serious errors (*8*). The values reported were the best analytic results obtained by the methods of Chaney (*9*), Barker (*10*), and Zak (*11*).
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Production of Milky-Disease Spores (*Bacillus popilliae* Dutky and *Bacillus lentimorbus* Dutky) on Artificial Media

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The larvae of two destructive soil-inhabiting insects, the Japanese beetle, *Popillia japonica* Newm., and the European chafer, *Amphimallon majalis* (Razoum.), are susceptible to milky disease (*1*). Dutky (*2*) described two species of spore-forming bacilli, *Bacillus popilliae* and *B. lentimorbus* that cause milky disease. These two species are able to invade the blood of larvae and produce large numbers of spores. After the larvae die, the spores are liberated in the soil, where they may infect other larvae.

To facilitate the natural spread of the disease, it has been feasible to inoculate the soil with milky-disease spore dust produced by a patented process (*3*) described by White and Dutky (*4*). Living larvae are used as the substrate. Dutky (*2, 5*) reported cultivation of both species of bacilli on artificial media in the vegetative state; however, they did not sporulate. The biological control of these insects could be considerably facilitated if a practical method for the production of milky-disease spores on artificial media were developed.

This article (*6*) presents the results of a project that was set up to study the *in vitro* characteristics of these bacteria with the objective of duplicating, as far as possible, the *in vivo* reactions, particularly sporulation.

A detailed study was undertaken of the growth of these organisms in the vegetative state on various types of media (*7*). Particular attention was given to *p*H, oxygen tension, growth factor, and the carbohydrate and nitrogen requirements of the organisms. Excellent vegetative growth was obtained, and spores formed occasionally. However, the spores were not typical of those formed within the living larvae.

Sporulation in a diseased larva occurs after the vegetative cells have become very numerous in the blood. This suggested that the supply of an essential nutrient might become deficient preceding sporulation. A nutrient deficiency might be a factor inducing sporulation (*8*). This possibility was investigated by growing the vegetative cells on a complete medium and then transferring them to a starvation medium on which further growth was impossible. Spores formed that were indistinguishable from those produced within the living larva. Details of the method follow.

An inoculum was prepared by sterilizing the surface of a diseased larva in 0.5-percent sodium hypochlorite solution, puncturing the hemacoele through the dorsal body region, and suspending the spores in sterile water. The spore suspension was heated at 70°C for 15 min. to destroy the vegetative cells. The spores were inoculated onto the surface of petri-dish cultures containing a complete medium made with the following ingredients: tryptone, 5 g; yeast extract, 3 g; K_2HPO_4, 3 g; glucose, 1 g; maltose, 1 g; soluble starch, 10 g; agar, 15 g; and distilled water, 1000 ml. The cultures were incubated at 32°C for approximately 4 days in order to insure good growth and germination of all spores.

The cells were then transferred as a paste from the surface of the complete medium to the surface of a starvation medium containing the following ingredients: $(NH_4)_2HPO_4$, 1 g; KCl, 0.2 g; $MgSO_4$, 0.2 g; yeast extract, 0.2 g; agar, 15 g; and distilled water, 1000 ml. Cells were incubated on the starvation medium at 32°, 37°, and 45°C. Although no further growth took place under these conditions, sporulation occurred within 24 to 72 hr at all three temperatures; the highest yield of spores was obtained at 37°C.

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FROM THE SECOND MEDICAL CLINIC, UNIVERSITY OF HELSINKI,
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Circulating Eosinophils, Blood Sugar and Blood Pressure after Ingestion of Casein

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In a previous paper (4) the authors showed that ingested casein produces a decrease in the number of circulating eosinophils similar to that noted at the Thorn test (3). The purpose of the present investigation was to study the effect of casein on blood sugar and blood pressure, and to corroborate the earlier result which dealt with its effect on circulating eosinophils.

Methods

The test subjects were 10 healthy voluntary males, 20—23 years of age, 63—82 kg in weight. Each was subjected to one casein test and one gelatin test. The tests, in varied order, were made in the morning after an overnight fast. For the duration of the tests the subjects were confined to bed.

The casein was the Merck vitamin-free preparation, with a nitrogen content of 12.54 per cent. The amount of casein preparation administered was adjusted to give 0.6 g of protein per kilogram of body weight. The casein quantity measured for each test subject was soaked for 12 hours in 150 millilitres of water. The subjects ingested this plus another 350 cc of water.

The gelatin used was the Gurr bacteriological preparation, with a nitrogen content of 14.26 per cent. The amount of the gelatin preparation

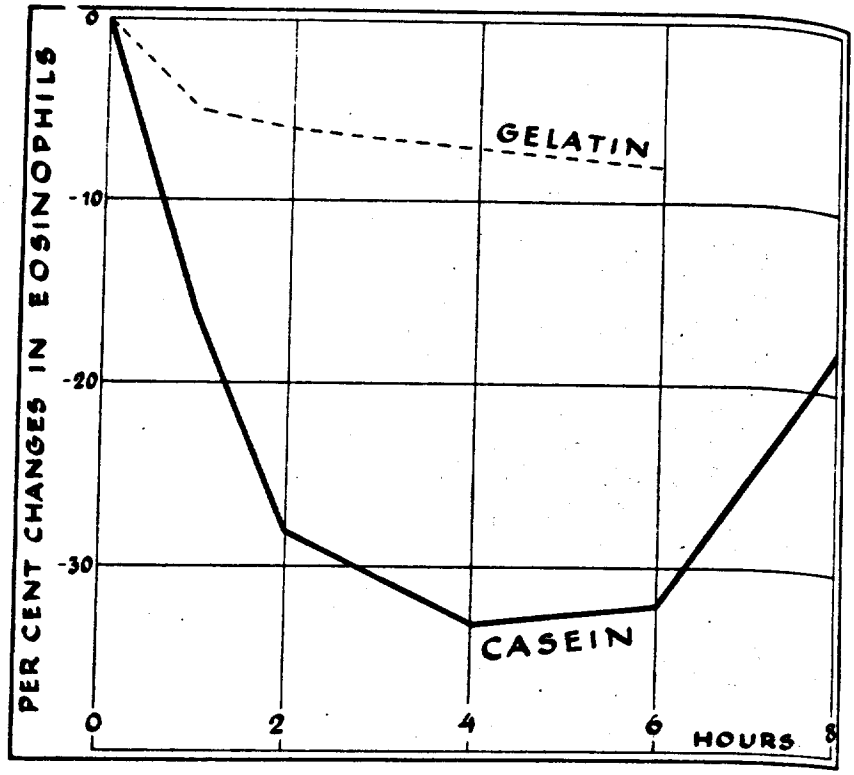

Fig. 1. — The mean percentile changes in the number of circulating eosinophils after ingestion of gelatin and casein, respectively (0.6 g of protein/Kg. of body wt.). Ten healthy subjects.

was also adjusted to give 0.6 g of protein per kilogram of body weight. Water (180 cc) was added to the weighed gelatin quantity and warmed until the gelatin dissolved. The solution was ingested warm, and in addition the test subjects had 175 cc of cold water. It was found that gelatin soaked in cold water swelled so much that it was difficult to swallow.

Circulating eosinophils were determined twice before the test and 1, 2, 4, 6, and 8 hours after its beginning. Double samples of blood were taken on each occasion. Randolph's technique (2) and Fuchs-Rosenthal counting chamber were employed.

The blood sugar was determined on the same occasions as the eosinophils, again from double samples, using the Hagedorn-Jensen method.

The blood pressure was measured with a mercury manometer, using the technique recommended by the American Heart Association and the Cardiac Society of Great Britain and Ireland (1). The recordings were

made twice before the test and 30 minutes, 1, 2, 3, 4, 5, 6, 7, and 8 hours after the beginning of protein ingestion.

The investigation was carried out jointly by three persons, one measuring the blood pressure, another making the eosinophil determinations and the third the blood sugar determinations. Thus the samples could be taken within ± 5 minutes on either side of the fixed hour.

Results

Casein induced a definite drop in the number of circulating eosinophils (Fig. 1). The effect of gelatin was negligible. The results are similar to those reported in our previous paper (4).

The blood sugar values (Table 1) were identical after casein and gelatin.

Table 1. — Blood sugar (mg per 100 ml) after the ingestion of casein or gelatin, respectively

	Before Ingestion	1 Hour after	2 Hours after	4 Hours after	6 Hours after	8 Hours after
Casein ..	98	97	96	95	91	88
Gelatin ..	98	97	96	94	91	88

Table 2. — Systolic blood pressure after the ingestion of casein or gelatin, respectively

	Before Inges-tion	30 Min. after	1 Hour after	2 H. after	3 H. after	4 H. after	5 H. after	6 H. after	7 H. after	8 H. after
Casein ..	131	127	130	126	126	128	130	127	128	127
Gelatin ..	130	127	127	128	126	124	121	122	125	124

The systolic blood pressures are shown in Table 2. A tendency to slightly lower values is noticeable after gelatin ingestion than after casein. The differences are not, however, statistically significant. No differences were observed in the values of the diastolic blood pressures after the administration of casein and gelatin.

Summary

After ten healthy test subjects had ingested casein (0.6 g of protein per kilogram of body weight), their circulating eosinophils, blood sugar and blood pressure were studied. In control tests the

same persons were given a corresponding quantity of gelatin. Casein induced a heavy drop in the number of circulating eosinophils, which reached a maximum 4 hours after ingestion. The results were similar to those arrived at in an earlier investigation by the authors.

No significant difference was found between the blood sugar and blood pressure values in casein tests and gelatin control tests.

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Studies on Xanthine Oxidase during Carcinogenesis by *p*-Dimethylaminoazobenzene*

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Protein and riboflavin deficiencies (11, 17, 1, 7) seem to be major dietary factors involved in the production of liver cancers by feeding *p*-dimethylaminoazobenzene. The quantity and type of fat in the diet also appear to be important in this process (14, 18), and other dietary factors, such as biotin, play a role under certain conditions (8, 22). Conversely, a major effect of feeding *p*-dimethylaminoazobenzene is the depletion of liver riboflavin (6, 10, 16). This effect may well be related to its carcinogenic action, since *p*-dimethylaminoazobenzene does not have a similar effect on other tissues, and noncarcinogenic dyes have little, if any, effect on the hepatic riboflavin.

In order for *p*-dimethylaminoazobenzene to be a potent carcinogen, it must act synergistically with a nutritional deficiency. Two important nutritional factors related to this process, protein and riboflavin deficiencies, are the same dietary factors that are capable of depleting liver xanthine oxidase (24, 19, 2). Xanthine oxidase is a complex flavoprotein enzyme (3, 5) and is the only oxidative enzyme involved in the formation of uric acid from nucleotides; it is the only one of the enzymes involved in nucleotide metabolism in rat liver that is appreciably affected by a low protein diet (20). Since these known interrelationships hold many implications of possible importance in the mechanism of *p*-dimethylaminoazobenzene carcinogenesis, a study of the relationship of xanthine oxidase to the production of cancer by *p*-dimethylaminoazobenzene was undertaken.

EXPERIMENTAL

The basic diet used for these studies was identical with the purified 12 per cent casein–5 per cent corn oil diet described by Kline *et al.* (13), except that 1 per cent cod liver oil was added to the diet at the expense of glucose. Two other control diets were the same, but one contained 8 per cent casein and the other 21 per cent casein; an appropriate adjustment of the glucose content was made in each case. These three dye-free diets, varying significantly only in protein content, were fed as controls to each of three groups of 24 Sprague-Dawley male rats weighing 175–200 gm. Three additional groups of 39 rats each were started on the same diets, to which had been added 0.06 per cent *p*-dimethylaminoazobenzene. At the end of 1, 2, and 4 months, six rats from each of the six groups were sacrificed by decapitation for blood and tissue analyses. All the dye-fed rats that survived for 18 weeks (six on 12 per cent casein; fifteen on 21 per cent casein; none on 8 per cent casein) were kept on the same diet without dye for an additional 6 weeks before sacrificing. At necropsy or upon death of the rat a liver section was placed in 10 per cent formalin for histological study; sections were stained with eosin and hemotoxylin. Body weights were recorded every 2 weeks.

Liver xanthine oxidase activity was determined by the method of Axelrod and Elvehjem (2) as previously reported (19). The enzyme activity in spleen, kidney, lung, and intestine was determined similarly (24). The xanthine oxidase activity in rat blood was determined by a procedure to be described, based upon the studies of Kalckar (9); it depended upon a measurement of the disappearance of xanthine and the formation of uric acid during the incubation of a xanthine solution with rat blood. The changes in concentration were determined by measuring the absorption at 270 mμ and 290 mμ.

Determination of xanthine oxidase in rat blood.—The reagents used were: phosphate buffer: 0.0585 M, ph 7.4, Na:K = 1:1; xanthine solution: the same phosphate buffer containing 40–50 γ xanthine per cc.; and 20 per cent perchloric acid.

Fresh phosphate buffer was prepared each time by diluting a concentrated solution. An aliquot of an 0.05 M xanthine solution in 0.05 M NaOH was added to the same buffer before completing the dilution, to obtain the freshly prepared xanthine solution.

1.0 cc. of the xanthine solution was added to a test tube containing 0.2 cc. of defibrinated rat

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blood. A blank was prepared by mixing 0.2 cc. of blood with 1.0 cc. of the phosphate buffer. Both tubes were incubated for 1 hour at 37°, with continuous shaking. Each was then diluted with 2.8 cc. of water, and the proteins were precipitated by the addition of 2.0 cc. of 20 per cent perchloric acid. After centrifuging thoroughly, to remove the protein precipitate, the supernatant was transferred to a 1-cm. quartz cell and read against water in a Beckman spectrophotometer at 270 mμ, 290 mμ, and 330 mμ. The density reading at 270 mμ indicated the xanthine concentration; the 290 mμ reading gave the uric acid concentration; and the reading at 330 mμ was subtracted from both the 270 mμ and 290 mμ readings, to correct for the turbidity or nonspecific absorption of the solution. The absorption of the blank was subtracted from the corresponding readings of the xanthine-containing solution in order to obtain the amount of xanthine remaining or the uric acid formed. The original xanthine concentration in the reagent used was determined by the same spectrophotometric measurements of a solution containing 1.0 cc. xanthine, 3.0 cc. water, and 2.0 cc. perchloric acid. The difference between these density readings and those calculated to be present after incubation gave the net density changes resulting from the conversion of xanthine to uric acid. The net density changes at 270 mμ and 290 mμ were divided by 0.034 and 0.071, respectively, to convert these figures into μg. of xanthine disappearing per cubic centimeter of filtrate. Multiplying by 6 gave the total μg. of xanthine converted by 0.2 cc. blood; a further multiplication by 5 gave the recorded values in terms of μg. xanthine oxidized by 1 cc. of blood per hour.

The values of 0.034 and 0.071 given above for the change in the density of the solution at 270 mμ and 290 mμ, respectively, that accompanies the oxidation of 1 μg. per cubic centimeter of xanthine to uric acid differ from those reported by Kalckar (9). The differences are due to the shift in the absorption spectra of both xanthine and uric acid in the acid medium of the perchloric acid filtrate. The absorption peak for xanthine in phosphate buffer of pH 7.4 is 277 mμ; in the perchloric acid filtrate it is 264 mμ; the absorption at 270 mμ changes very little with acidification, because the two curves cross at this point. The peak of absorption for uric acid at pH 7.4, 299 mμ, is shifted to 290 mμ in the perchloric acid solution. The values of 0.034 and 0.071 were calculated from the absorption curves for xanthine and uric acid in a 6⅔ per cent perchloric acid solution.

The phosphate buffer and perchloric acid did not contribute to the absorption at the wave lengths studied. Rat blood filtrates contained considerable material absorbing at 270 mμ and 290 mμ, most of which came from the red cells. Upon incubation of whole blood or serum with phosphate buffer alone for 1 hour there was a slight increase in the absorption at both 270 mμ and 290 mμ. Incubation for 3 hours resulted in a slight decrease in the absorption at 270 mμ and a slightly increased absorption at 290 mμ. These changes indicated a relatively slow conversion of some pre-existing blood constituent to xanthine and then uric acid, but the changes were relatively small after 1 hour of incubation. Xanthine added to blood was recovered quantitatively in the filtrate when the proteins were precipitated immediately. The xanthine concentration in the reagent was unaffected by incubation of the buffer solution alone.

The 330-mμ reading was unaffected by the xanthine or uric acid concentration and was at a point where the absorption by a blood filtrate was minimal and flat. In most cases the correction introduced by the 330-mμ reading was negligible, because both the blank and xanthine-containing solutions from any one blood sample gave nearly identical readings. Filtrates from different blood samples varied appreciably in the amount of absorption at 330 mμ.

Theoretically, the xanthine disappearance could be calculated from the density change at either 270 mμ or 290 mμ, and the two values so obtained should agree. In most cases reasonably good agreement (15 per cent) was observed, and the two values were averaged. In some cases, however, there was a much greater formation of uric acid (Δ at 290) than could be accounted for by the small amount of xanthine disappearing (Δ at 270). In such instances the values were calculated from the density change at 290 mμ only. The reasons for placing greater reliance upon the 290 mμ readings are: (*a*) The change at 270 mμ may be the resultant of some xanthine being formed as well as disappearing during the incubation; (*b*) The blood filtrate blank at 270 mμ is usually much greater than the blank at 290 mμ, and the density change at 290 mμ/μg xanthine oxidized is more than twice that observed at 270 mμ; hence, the change at 290 mμ is relatively greater and more accurate. Repeat determinations on six blood samples that had been stored in the refrigerator for 24 hours gave values that differed from the original determination by 10 per cent or less.

RESULTS

At the time the rats were started on the various diets, nine additional rats from the same group were sacrificed immediately and found to contain 1,188 ± 91 units of xanthine oxidase in the liver.

Studies on xanthine oxidase during carcinogenesis by *p*-dimethylaminoazobenzene. W. W. Westerfeld, Dan A. Richert, and Martin F. Hilfinger (Syracuse Univ., Syracuse, N.Y.). *Cancer Research* 10, 486-91(1950); cf. *C.A.* 43, 8037*f*.—By comparison with chow-fed rats, rats on a purified diet contg. 21% casein showed decreased xanthine oxidase (I) values in the liver, spleen, lung, and intestine, not in the kidney. A diet contg. 8% casein gave a further marked decrease in the liver I only, and a marked increase in the blood I. *p*-Dimethylaminoazobenzene (II) decreased I in kidney and blood, irrespective of diet, and decreased I in liver synergistically with a low-protein diet. I activity of liver homogenates was not altered by dialysis. The activity of added milk I was increased slightly more in the liver homogenates of II-free rats than in II-fed rats. Methylene blue added to the liver homogenate in the aerobic test system increased the I activity more in the II-fed rats than in the controls; this effect was lost on dialysis. These results indicated that the livers of II-fed rats contained a small amt. of nondialyzable inhibitor of I that affected primarily the autoxidizable rather than the dehydrogenase portion of the enzyme. The effect of II in decreasing the endogenous respiration of liver homogenate was due to a decreased I activity of the liver. The low-protein diets caused fatty infiltration and hydropic degeneration of the liver. II feeding led to bile-duct proliferation and cystadenomas in the group on 21% casein, and to bile-duct carcinomas in the groups on 12 or 8% casein.

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The changes in the activity of this enzyme in the various tissues that resulted from the diets alone as well as from the inclusion of *p*-dimethylaminoazobenzene in the diets are shown in Table 1. The growth response for each group is also recorded. The effect of each diet is evident at the end of 1 month, and the longer dietary periods do not alter the picture appreciably. Hence, the results will be discussed in approximate over-all average terms; the specific figures, together with the standard errors for the groups, are recorded in the table.

The 21 per cent protein diet maintained the liver xanthine oxidase at about 1,000 units; the dye decreased this value to about 700 units. The 12 per cent protein diet gave levels of approximately 600 units, while only 135 units were found in the corresponding dye-fed rats. The 8 per cent protein diet alone decreased the liver xanthine oxidase to 250 units, and the dye further decreased it to about 100 units. Feeding the dye acted synergistically with a dietary protein deficiency in depressing the xanthine oxidase level in the liver.

The effect of the protein level of the diet on the concentration of xanthine oxidase in the liver is evident and essentially in agreement with previous findings (23, 24). The activity found in the rats fed the 8 per cent protein diet was higher than anticipated, since values approaching zero have usu-

TABLE 1

THE XANTHINE OXIDASE ACTIVITY OF TISSUES OF RATS FED DIETS OF DIFFERENT PROTEIN CONTENT WITH AND WITHOUT *p*-DIMETHYLAMINOAZOBENZENE

Diet	Body wt. (gm.) Start	End	Xanthine oxidase activity (mean ± S.E.)* Liver	Kidney	Lung	Spleen	Intestine	Blood
			(On diet 4 weeks)					
21 per cent Protein	188	332	1312 ±123					60 ±3
"+Dye	182	267	730 ±142					35 ±6
12 per cent Protein	184	267	718 ±153					67 ±5
"+Dye	184	233	175 ±56					32 ±4
8 per cent Protein	183	243	318 ±68					91 ±4
"+Dye	181	191	49 ±21					40 ±7
			(On diet 8–9 weeks)					
21 per cent Protein	193	356	926 ±42	222 ±20	295 ±24	461 ±41	82 ±36	49 ±3
"+Dye	188	324	768 ±117	65 ±36	366 ±46	610 ±98	145 ±69	25 ±2
12 per cent Protein	181	324	413 ±87	156 ±18	261 ±42	368 ±46	15 ±15	53 ±7
"+Dye	191	227	105 ±25	10 ±10	168 ±18	481 ±65	146 ±56	18 ±7
8 per cent Protein	181	233	335 ±48	160 ±55	350 ±38	370 ±57	74 ±26	87 ±9
"+Dye	178	201	114 ±30	7 ±7	225 ±36	435 ±77	81 ±41	19 ±8
			(On diet 16–18 weeks)					
21 per cent Protein	186	449	990 ±34	198 ±29	189 ±28	454 ±22	79 ±31	58 ±1
"+Dye	178	395	616 ±80	7 ±7	165 ±21	431 ±87	29 ±19	42 ±2
12 per cent Protein	182	381	630 ±61	247 ±19	255 ±20	435 ±67	37 ±29	67 ±5
"+Dye	192	312	122 ±15	46 ±19	190 ±24	625 ±40	45 ±45	35 ±4
8 per cent Protein	177	253	82 ±48	200 ±27	244 ±26	421 ±57	54 ±28	91 ±10
"+Dye	202	208	129 ±33	81 ±19	219 ±41	641 ±69	150 ±36	58 ±10

* Tissue xanthine oxidase activity in c. mm. O_2 per gram dry wt. per hour; blood activity in μg. xanthine oxidized per cc. blood per hour. Each figure is the mean for six rats.

ally been obtained with the 8 per cent protein diet used in previous studies (23). The difference is possibly due to the choline deficiency of the diets used for these studies.

The low-protein diets alone did not decrease the xanthine oxidase activity of the kidney, the average level in this series being about 200 units. Feeding the dye decreased this activity to very low levels, an average of 35 units being found for all the diets. The protein content of the diet had no particular influence on the effect of the dye in this tissue. All three diets gave approximately the same results on the lung enzyme—levels of 200-300 units. Inclusion of the dye in the diet gave slightly lower values (approx. 15 per cent). Neither the protein content of the diet nor the dye had much effect on the lung enzyme.

The protein content of the diet had little effect on the xanthine oxidase activity of the spleen, levels of about 400 units being found on all the diets. When the dye was also fed, the enzyme activity of the spleen was generally higher, with an average of 540 units. The dye-fed rats not only maintained normal levels of this enzyme in the spleen, but the spleen was also greatly enlarged over that found on the diets alone. All three diets gave essentially the same low order of activity in the intestine—approximately 60 units. The dye had no additional effect on the intestinal enzyme: occasional increased values were small and of doubtful significance.

The protein content of the diet had an effect on the xanthine oxidase content of rat blood opposite to that of the liver. As the liver enzyme was depleted by the low protein diets, the blood enzyme increased. However, when the dye was also fed, the blood enzyme activity was depressed and held at the same low figure irrespective of the diet. These results are graphically illustrated in Figure 1.

By comparison with the values found for rats maintained on dog chow (24), the purified 21 per cent casein diet decreased the enzyme somewhat in the liver, spleen, lung, and intestine, but not in the kidney. Feeding an 8 per cent casein diet gave a further marked decrease in the liver enzyme but had no additional effect, beyond that of the purified diet alone in the other tissues. The blood enzyme was markedly increased by a low protein diet. *p*-Dimethylaminoazobenzene decreased the enzyme in kidney and blood, irrespective of diet, and decreased the enzyme in liver synergistically with a low protein intake; the dye had no depressant effect on the enzyme in lung, spleen, and intestine. It should be noted that lung and spleen are the two tissues whose xanthine oxidase activities are not increased appreciably by methylene blue (24).

Studies on liver xanthine oxidase.— The following studies were made to assess the method of determining xanthine oxidase in the livers of the dye-fed rats to see if the lower levels of activity were due to an actual absence of the enzyme from the tissue or to the presence of an inhibitor in the liver.

The livers were analyzed in the usual manner and also in the presence of 0.15 cc. of 0.0113 M

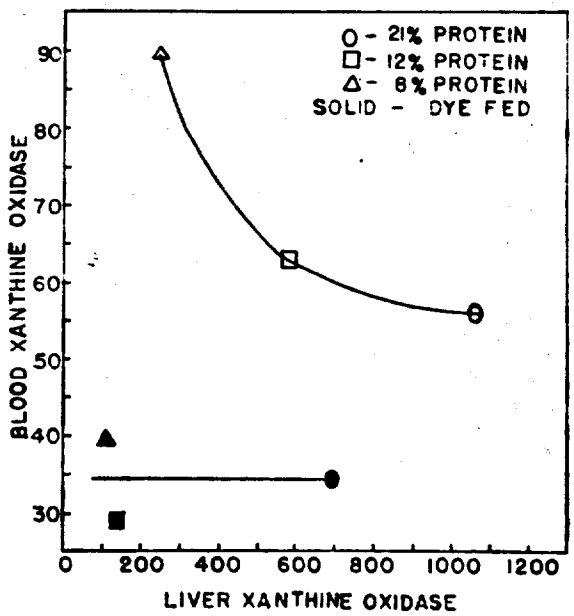

FIG. 1.—The relationship between blood and liver xanthine oxidase in rats maintained on three different diets, with and without *p*-dimethylaminoazobenzene.

Each point represents the average of eighteen rats analyzed after 1, 2, or 4 months on the diets. Liver xanthine oxidase units = c. mm. O_2 per gram dry liver per hour; blood xanthine oxidase units = μg. xanthine oxidized by 1 cc. blood per hour.

methylene blue added to the aerobic system. Purified milk xanthine oxidase (3) was added to the liver homogenate to see if its activity was increased to the same extent in the dye-fed rats as in those on the diets alone. A portion of the liver was homogenized with 2 volumes of water and dialyzed for 3-4 hours against running tap water, and an additional 16-18 hours in a large volume of cold distilled water. The dialyzed homogenate was then diluted with concentrated phosphate buffer and water to give the same proportions used with the original liver. The activity of the dialyzed preparation, as well as the effect of methylene blue and

added xanthine oxidase, was then again determined. Similar studies on normal rat livers were previously reported (19).

The same results were obtained with the rats fed the dye-containing diets for 8–9 weeks and with the rats sacrificed after 16–18 weeks. The latter data, together with the calculated results, have been summarized in Table 2. The following points may be noted: (*a*) Essentially the same enzyme activity was found after dialysis as was present in the original liver in both the dye-fed and dye-free groups. The finding of increased amounts of activity after dialysis in both 8 per cent protein groups cannot be considered unusual because of the inaccuracy of the method in determining the small amounts of activity in these livers, and because the livers regularly show some activity after dialysis when none was apparent in the original homogenate (19). The responses of the dye-fed and dye-free groups were similar in this respect, so that no evidence was obtained for the presence of a dialyzable inhibitor of xanthine oxidase in the livers of dye-fed rats. (*b*) The activity of the purified milk xanthine oxidase was increased by a factor of 1.50 in the dye-free rats and 1.27 in the dye-fed rats; these figures were unchanged by dialysis. The difference was small but consistent in each group and may indicate the presence of a very small amount of nondialyzable inhibitor of xanthine oxidase in the livers of the dye-fed rats. (*c*) The xanthine oxidase activity of the undialyzed liver homogenate was increased appreciably by the addition of methylene blue to the aerobic system; this increase was consistently greater in the dye-fed rats than in those on the dye-free diets, and the difference was statistically significant in two of the three groups. These results suggest that the auto-oxidizability of this enzyme is somewhat impaired by the dye feeding, since methylene blue acts as an accessory hydrogen transport mechanism between the dehydrogenating portion of the enzyme and oxygen. If the enzyme activities of the livers were considered on the basis of the determinations in the presence of methylene blue, the effect of each diet and the dye would be the same as that already described, but the differences would be smaller. (*d*) The effect of methylene blue on the xanthine oxidase activity of dialyzed liver, which was studied for the first time in this series, was unexpected. For, while there was no loss of enzyme as determined by the usual procedure, methylene blue gave significantly less increase in the activity of the enzyme in the two groups fed 21 per cent protein, and it actually

TABLE 2

THE EFFECT OF METHYLENE BLUE, XANTHINE OXIDASE, AND DIALYSIS ON THE XANTHINE OXIDASE ACTIVITY OF LIVERS FROM RATS FED THE CONTROL AND *p*-DIMETHYLAMINOAZOBENZENE DIETS*†

Diet	Undialyzed				Dialyzed			
	Liver	Liver + M. blue	Added X.O.	Liver + X.O.	Liver	Liver + M. blue	Added X.O.	Liver + X.O.
21 per cent Protein	28	53	18	56	29	35	18	58
"+Dye	18	45	15.5	34	14	27	17	41
12 per cent Protein	18	29	21	47	19	11	18	43
"+Dye	3.5	15	27	37	5	4	20	24
8 per cent Protein	2.3	6.6	24	41	6.5	2.6	23	40
"+Dye	3.0	11	21.5	33	7	3.5	20.5	35.5

CALCULATED DATA

Diet	Liver Activity	Liver + M.B.	Undialyzed		Dialyzed	
	Dial./undial.	Dial./undial.	Liver+M.B. / liver	X.O. recovered	Liver+M.B. / liver	X.O. recovered
21 per cent Protein	1.04 ±0.04	0.65 ±0.05	1.90 ±0.04	1.52 ±0.04	1.17 ±0.09	1.69 ±0.14
"+Dye	0.76 ±0.15	0.61 ±0.08	2.71 ±0.23	1.08 ±0.14	2.08 ±0.29	1.58 ±0.13
12 per cent Protein	1.10 ±0.09	0.39 ±0.09	1.55 ±0.10	1.41 ±0.09	0.57 ±0.12	1.29 ±0.06
"+Dye	1.51 ±0.35	0.33 ±0.09	4.35 ±0.74	1.31 ±0.13	0.94 ±0.22	0.97 ±0.10
8 per cent Protein	3.52 ±0.90	0.48 ±0.11	3.42 ±0.66	1.59 ±0.09	0.56 ±0.19	1.48 ±0.08
"+Dye	4.10 ±1.60	0.32 ±0.07	5.40 ±2.00	1.41 ±0.14	0.56 ±0.20	1.38 ±0.24

* All original data figures are in c. mm. O_2 per 20 minutes; calculated data are given as ratios.

† Each figure is the average of six rats per group fed the indicated diet for 16–18 weeks. The standard error of the mean is also given for the calculated data.

inhibited the activity of the enzyme in all groups receiving 12 per cent or 8 per cent casein. When the liver homogenates from rats on the 21 per cent casein control diets were dialyzed for 44 hours, some loss of dehydrogenase activity occurred, but the same values were obtained in the presence or absence of methylene blue. This loss of the methylene blue effect after dialysis is not a characteristic of the enzyme, since both the purified milk and liver enzymes show an increased activity with methylene blue after dialysis.

The results clearly show that no dialyzable inhibitor of xanthine oxidase accumulates in the dye-fed rats. There is some indication that a small amount of a nondialyzable inhibitor is present and that it affects primarily that portion of the enzyme which is responsible for its reaction with air rather than the dehydrogenase portion of the enzyme. Dye metabolites are known to be protein-bound in the liver (15). It must also be concluded that under the influence of either a low-protein diet or the dye the liver actually contains less enzyme. The oxidation of urate by all these livers was not inhibited appreciably, especially when the urate was tipped in after 40 minutes (19).

The *in vitro* addition of *p*-dimethylaminoazobenzene to a normal rat liver homogenate had no effect on its xanthine oxidase activity. The metabolism of *p*-dimethylaminoazobenzene by the rat is known to give rise to compounds related to *p*-aminophenol (21); the oxidized or quinimine form of *p*-aminophenol is a potent and relatively specific inhibitor of xanthine oxidase (4).

Endogenous respiration.—The endogenous respiration of the liver homogenate varied with its xanthine oxidase activity as previously described (19) and therefore varied with the protein content of the diet and the presence or absence of the dye in the diet. Figure 2 shows the average oxygen consumption curves for the rats maintained on 12 per cent casein and 12 per cent casein plus dye for 16–18 weeks. The decreased oxygen consumption of the livers from the dye-fed rats is apparent, and it is due to the lowered xanthine oxidase activity in the dye-fed rats. This was determined by a comparison of the average endogenous curve of the livers from rats fed the 21 per cent casein diet plus dye with the curve in Figure 2 for the 12 per cent casein diet alone; both groups of rats had the same average xanthine oxidase activity (17 c mm O_2/20 min), and both had identical endogenous respiration curves. The feeding of the dye had no additional effect on the endogenous respiration curve beyond that which could be accounted for by the decreased xanthine oxidase activity of the livers. Similarly, the rats on 12 per cent casein plus dye, 8 per cent casein, and 8 per cent casein plus dye all had the same low xanthine oxidase activity (approximately 3 c mm O_2 20 min), and all had essentially the same low endogenous curve. The endogenous respiration curves for kidney, spleen, lung, and intestine were not appreciably affected by

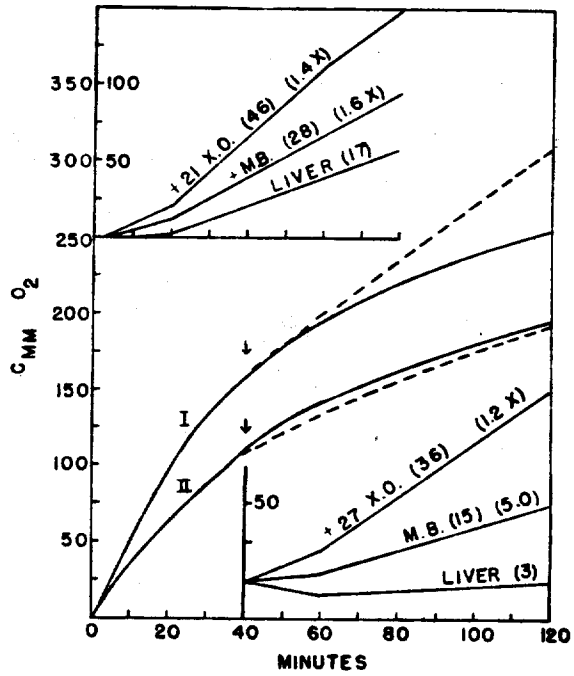

FIG. 2.—The average oxygen uptake curves for liver homogenates from rats fed 12 per cent casein diets for 16–18 weeks.

Each curve is the average for six rats. Curve I = 12 per cent casein diet; Curve II = 12 per cent casein plus 0.06 per cent *p*-dimethylaminoazobenzene. Xanthine was tipped in at the arrows to give the dotted lines. Each Warburg flask contained 1.7 cc. of a 1 + 5 liver homogenate, or 283 mg. fresh liver.

Upper inset shows the net oxygen uptake due to the oxidation of xanthine for Curve I. The effect of added purified milk xanthine oxidase (XO) and methylene blue (MB) to the body of the Warburg flask is also shown.

Numbers along curves refer to c.mm. O_2 per 20 minutes and the ratios by which the activity of the milk enzyme was increased in the liver, or the activity of the liver was increased by methylene blue.

Lower inset shows corresponding values for Curve II.

either the low protein diets or the feeding of the dye. Some small differences of questionable significance were noted.

Pathology.—The liver pathology of rats sacrificed after 1, 2, 4, and 6 months can be summarized as follows. The rats on the 21 per cent casein control diet had essentially normal livers throughout the duration of the experiment. About one-half of the rats on the 12 per cent casein diet had a sig-

nificant amount of fatty infiltration. All the rats on the 8 per cent casein diet had some fatty infiltration with marked fat deposition in over one-half of this group, particularly after 2 months on the diet. Changes characteristic of so-called hydropic degeneration were seen in a few of the animals on the 12 per cent casein diet and in over one-half of those on the 8 per cent casein diet, especially after 2 months. Early progressive cirrhosis was seen after 2 months in only two of the rats on the 8 per cent casein diet. All the above changes are characteristic of what might be expected from the low choline diets of variable protein content.

The most characteristic finding in the group of rats fed the 21 per cent casein diet plus dye was a proliferation of the smaller bile ducts out from the portal area between parenchymal liver cells. This was not accompanied by any inflammatory or fibrotic reaction, and the proliferating cells were non-neoplastic in appearance. This feature was noted with increasing frequency after 1 month on this dye-containing diet. After 4 months of dye-feeding, some animals were continued for 6 weeks more on the dye-free diet alone, and this proliferation was then seen with less frequency, although it still could be seen in a few animals. At the end of 6 months, four of fourteen rats had biliary cystadenomas, and one of these also showed a well developed hepatoma plus a separate bile-duct carcinoma without cirrhosis.

Similar duct proliferation, although with less frequency, was seen in the group fed the 12 per cent casein diet plus dye, and this was accompanied by a moderate amount of fatty infiltration. After 4 months on this dye-containing diet and 6 weeks more on the diet alone, duct proliferation was no longer present, although the fatty change without cirrhosis was present. One animal in this group showed several foci of bile-duct carcinoma without cirrhosis after 6 months.

The addition of dye to the 8 per cent casein diet produced some duct proliferation in addition to the fatty and hydropic changes noted on the diet alone. The duct proliferation was less frequent than was observed with the 12 per cent and 21 per cent casein groups. Occasional progressive cirrhosis was seen after 2 months, and two bile-duct carcinomas were obtained, one at 2 months with slight cirrhosis and one at 4 months with no cirrhosis. No animals in this group survived for a 6-month study.

Changes common to all animals on the various dye-containing diets were seen in the liver parenchymal cells, especially those near the portal area. These cells were characterized by increased nuclear as well as cell size, prominent nucleoli, and a decrease of cytoplasmic granules with increased eosinophilic staining quality of the cytoplasm. These changes were seen with increasing frequency as the time on the diets lengthened. After the dye was discontinued, most of these changes had disappeared at the end of an additional 6 weeks. A few areas of apparently regenerating parenchymal cells were seen, but in no instance could these, with assurance, be considered neoplastic.

DISCUSSION

The synergistic effect of diet plus dye in depleting liver xanthine oxidase activity may be nothing more than a reflection of a more general effect on liver flavoproteins. The depletion of xanthine oxidase and other similar liver flavoprotein enzymes by the diet-dye combination might be the primary effect that allows the carcinogenic process to be initiated. One mechanism by which such enzyme inhibitions could affect the carcinogenic process is through a blocking of the normal degradation of *p*-dimethylaminoazobenzene into noncarcinogenic metabolites (12).

Xanthine oxidase might also be related to the carcinogenic process as one of the enzyme systems involved in nucleoprotein metabolism. With increasing attention being given to the role of the nucleoproteins in cancer, an inhibition of a key enzyme in their metabolism might have more than casual importance in the carcinogenic process. However, the following points do not support this concept: (*a*) this enzyme can be eliminated almost completely from the liver by dietary means alone, without cancer resulting; (*b*) the rats that developed cancer on the 21 per cent casein diet had sub-normal but not depleted levels of liver xanthine oxidase; and (*c*) all these rats excreted normal levels of uric acid and allantoin, showing that nucleoprotein catabolism in the entire animal proceeded adequately in spite of the marked depletion of this enzyme in certain tissues.

Changes in the blood xanthine oxidase suggest another possible mechanism for the action of the dye. As the liver enzyme is depleted by the low protein diet alone, the blood enzyme increases. It seems reasonable to believe that this represents a "leakage" of the liver enzyme into the blood stream. The dye-fed rats show a more severe depletion of their liver enzyme without any increase in the blood enzyme. Does this mean that a protein-deficient diet alters the liver in such a way that it cannot retain an enzyme such as xanthine oxidase, but that the enzyme is being synthesized continuously, nevertheless? Does the lack of elevation

of the blood enzyme in the dye-fed rats signify that the enzyme is not being made? Does this indicate that a fundamental action of the dye is an inhibition of the synthesis of certain liver enzymes, such as xanthine oxidase? Other explanations of these results are equally possible, e.g., the blood of dye-fed rats may contain an inhibitor of xanthine oxidase, or the dye may affect the kidney in such a way that the blood enzyme is excreted.

A comparison of the blood and liver xanthine oxidases, when both are calculated as μg. xanthine oxidized per hour per gram dry weight, shows that a liver activity of 1,000 units is about 24 times as active as the 56 blood units present in the same rats. When the liver enzyme has been depleted to 250 units by a low protein diet, the blood has increased to 90 blood units at the same time, and the liver is then about 4 times as active.

SUMMARY

Rats were fed purified diets containing 21 per cent, 12 per cent, or 8 per cent casein with or without added *p*-dimethylaminoazobenzene. At the end of 1, 2, and 4 months, six rats from each of the six dietary groups were analyzed for xanthine oxidase in the liver, kidney, lung, spleen, intestine, and blood. A method for determining blood xanthine oxidase was described.

By comparison with chow-fed rats, the purified 21 per cent casein diet alone decreased the xanthine oxidase somewhat in the liver, spleen, lung, and intestine, but not in the kidney. The 8 per cent casein diet gave a further marked decrease in the liver enzyme only, and a marked increase in the blood xanthine oxidase.

p-Dimethylaminoazobenzene decreased the xanthine oxidase in kidney and blood, irrespective of diet, and decreased the enzyme in liver synergistically with a low protein intake; the dye had no depressant effect on the xanthine oxidase in lung, spleen, or intestine.

The same xanthine oxidase activity was found in the liver homogenates after dialysis. The activity of added milk xanthine oxidase was increased slightly more in the liver homogenates of dye-free rats than in dye-fed rats. Methylene blue added to the liver homogenate in the aerobic test system increased the xanthine oxidase activity more in the dye-fed rats than in the controls. The effect of methylene blue on the xanthine oxidase activity of the liver homogenate was lost on dialysis, especially with livers from the low protein groups. These results indicated that the livers of dye-fed rats contained a small amount of non-dialyzable inhibitor of xanthine oxidase that affected primarily the auto-oxidizable rather than the dehydrogenase portion of the enzyme. The livers also contained less enzyme than the dye-free controls. The effect of the dye in decreasing the endogenous respiration of the liver homogenate was due to a decreased xanthine oxidase activity of the liver.

The low protein diets were characterized by fatty infiltration and hydropic degeneration of the liver. Dye feeding led to bile-duct proliferation and cystadenomas in the 21 per cent casein group and bile-duct carcinomas in the other dietary groups.

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CIRCULATING ANTIBODIES TO COW'S MILK PROTEINS AND GLUTEN IN THE NEWBORN

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BY

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Recent studies have shown that in certain diseases of the alimentary tract abnormally high concentrations of circulating antibodies to cow's milk proteins may occur. Thus Taylor and Truelove (1961) found that such antibodies were more likely to be present in high titres in patients with ulcerative colitis than in healthy subjects, and this has been confirmed by other workers (Gray, 1961; Davidson, 1961). Similarly, patients with coeliac disease and idiopathic steatorrhoea are much more likely to have circulating antibodies in high titres to cow's milk proteins and to partly proteolysed wheat gluten (Taylor *et al.*, 1961).

These high antibody titres might be an expression of pre-existing states of hypersensitivity to common dietary proteins, which may serve as aetiological factors in these diseases, or they might indicate that in certain alimentary diseases abnormal absorption of such proteins occurs, with enhanced antibody formation.

Acheson and Truelove (1961) have found that patients with ulcerative colitis are more likely to have been bottle-fed during the first few weeks of life than healthy matched controls. This suggests that there may be a relationship between early exposure to potentially antigenic material and later development of a disease which could possibly be caused wholly or in part by a disturbance of normal immunological response. That milk proteins may be involved in such a way is strongly supported by clinical observations which have shown that some patients with ulcerative colitis become symptom-free when put on a milk-free diet and relapse in a few days or weeks after reintroduction of cow's milk into the diet (Andresen, 1942; Rowe, 1942; Rider *et al.*, 1960; Truelove, 1961).

These considerations led us to study the levels of circulating antibodies to cow's milk proteins and wheat gluten in various age-groups, and we report here our findings in the cord blood of newly delivered infants and in the blood of their mothers.

Materials and Methods

Specimens of cord blood from 100 babies were collected from the labour wards of the Radcliffe Infirmary, and specimens of blood from the 100 corresponding mothers were obtained by venepuncture within 24 hours of the time of delivery. The sera were stored at −20° C.

The test employed was the coated tanned red-cell test of Boyden (1951) as modified by Witebsky and Rose (1956) and Gunther *et al.* (1960). The test was read and scored as described previously (Taylor and Truelove, 1961). With each individual antigen, all the sera of mothers and babies were tested as a single batch on the same day to minimize technical errors.

As in our previous studies, purified cow's milk proteins (casein, α-lactalbumin, and β-lactoglobulin) were used as the milk antigens. A soluble product of proteolysed gluten (fraction III of Frazer *et al.*, 1959) was used as the gluten antigen.

Results

Table I and Fig. 1 show the overall results for the 100 pairs of sera. It is easily seen from the Chart that the comparative distributions in mothers and babies of titres of circulating antibodies vary according to the particular antigen. With casein and α-lactalbumin the frequency distributions of levels of circulating antibody are approximately the same in mothers and babies. With β-lactoglobulin and especially with gluten fraction III the distributions are different. Moreover, the differences are in a direction which at first sight appears surprising—namely, that the infants on average possess higher levels of circulating antibodies than the mothers.

TABLE I.—*Titres of Serological Reactions in 100 Specimens of Maternal and 100 Corresponding Specimens of Cord Blood*

Antigen	Specimen	Serological Reaction					Total	Statistical Significance
		Neg.	1/20	1/200	1/2,000	1/20,000		
Casein	Maternal	12	44	29	9	6	100	Combining the values for titres of 1/2,000 and 1/20,000, $\chi^2 = 3{\cdot}9$. $n=3$. $P>0{\cdot}05$. Not significant
	Cord	9	58	22	9	2	100	
α-Lactalbumin	Maternal	14	47	31	6	2	100	Combining the values for titres of 1/2,000 1/20,000. $\chi^2=7{\cdot}0$. $n=3$. $P>0{\cdot}05$. Not significant
	Cord	24	39	22	7	8	100	
β-Lactoglobulin	Maternal	60	25	10	3	2	100	Combining the values for titres of 1/2,000 and 1/20,000, $\chi^2 = 15{\cdot}3$. $n=3$. $P<0{\cdot}01$. Significant
	Cord	34	37	24	5	—	100	
Gluten fraction III	Maternal	68	30	2	—	—	100	Combining all values for titres of 1/200 and above, $\chi^2 = 60{\cdot}7$. $n=2$. $P<0{\cdot}001$. Highly significant
	Cord	15	67	17	1	—	100	

There is no close correspondence between the titres of these antibodies in the sera of mother and child. This can be seen when the data are set out in contingency tables as in Table II. The considerable measure of difference which often exists between mother and child is obvious. Statistically, there are feebly positive coefficients of correlation with three of the antibodies. A significantly positive coefficient of correlation is present only in the case of α-lactalbumin, but even with this there are many examples of poor correspondence between mother and child.

Further Examination of Data.—We have further examined the data and have made additional tests to exclude the possibility of extraneous factors being responsible for the differences between mothers and babies. Some of the sera obtained from cord blood showed evidence of haemolysis. Exclusion of the results

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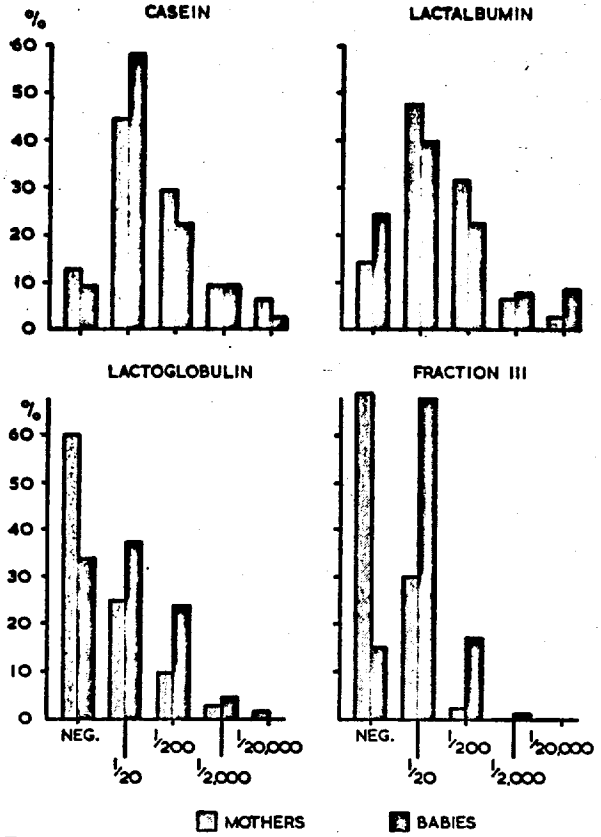
obtained with these sera and with the sera of the corresponding mothers left the results for the remaining 76 pairs of sera essentially similar to those of the full 100 pairs.



Frequency distribution of serological reactions at various titres in mothers and their babies in relation to four dietary antigens. (Coated tanned red-cell test.)

TABLE II.—*Correlation Between the Serological Reactions in Maternal and Cord Blood*

Maternal Blood	Cord Blood						Statistical Significance
	Neg.	1/20	1/200	1/2,000	1/20,000	Total	
				Casein			
Neg. ..	2	6	4	—	—	12	
1/20 ..	4	28	6	4	2	44	r = +0·075
1/200 ..	2	15	9	3	—	29	P > 0·05
1/2,000	1	5	2	1	—	9	Not significant
1/20,000	—	4	1	1	—	6	
Total	9	58	22	9	2	100	
				α-Lactalbumin			
Neg. ..	7	5	1	—	1	14	
1/20 ..	13	20	8	5	1	47	r = +0·305
1/200 ..	4	11	10	2	4	31	P < 0·01
1/2,000	—	1	3	—	2	6	Significant
1/20,000	—	2	—	—	—	2	
Total	24	39	22	7	8	100	
				β-Lactoglobulin			
Neg. ..	22	24	12	2	—	60	
1/20 ..	9	7	6	3	—	25	r = +0·110
1/200 ..	2	3	5	—	—	10	P > 0·05
1/2,000	—	3	—	—	—	3	Not significant
1/20,000	1	—	1	—	—	2	
Total	34	37	24	5	—	100	
				Gluten Fraction III			
Neg. ..	9	48	10	1	—	68	
1/20 ..	6	18	6	—	—	30	r = +0·021
1/200 ..	—	1	1	—	—	2	P > 0·05
1/2,000	—	—	—	—	—	—	Not significant
1/20,000	—	—	—	—	—	—	
Total	15	67	17	1	—	100	

The sex of the baby had no influence on the results. Likewise, the parity of the mother appeared to be of no consequence.

Some of the pregnancies were abnormal, being premature, post-mature, or associated with pre-eclamptic toxaemia. When the results for these are considered it appears that this abnormal group was more prone to show high titres of circulating antibodies than the normal group, although the relationship between mother's blood and cord blood was similar to that found in normal pregnancies. The whole question of the levels of circulating antibodies in abnormal pregnancies is an issue which deserves further study.

Additional Tests.—Repeat tests with these antigens have shown close reproducibility of results. In order to test the specificity of the reactions, inhibition tests were performed with casein, β-lactoglobulin, and gluten fraction III. Specific inhibition was demonstrated. In the case of α-lactalbumin, inhibition tests were not performed because the antigen was scarce. Maternal sera which showed no reactions to casein, to β-lactoglobulin, and to gluten fraction III at a titre of 1/20 were tested at titres of 1/4 and 1/10 against the appropriate antigen (Table III). The majority of the sera showed reactions at a titre of 1/10, and all showed reaction at a titre of 1/4 apart from six sera which showed no reaction to lactoglobulin. Inhibition tests showed that these reactions at low titre were specific.

TABLE III.—*Results of Testing Maternal Sera at Lower Titres than were Employed in the Original Test*

Antigen	No. of Maternal Sera Negative at 1/20	No. Negative at 1/10	No. Negative at 1/4
Casein	12	2	0
β-Lactoglobulin ..	60	10	6
Gluten fraction III ..	30*	3*	0*

* Only 30 of the 68 maternal sera negative at 1/20 were tested.

Discussion

The present study appears to be the first in which titres of circulating antibodies to purified cow's milk proteins and to gluten fraction III have been studied in maternal and cord blood, although the presence in cord blood of circulating antibodies to casein or to whole milk proteins has already been demonstrated (Berger and Bauer, 1959; Gunther *et al.*, 1960). With the present data on 100 mothers and their infants, the relationship between the findings in maternal and cord blood can be examined statistically for each of the antigens studied.

A conspicuous feature is the lack of close correlation between the titres of maternal and cord blood, although this varies from antigen to antigen, there being a significant positive correlation with α-lactalbumin but not with the other antigens. Another conspicuous feature is that higher titres of circulating antibody are more likely to be present in cord blood than in maternal blood in the case of β-lactoglobulin and gluten fraction III.

Transmission of antibodies from mother to foetus has been demonstrated in a variety of animals. In man, the route of transmission appears to be across the placenta (Dancis *et al.*, 1961), and the same is true in other primates (Bangham *et al.*, 1958), whereas in rodents the yolk-sac is the important route (Brambell, 1958).

Published observations suggest that this transfer of antibodies across the placenta is selective. Thus Arnon *et al.* (1959) have shown that antibodies to *Escherichia*

coli and *Shigella sonnei* are poorly transmitted, whereas the transfer of diphtheria antitoxin takes place with ease. Our own observations show that comparable differences occur with antibodies to different cow's milk proteins and gluten fraction III. The reason for such differences is not clear, and such factors as molecular size or valency of antibody do not appear to be decisive (Hemmings and Brambell, 1961; Vahlquist, 1958).

Antibodies to most antigens are present in the blood of the offspring at the same or a lower concentration than in the maternal blood (Vahlquist, 1958). However, with some antibodies, such as those to streptolysin-O, the titre is higher in cord blood than in maternal blood (Murray and Calman, 1953; Stück *et al.*, 1957). The present study has shown that antibodies to milk proteins and gluten fraction III are often present at higher titre in cord blood than in maternal blood, especially in the case of antibodies to β-lactoglobulin and fraction III.

Such higher levels of antibody in cord blood could be due to one or more of the following possibilities. First, there may be selective concentration of antibodies by the placenta. Secondly, there may be active formation of antibodies by the foetus *in utero* in response to antigen transmitted from the mother. Thirdly, it is conceivable that a fall in the titre of antibodies in the maternal circulation occurs shortly before or after delivery, but this seems unlikely because the results with maternal sera appear to match those we have obtained with non-pregnant healthy subjects.

As between the first two possibilities—namely, transplacental concentration of antibodies or placental transfer of antigen with foetal imunological competence—our data do not permit of any conclusion. Ratner *et al.* (1953) believe that the foetus may be sensitized *in utero* either actively or passively when pregnant women consume large amounts of highly antigenic foods, but their evidence appears inconclusive.

Whether the titres in cord blood of antibodies to various dietary proteins provide any index of predisposition to disease in infancy or later life is problematical. If such antibodies represent an active immunological response by the infant, then it is conceivable that they might be a manifestation of a state of hypersensitivity to common dietary proteins, and this might be of some importance in later life. If, on the other hand, these foetal antibodies have been acquired passively from the mother, they may exert a protective effect. It is well known that passively transmitted circulating antibodies interfere with active immunization during early infancy (Christie and Peterson, 1951; Osborn *et al.*, 1952; Uhr and Baumann, 1961). It is possible that passively transmitted antibodies to dietary proteins may similarly modify or prevent sensitization of the infant at the time of its first contact with these proteins after birth.

Summary

The levels of circulating antibodies to cow's milk proteins and proteolysed wheat gluten (fraction III) have been determined in the cord blood of 100 newly delivered infants and in the blood of their mothers.

The conspicuous finding was the lack of close correlation between the titres of maternal and cord blood.

In the case of β-lactoglobulin and fraction III it was usual to find higher titres of circulating antibody in cord blood than in maternal blood.

The source of the antibodies in cord blood and their possible influence on subsequent development of hypersensitivity to dietary proteins are discussed.

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"In general, however, looking at [the food of] other cultures, it is the restriction of choice that is more striking, particularly when one fails to find the food one is used to oneself. Stefansson's Eskimos resented having to eat berries and vegetables, and only did so in times of dire necessity, before eating their dogs, the stage that immediately preceded cannibalism. Other cultures have even stronger taboos. Beef is not appreciated in India, nor is pork in the Arab countries, nor is horse in the English-speaking countries. Preference, habit and taboo may indeed extend the realm of hunger. During the last war, in starving Malta, people threw away powdered eggs in the street rather than eat them. Many other examples could be cited. For most people, it appears, food to be eaten must be good food. Mere nutritiousness is not enough. But in the peculiar conquest of hunger now proceeding in the well-fed countries among those intent on slimming—in the interests of fashion perhaps rather than of health—a new consciousness of nutritive values is growing up and is already affecting taste. There is a sort of perverse injustice about the same world having two kinds of slim people, one kind because they eat only the titbits or imitate the primitive belt-tightening by alternate overeating and starving, the other because their food is inadequate." (*World Health*, September–October, 1962. Special issue on food.)